Experimental Diabetes Research

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Guest Editors: Muthuswamy Balasubramanyam, Lalith P. Singh, and Sampathkumar Rangasamy

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Editorial Board

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Editorial

Molecular Intricacies and the Role of ER Stress in Diabetes

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Diabetes mellitus is a metabolic disease caused by both genetic and environmental factors. The pathogenic mechanism(s) of diabetes are complex, and the complicated networks related to this disease involve distinct signaling pathways. Evidence has recently been provided that ER stress might be involved in the pathogenesis of diabetes and its complications. Early steps in the maturation of secretory proteins take place in the ER, for example, the folding of the nascent polypeptide chains and posttranslational modifications important for proper folding and function of the protein. At a stage (due to several metabolic disturbances), when unfolded polypeptide exceeds the folding and/or processing capacity of the ER, cells are susceptible to a phenomenon referred to as "ER stress." Under these conditions, specific signaling pathways, termed the unfolded protein response (UPR), are activated to return the ER to its normal physiological state. Prolonged activation of the ER stress and the UPR can lead to cell pathology and subsequent tissue dysfunction. There is now ample evidence that the UPR is chronically activated in many disease states including diabetes and its complications. Therefore, a better understanding of the pathways regulating ER stress and UPR is warranted in order to be instrumental for the design of novel therapies for diabetes and its complications.

In this focused issue of the journal, we have assembled several invited reviews, from well-recognized experts in their fields, as well as original research articles. These reviews provide state-of-the-art knowledge dealing with several mechanisms not only related to the genesis of diabetes but also to its progression to diabetic complications, all of which potentially originate or converge from chronic ER stress. In addition, several excellent original research articles demonstrate novel pathophysiologic aspects of diabetes with mechanistic studies central to ER stress and give hope and directionality for identifying new drug targets and developing newer therapeutic measures.

Of all the professional secretory cells we possess, β-cells are the most sensitive to ER stress because of the large fluctuations in protein synthesis (including insulin) they face daily. M.-K. Kim et al. have reviewed how this "protein quality-control machinery" of the cell is responsible for appropriate insulin biosynthesis and how ER stress plays an important role in the impairment of insulin biosynthesis. J. Zhong et al. have summarized the status on how ER stress plays an essential role in autoimmune-mediated β-cell destruction and also pointed out how ER stress regulates the functionality of immune cells relevant to autoimmune progression during Type 1 diabetes development. In an attempt to improve islet transplantation in humans, the molecular mechanism of apoptosis in β cells of islets in the transplantation setting needs to be clearly understood. In this context, M. Wang et al. have discussed their original research work on human islets subjected to multiple stressors and delineated several apoptotic pathways originating from oxidative stress, autophagy, and ER stress.

While ER stress is emerging as a unifying paradigm in diabetes and its complications, several recent studies, emphasized a definite role of ER stress in retinal, podocyte, and neuronal cell apoptosis. G. Jing et al. have summarized the recent progress on ER stress and apoptosis in retinal diseases, focusing on various proapoptotic and antiapoptotic pathways that are activated by the UPR and discussed how

these pathways contribute to ER stress-induced apoptosis in retinal cells. Considering the fact that ER stress is initially an adaptive response, studying ER stress-related factors appear to unravel novel drug targets to prevent and treat diabetic retinopathy. In this connection, W. -K. Hu et al. have explained the role of P58IPK and ER-associated degradation (ERAD) of unfolded protein which prevents ER stress and reduce retinal vascular leakage under high-glucose conditions. While thioredoxin interacting protein (TXNIP) has been recently identified as an early response gene highly induced by diabetes and hyperglycemia, its role in the pathogenesis of diabetic retinopathy is not clearly understood. Using appropriate animal model and retinal Muller cell line and several molecular biology techniques, T. S. Devi et al. have described how upregulation of TXNIP evokes a program of cellular defense and survival mechanism(s) that ultimately lead to oxidative stress, ER-stress, inflammation, and apoptosis.

Despite a great deal of research, the mechanisms that may link high-glucose concentrations to the molecular and cellular pathways of diabetic atherogenesis are not fully understood. D. R. Beriault and G. H. Werstuck have summarized the current state of our knowledge of pathways and mechanisms that may link diabetes and hyperglycemia to atherogenesis highlighting the recent work from their lab (and others) that supports a role for ER stress in these processes. Although recent studies have shown that perturbations in lipid metabolism cause an ER stress response, very little is known about the mechanism of UPR activation by perturbations in glucose and lipid metabolism. Moreover, it has been demonstrated that 4-phenylbutyrate (4-PBA) and tauroursodeoxycholic acid (TUDCA), which are two different chemical structures having chemical chaperone activity in common, relieve ER stress. Using THP-1 human monocytes as a surrogate cell model and utilizing several molecular biology techniques, R. Lenin et al. have demonstrated that monocytes subjected to glucolipotoxicity exhibited increased UPR responses (as evidenced by increased mRNA expression of several ER stress markers) along with increased oxidative stress and apoptosis. Interestingly, ER stress inducted by glucolipotoxicity was shown resisted by PBA. These observations constitute an important proof of principle that manipulation of the ER system to decrease ER stress by chemical agents may have therapeutic implications for diabetes and its complications.

Lastly, the prevalence of nonalcoholic fatty liver disease (NAFLD) has increased in parallel with the epidemics of obesity and type 2 diabetes, which are risk factors for NAFLD. Whereas the association of type 2 diabetes with microvascular complications and macrovascular disease is well established, the association of type 2 diabetes with NAFLD is only recently recognized and so are the inter-related pathogenic mechanisms. Using steatohepatitis animal model and HepB3 cells, M. K. Chae et al. have demonstrated that Pentoxifylline (a known anti-inflammatory agent) attenuates methionine and choline-1-deficient diet-induced steatohepatitis by suppressing ER stress.

These papers, hopefully, will provide better understanding of ER stress and UPR pathway involvement in the pathogenesis of diabetes and its complications and bring forward new and innovative ideas with respect to the development of efficient and adjuvant treatment modalities. Considering the involvement of ER stress in multiple tissues and their convergence in multiple pathogenic pathways (oxidative stress, inflammation, apoptosis, autophagy, and proteasomal degradation), targeting the ER stress pathway appears as a promising therapeutic strategy. The significant side effects with existing drugs and the demand for newer molecules with improved safety and a different mode of action justifies this directionality. In fact, it has been reported that chemical ER chaperones can reduce ER stress, suggesting that small molecules can affect ER stress signaling in disease states. There is also much hope in investigating the traditional plant principles of medicinal claims to see whether they act as beneficial ER stress modulators. Given the possible development of novel UPR-targeted therapies for diabetes and its complications, it is essential to know which components of the ER stress response to target and which particular disease stage will be most amenable to therapy.

Although there is an enormous progress in studying the ER stress aspects, the list of unresolved queries in the stress mediated pathway of ER dysfunction in diabetes and its complications warrant continued research efforts. Obviously not all aspects of this exciting ER stress field could be addressed in one issue and we extend our apologies to many contributors of this field whose work has not been covered. We thank all the authors who contributed to this special issue of EDR and the reviewers for the highly constructive and helpful comments.

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Research Article

Amelioration of Glucolipotoxicity-Induced Endoplasmic Reticulum Stress by a "Chemical Chaperone" in Human THP-1 Monocytes

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Chronic ER stress is emerging as a trigger that imbalances a number of systemic and arterial-wall factors and promote atherosclerosis. Macrophage apoptosis within advanced atherosclerotic lesions is also known to increase the risk of atherothrombotic disease. We hypothesize that glucolipotoxicity might mediate monocyte activation and apoptosis through ER stress. Therefore, the aims of this study are (a) to investigate whether glucolipotoxicity could impose ER stress and apoptosis in THP-1 human monocytes and (b) to investigate whether 4-Phenyl butyric acid (PBA), a chemical chaperone could resist the glucolipotoxicity-induced ER stress and apoptosis. Cells subjected to either glucolipotoxicity or tunicamycin exhibited increased ROS generation, gene and protein (PERK, GRP-78, IRE1α, and CHOP) expression of ER stress markers. In addition, these cells showed increased TRPC-6 channel expression and apoptosis as revealed by DNA damage and increased caspase-3 activity. While glucolipotoxicity/tunicamycin increased oxidative stress, ER stress, mRNA expression of TRPC-6, and programmed the THP-1 monocytes towards apoptosis, all these molecular perturbations were resisted by PBA. Since ER stress is one of the underlying causes of monocyte dysfunction in diabetes and atherosclerosis, our study emphasize that chemical chaperones such as PBA could alleviate ER stress and have potential to become novel therapeutics.

1. Introduction

Cardiovascular complications due to atherosclerotic disease are a frequent cause of morbidity and mortality in patients with diabetes [1]. A role for immune cells such as monocytes and lymphocytes has been implicated in the pathogenesis of diabetes and its various complications including atherosclerosis. Clinical and experimental evidence indicates that inflammatory processes in the vascular wall are the decisive factor that accounts for the rate of lesion formation and clinical development in patients suffering from atherosclerosis [2]. Monocytes and macrophages, in an attempt to engulf lipid laden cells along the blood vessel, will get exposed to hyperglycemia and hyperlipidemia, a combined pathophysiological situation referred to as "glucolipotoxicity." The exact mechanisms by which glucolipotoxicity triggers monocyte activation and atherosclerotic processes are not clearly understood. However, evidence indicates that ER stress plays an important role in these mechanisms.

ER stress is known to activate a series of signals that comprise the unfolded protein response (UPR). The UPR includes at least three signaling pathways initiated by the kinases IRE1 and PERK and the transcription factor ATF6 [3] signals that coordinate the cellular response to unfolded proteins, which includes (a) downregulation of protein translation, (b) enhanced expression of ER chaperone proteins that promote protein refolding, and (c) activation of proteases involved in the degradation of misfolded proteins.

Conversely, prolonged or severe ER stress can lead to the activation of apoptotic cell death. Recent studies have suggested that chronic ER stress originating from glucolipotoxicity is one of the major culprits that can very well explain the underlying causes of both insulin resistance and β-cell dysfunction [4–7]. ER stress has been linked to insulin resistance in diabetes and also expansion of ER was detected in β-cells from type 2 diabetic patients [8, 9]. Furthermore, increased expression of ER stress markers has been demonstrated in db/db mouse islets and β-cells of type 2 diabetes patients [9, 10]. Recent studies also imply that β-cell dysfunction by glucolipotoxicity-induced ER stress is mediated by increased oxidative stress and apoptosis [11]. While the recruitment of monocytes and their retention within atherosclerotic lesions contribute to plaque development [12], macrophage apoptosis within advanced atherosclerotic lesions is also known to increase the risk of atherothrombotic disease [13]. Although glucolipotoxicity is known to trigger ER stress, its effect on various arms of the ER stress machinery in monocytes has not been clearly defined. Therefore, we investigated the effect of glucolipotoxicity on monocytes with reference to ROS generation, transcriptional alterations of various ER stress markers, and apoptotic indicators. We also investigated the effect of 4-phenyl butyric acid (PBA, a chemical chaperone) whether it resists glucolipotoxicity-induced ER stress and apoptosis.

2. Materials and Methods

2.1. Cell Culture and Treatment. The human monocyte THP-1 cells were obtained from the National Centre for Cell Science (NCCS, Pune, India). THP-1 cells were maintained in endotoxin-free RPMI-1640 containing 5.5 mM glucose, 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/L amphotericin B, 2 mM L-glutamine, 1 mM sodium pyruvate and 10 mM HEPES, pH 7.0–7.4, under a humidified condition of 5% CO_2 at 37°C. For all the experiments, cells were subjected to either 5.5 mM glucose (basal) or glucolipotoxicity (25 mM glucose plus 0.5 mM palmitic acid) or tunicamycin (4 μM) treatment for 24 h in the presence and absence of 4-PBA (1 mM), in serum-free medium. All experiments were independently performed at least thrice.

2.2. Intracellular Reactive Oxygen Species (ROS) Measurement. Intracellular ROS generation was measured in THP-1 monocytes as described earlier [14] with minor modifications as to the confocal application. Briefly, 1×10^3 cells were seeded in confocal chambered slides (LabTek II) precoated with 0.01% poly-L-lysine. After the treatment conditions, 2′,7′-dichlorodihydrofluorescein diacetate (H2-DCFDA, 10 μM) dye was added to each well and incubated for 30 min at 37°C. Cells were then washed twice with PBS. To capture the change in fluorescence, cells were excited at a wavelength of 485 nm and the fluorescence emission was read at 530 nm using Carl Zeiss-LSM 700 confocal microscope with an objective of 20x. Change in mean fluorescence intensity was represented as arbitrary units (AU).

2.3. Real-Time PCR

2.3.1. RNA Isolation. Total RNA from cells was isolated as described previously [15]. The RNA quality and concentration of total RNA were measured using nanodrop. 1 μg of RNA was converted to cDNA using 100 units reverse transcriptase enzyme, 40 μM Oligo-dT18 primer (New England Biolabs), 10xRT buffer, 20 U RNase inhibitor (Amersham Biosciences), and 2.5 mM each of dNTPS and incubated at 42°C for 1 h.

2.3.2. Quantitative Real-Time PCR. Quantitative real-time PCR was performed for specific genes using SYBR green master mix (Finnzymes). PCR amplification was carried out using ABI-7000 (Applied Biosystems) with cycle conditions (initial cycle: 50°C for 2 min, Initial denaturation 95°C for 15 sec, 40 cycles of denaturation 95°C for 15 sec, and annealing/extension of 60°C for 1 min). The expression level of RNA was determined using $2^{-\mathrm{DDCt}}$ and normalized using β-actin. The primer sequences of specific genes are listed in Table 1.

2.4. Protein Expression. After specific treatments, cells were lysed using RIPA buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.2% sodium azide, 1% Triton X-100, 0.25% sodium deoxycholate, and 1x protease inhibitor). In brief, cells were sonicated and incubated for 1 hr in ice and centrifuged at 16,000 g for 5 minutes at 4°C. The supernatant collected was quantified for protein by Bradford method. 15 μg protein was resolved on a 10% SDS-PAGE and transferred to polyvinylidine fluoride (PVDF) membrane. After 1 hour blocking in 5% bovine serum albumin (BSA) and incubation with the appropriate primary antibodies and HRP-conjugated secondary antibodies, detection was performed using enhanced chemiluminescence kit (GE Healthcare). β-actin was used as internal control. Mean densitometry data from independent experiments were normalized to control using Image-J software and represented as the ratio of test protein and β-actin.

2.5. DNA Damage—Comet Assay. DNA damage was evaluated with the Comet assay as described previously [16]. Briefly, the treated cells were mixed with 200 μL of 0.5% low-melting point agarose and layered over clear microscope slides precoated with 1% normal melting agarose. Lysis, electrophoresis, and neutralization were followed by staining with ethidium bromide. The slides were examined under a fluorescent microscope. They were scored using an image analysis system (Comet Imager 1.2.13) attached to a fluorescent microscope (Carl-Zeiss, Germany) equipped with appropriate filter.

2.6. Caspase 3 Activity Assay. Caspase-3 activity was determined by colorimetric assay using the caspase-specific peptide containing amino acid sequence Asp-Glu-Val-Asp (DEVD) that is conjugated to the color reporter molecule p-nitroanilide (pNA) (RandD systems). The cleavage of the peptide by the caspases releases the chromophore pNA,

Table 1: Primer sequence of specific genes.

GRP78	Forward CTG CCA TGG TTC TCA CTA AAA TG
	Reverse TTA GGC CAG CAA TAG TTC CAG
PERK	Forward GAA CCA GAC GAT GAG ACA GAG
	Reverse GGA TGA CAC CAA GGA ACC G
IRE1	Forward GCG AAC AGA ATA CAC CAT CAC
	Reverse ACC AGC CCA TCA CCA TTG
XBP1	Forward TGG ATT CTG GCG GTA TTG AC
	Reverse TCC TTC TGG GTA GAC CTCTG
ATF-6	Forward CCT GTC CTA CAA AGT ACC ATG AG
	Reverse CCT TTA ATC TCG CCT CTA ACC C
CHOP/GADD	Forward GTA CCT ATG TTT CAC CTC CTG G
	Reverse TGG AAT CTG GAG AGT GAG GG
TRPC6	Forward TTT GAG GAG GGC AGA ACA CTT CCT
	Reverse TAT GGC CCT GGA ACA GCT CAG AAA
β-actin	Forward GTC TTC CCC TCC ATC GT
	Reverse CGT CGC CCA CAT AGG AAT

which is quantified spectrophotometrically at 405 nm. Cells harvested after treatment were lysed and 10 μg protein was aliquoted from each sample into a 96 well plate. 0.5 μL DTT was added to all the wells followed by addition of 50 μL of 2x reaction buffer and 3.5 μL of Caspase-3 colorimetric substrate. The plate was incubated at 37°C for 1 hr and read at 405 nm using microplate reader. Caspase-3 activity was expressed as mean ± SEM of optical density.

2.7. Statistical Analysis. Statistical Package for Social Sciences (SPSS) Windows, (Version 16.0, Chicago, IL), was used for statistical analysis. Data were expressed as Mean ± SEM. Comparisons between groups were performed using student's t-test and a P-value < 0.05 was considered statistically significant.

3. Results

We have applied confocal microscopy to measure the fluorescent intensity of DCF which indicates the extent of intracellular ROS generation in monocytes. While representative fluorescent images related to ROS generation were depicted in Figure 1(a), cumulative data on ROS generation were presented in Figure 1(b). Cells subjected to either glucolipotoxicity or tunicamycin exhibited increased ROS generation compared to the untreated cells (Figure 1(b)). Pretreatment of cells with 4-phenyl butyric acid showed significant reduction in ROS generation ($P < 0.05$). A complete mRNA expression pattern of ER stress machinery was determined by real-time PCR and the cumulative data on mRNA pattern of ER stress markers in relation to their ratio with the house keeping β-actin was depicted in Figure 2. Compared to untreated cells, cells treated with glucolipotoxicity or tunicamycin showed an increased mRNA expression of PERK, GRP78, IRE1α, XBP1, ATF6, and CHOP (Figures 2(a)–2(f)). It is also interesting to note that the extent increase in ER stress markers under glucolipotoxicity was higher than that induced by tunicamycin. PBA treatment significantly reduced all the transcriptional expression of ER stress markers most likely due to its chaperone activity. Figure 3(A) depicts the representative protein expression of ER stress markers along with β-actin. Consistent with the mRNA results, protein expression of ER stress markers, namely, PERK, GRP78, IRE1α, and XBP1 were also increased in cells subjected to glucolipotoxicity and tunicamycin treatment (Figure 3(B)). 4-PBA treatment significantly reduced the protein expression of all the ER stress markers.

Since the programming of apoptosis by both the oxidative and ER stress signals depend on the load of intracellular Ca^{2+}, we investigated the transcriptional level of TRPC-6, which is an important driving force for increased Ca^{2+} influx. Cells treated with glucolipotoxicity or tunicamycin resulted in several folds of increase in TRPC-6 mRNA levels (Figure 4) and this was significantly normalized by PBA.

Compared to untreated cells, cells treated with glucolipotoxicity or tunicamycin showed increased DNA damage (4.6 and 3 folds, resp.). Interestingly, PBA treatment significantly reduced the DNA damage induced either by glucolipotoxicity or tunicamycin (Figure 5(a)). Since CHOP (an intermediate in caspase-dependent apoptosis) mRNA and protein expression were increased in cells subjected to glucolipotoxicity or tunicamycin, we estimated the activity of caspase-3 enzyme. As expected, cells treated with glucolipotoxicity or tunicamycin showed significantly increased caspase-3 activity (Figure 5(b)) implying that uncontrolled ER stress might have programmed the cells towards apoptosis. Increased caspase-3 activity either due to glucolipotoxicity or tunicamycin was significantly ($P < 0.05$) reduced by 4-PBA.

4. Discussion

The following are the nutshell findings of the study. First, monocytes subjected to glucolipotoxicity showed increased ROS generation and increased mRNA expression of several genes of the ER stress machinery. Secondly, these monocytes exhibited features of chronic ER stress as evidenced by increased mRNA expression of TRPC-6, DNA damage, and caspase-3 activity. Thirdly, PBA—a chemical chaperone, resisted all the glucolipotoxicity-induced cellular and molecular alterations in monocytes emphasizing the benefits of ER stress alleviation.

Monocyte activation, adhesion to the endothelium, and transmigration into the subendothelial space are key events in early pathogenesis of atherosclerosis. The mechanisms by which glucose and lipid toxicity induces monocyte-associated atherosclerosis are only partially known. Mononuclear blood cells from patients with diabetes show increased generation of reactive oxygen species and altered redox signaling [16–21]. In our study, monocytes subjected to glucolipotoxicity exhibited increased generation of ROS. ROS could activate cell death processes directly by the oxidation of proteins, lipids, and/or nucleic acids or could act as initiators or second messengers in the cell

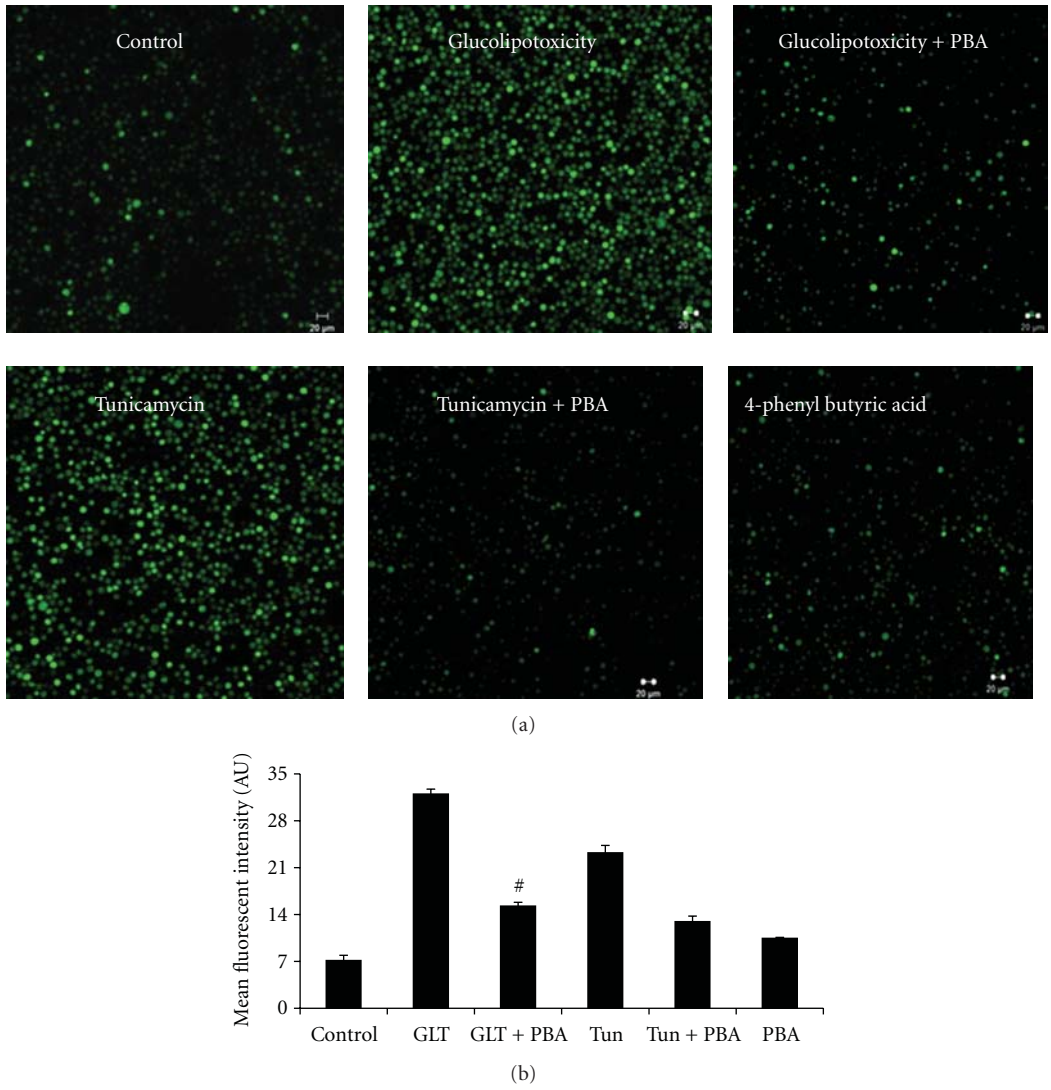

Figure 1: (a) Representative confocal microscopy images of Intracellular ROS generation in cells treated under various conditions. (b) Mean (±SEM) fluorescence intensities of ROS under different experimental maneuvers, namely, control, glucolipotoxicity (GLT), glucolipotoxicity + 4-phenyl butyric acid (GLT + PBA), tunicamycin (Tun), tunicamycin + 4-phenyl butyric acid (Tun + PBA), 4-phenyl butyric acid (PBA). $^{*}P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to GLT, $^{**}P < 0.05$ compared to tunicamycin.

death process. Accumulating evidence suggests that protein folding and generation of reactive oxygen species (ROS) as a byproduct of protein oxidation in the ER are closely linked events. It has also become apparent that activation of the UPR on exposure to oxidative stress is an adaptive mechanism to preserve cell function and survival. However, persistent oxidative stress and protein misfolding initiate apoptotic cascades and are now known to play predominant roles in the pathogenesis of multiple human diseases including diabetes and atherosclerosis [22–24].

A number of hypotheses have been conceived to explain advanced lesional macrophage apoptosis and atherosclerosis, and undoubtedly more than one mechanism is involved. Recent mechanistic data in cultured cells and correlative and genetic causation evidence *in vivo* support a role for endoplasmic reticulum (ER) stress in advanced lesional macrophage apoptosis and its major consequence of plaque necrosis [13, 25]. Consistent with previous findings, glucolipotoxicity in our study showed increased transcription of PERK, GRP78, IRE1α, XBP-1, ATF6, and CHOP in monocytes. This was also corroborated by the increased protein expression of ER stress markers. The fact that increased expression of ER stress markers under glucolipotoxicity is similar to that induced by tunicamycin (a known inducer of ER stress) emphasizes that glucolipotoxicity inducts ER stress in monocytes.

Increased TRPC-6 mRNA expression under glucolipotoxicity in our study implies a role for increased calcium levels in monocyte dysfunction and ER stress. Calcium is an essential intracellular messenger and serves critical cellular functions in both excitable and nonexcitable cells. Available data on transient receptor potential conical (TRPC) protein

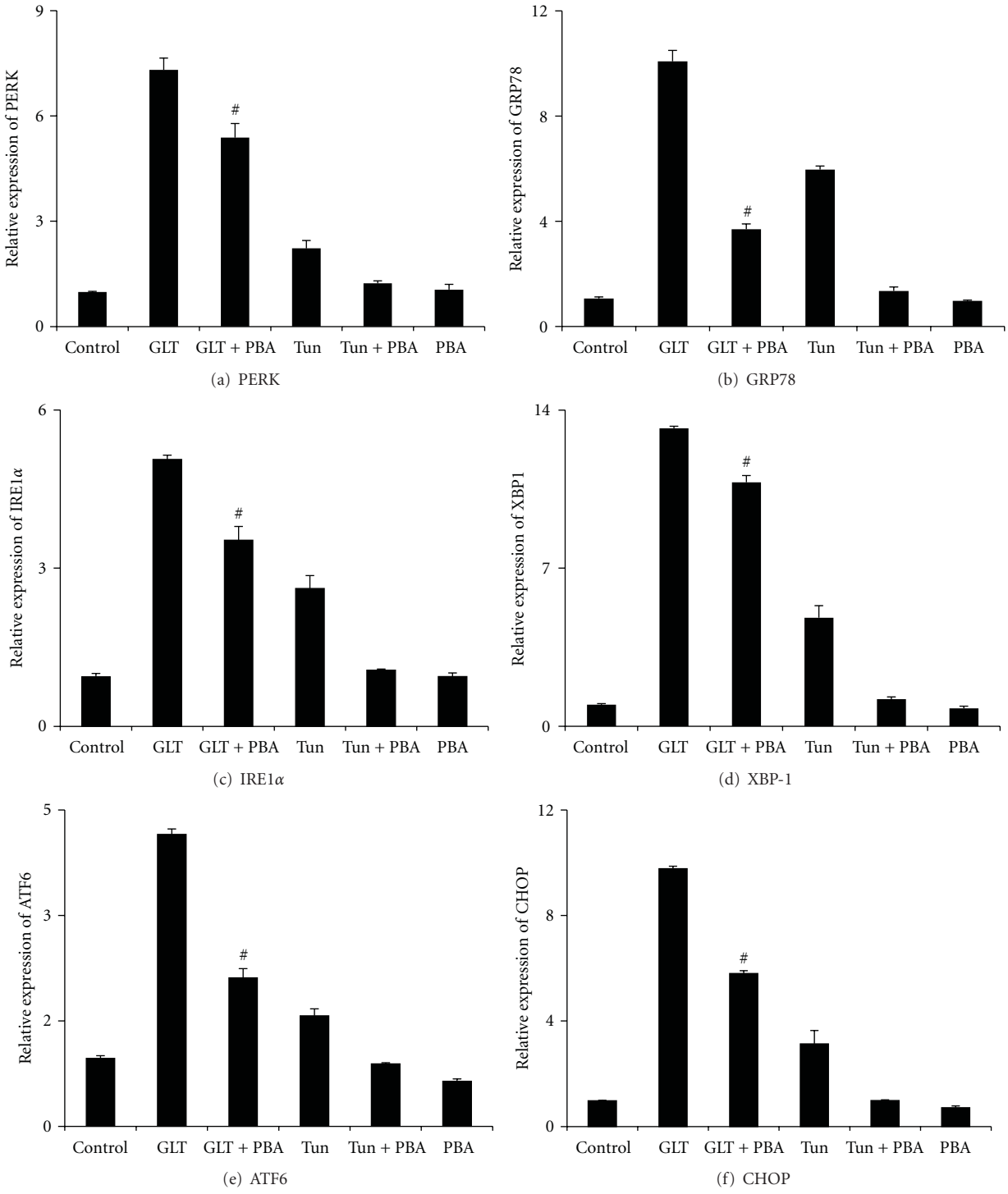

Figure 2: Relative gene expression Mean (±SEM) data of ER stress markers namely, PERK (a), GRP78 (b), IRE1α (c), XBP-1 (d), ATF6 (e), CHOP (f). $^{*}P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to GLT, $^{**}P < 0.05$ compared to tunicamycin.

indicate that these proteins initiate Ca^{2+} entry pathways and are essential in maintaining cytosolic, ER, and mitochondrial Ca^{2+} levels [26]. Alterations in Ca^{2+} homeostasis have been suggested in diabetes and associated complications [27, 28]. Zhu et al. [28] have recently reviewed a role for TRP channels and their implications in metabolic diseases. Activity of TRPC is physiologically important as Ca^{2+} concentrations within the ER must be maintained at sufficient levels in order for the organelle to carry out many of its fundamental functions including protein folding and trafficking. However, loss of Ca^{2+} homeostasis due to improper TRPC activation could lead to ER stress responses, and even apoptosis [29]. While

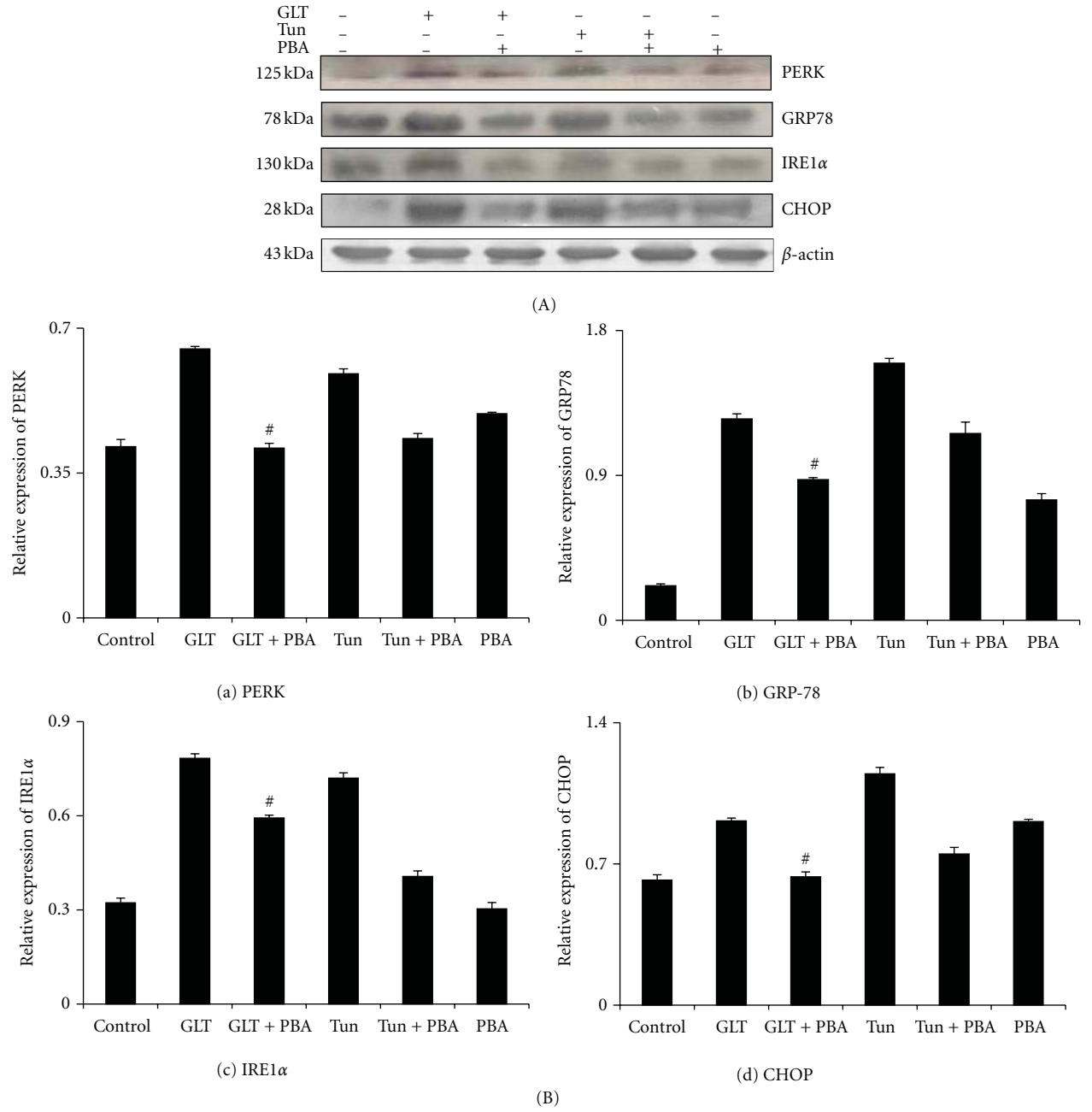

Figure 3: (A) Representative protein expression data on ER stress protein markers, namely, PERK (1), GRP78 (2), IRE1α (3), and CHOP (4). (B) Cumulative histogram data Mean (±SEM) of ER stress markers, namely, PERK (a), GRP78 (b), IRE1α (c) and CHOP (d). $^{*}P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to GLT, $^{**}P < 0.05$ compared to tunicamycin.

oxidative stress could result in cellular defects including a defect in ER Ca^{2+} uptake and Ca^{2+} efflux, thereby increasing $[Ca^{2+}]_i$ and Ca^{2+} influx [30], several TRPC families have also been shown directly activated in response to oxidative stress [31]. In our study, both glucolipotoxicity and tunicamycin resulted in increased ROS generation in monocytes and, at the transcription level, we have also witnessed the increased TRPC6 mRNA expression. Among the TRPC subfamily of TRP channels, TRPC6 is gated by signal transduction pathways that activate C-type phospholipases as well as by direct exposure to diacylglycerols [32]. Recent studies emphasize a pathophysiological role of TRPC6 in several disease states [33, 34] and it appears to be an emerging drug target. Altered TRPC6 regulation and impaired capacitative calcium entry have been shown in vessels of diabetic patients [35]. While a role for oxidative stress has been implicated in the pathogenesis of Type 2 diabetes and its micro- and macrovascular complications [20], TRPC6 mRNA was also

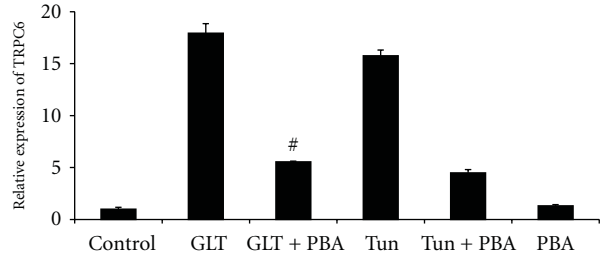

Figure 4: Mean (±SEM) mRNA expression of TRPC-6. $^{*}P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to GLT, $^{**}P < 0.05$ compared to tunicamycin.

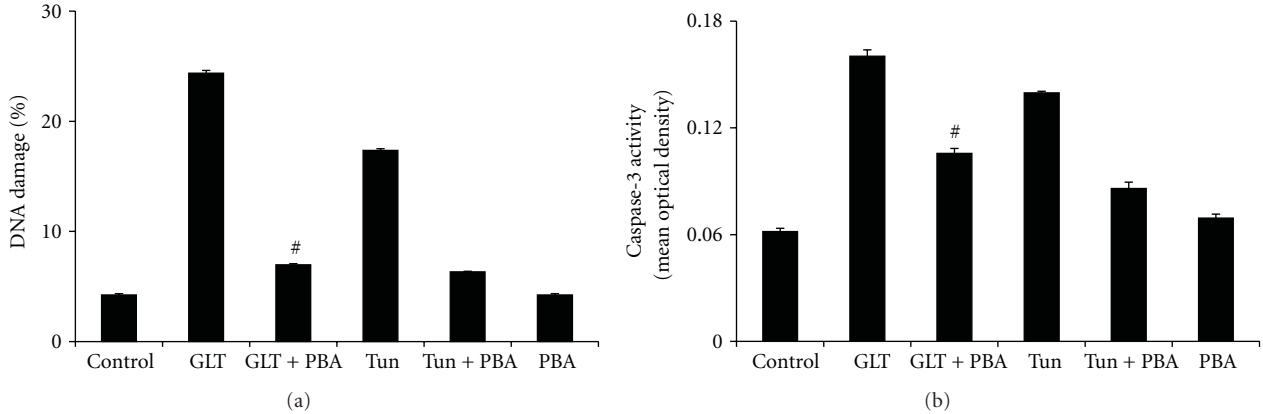

Figure 5: (a) Mean (±SEM) percentage DNA damage under different experimental conditions. $^{*}P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to GLT, $^{**}P < 0.05$ compared to tunicamycin. (b) Mean (±SEM) Caspase 3 activity under different experimental conditions. $^{*}P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to GLT, $^{**}P < 0.05$ compared to tunicamycin.

shown significantly higher in monocytes from patients with type 2 diabetes [36] pointing to a novel pathway for increased activation of monocytes and hence atherosclerosis in patients with diabetes.

ER stress is one of the damaging stresses resulting from glucolipotoxicity which can lead to apoptosis when the stress is prolonged or uncontrolled. Komura et al. [37] have shown that monocytes of diabetic patients are not as efficient in phagocytosing as in normal healthy people and electron microscopic examination of monocytes revealed morphologic alterations in the ER of cells derived from patients with diabetes. Although it has been known that activation of the CHOP pathway of the UPR can cause apoptosis, the molecular mechanisms linking CHOP to death execution pathways is poorly understood. Our results here show that apoptosis might be executed by the increased activation of caspase-3 under glucolipotoxicity. Since autophagy has been proposed to operate as an alternative cell death mechanism or act upstream of apoptosis [38], it is plausible that glucolipotoxicity-induced ER stress could also promote cell death via autophagy [39, 40]. ER stress-induced UPR activation can also influence the expression of certain inflammatory cytokines [41] rendering the monocyte intrinsically proinflammatory which might have drastic consequences when monocytes infiltrate the intima of the vessel wall. Recent work has provided evidence for a calcium dependent mechanism in ER stress-induced macrophage apoptosis [42]. The increased mRNA levels of TRPC-6 seen in our study also support this. Since caspase and the downstream apoptosis effector molecules are Ca^{2+}-dependent, future studies should also focus on the involvement of intracellular calcium in the induction of apoptosis in ER-stressed cells under glucose and lipid dyshomeostasis.

In our study, glucolipotoxicity-induced ROS generation and increased ER stress markers seen under glucolipotoxicity were normalized by PBA. Drugs that interfere with ER stress have wide therapeutic potential and recently chemical chaperones like tauroursodeoxycholic acid (TUDCA), and PBA received much attention because of their ER stress alleviating activities as these compounds improve ER folding capacity and help in stabilizing protein conformation [43]. PBA was found to be protective in *in vitro* and *in vivo* models of diabetes [43–45]. Accumulating evidence suggests that protein folding and generation of reactive oxygen species (ROS) as a byproduct of protein oxidation in the ER are closely linked events. While facilitating appropriate protein folding, PBA helps maintaining the balance between ER oxidoreductin 1 (ERO1) and protein disulfide isomerase (PDI) and thereby reduces the ROS levels generated during protein oxidation [46]. It has also been shown that PBA counteracts oxidative stress by upregulating the expression and activity of superoxide dismutase (SOD) [47, 48]. Luo

et al. [49] have also demonstrated PBA inhibition of NADPH oxidase activity and emphasized a dual regulation ER stress and oxidative stress by PBA. Erbay et al. [50] demonstrated that mitigation of ER stress with PBA protected macrophages against lipotoxic death and atherosclerosis by suppression of XBP1 splicing and CHOP expression. PBA has been shown to regulate ER stress and offer potential therapeutic benefits in several preclinical models of human diseases including type 2 diabetes [43, 51–55]. As an orally bioavailable terminal aromatic substituted fatty acid, PBA has been used for the treatment of urea cycle disorders [56]. More importantly, oral treatment of PBA was recently shown preventing lipid-induced impairment in insulin sensitivity and b-cell function in humans [57].

To conclude, our study exposes the convergence of ER stress, oxidative stress and apoptosis in the presence of glucolipotoxicity in monocytes and points out ER stress network as a novel drug-targetable pathway. Our results also emphasize that chemical chaperones enhance the adaptive capacity of the ER, and on further evaluation in appropriate clinical trials, could serve as potent antidiabetic/antiatherosclerotic modalities with potential application in the treatment of type 2 diabetes and its vascular complications like atherosclerosis.

Abbreviations

GRP-78: Glucose regulated protein-78
PERK: PKR like ER kinase
IRE-1α: Inositol Requiring enzyme-1α
XBP-1: X box binding Protein
ATF-6: Activating transcription factor-6
CHOP: CCAAT/enhancer-binding Homologous protein
GADD: Growth arrest and DNA damage protein
TRPC-6: Transient receptor protein channel-6.

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Research Article

TXNIP Links Innate Host Defense Mechanisms to Oxidative Stress and Inflammation in Retinal Muller Glia under Chronic Hyperglycemia: Implications for Diabetic Retinopathy

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Thioredoxin Interacting Protein (TXNIP) mediates retinal inflammation, gliosis, and apoptosis in experimental diabetes. Here, we investigate the temporal response of Muller glia to high glucose (HG) and TXNIP expression using a rat Muller cell line (rMC1) in culture. We examined if HG-induced TXNIP expression evokes host defense mechanisms in rMC1 in response to metabolic abnormalities. HG causes sustained up-regulation of TXNIP (2 h to 5 days), ROS generation, ATP depletion, ER stress, and inflammation. Various cellular defense mechanisms are activated by HG: (i) NLRP3 inflammasome, (ii) ER stress response (sXBP1), (iii) hypoxic-like HIF-1α induction, (iv) autophagy/mitophagy, and (v) apoptosis. We also found *in vivo* that streptozocin-induced diabetic rats have higher retinal TXNIP and innate immune response gene expression than normal rats. Knock down of TXNIP by intravitreal siRNA reduces inflammation (IL-1β) and gliosis (GFAP) in the diabetic retina. TXNIP ablation *in vitro* prevents ROS generation, restores ATP level and autophagic LC3B induction in rMC1. Thus, our results show that HG sustains TXNIP up-regulation in Muller glia and evokes a program of cellular defense/survival mechanisms that ultimately lead to oxidative stress, ER stress/inflammation, autophagy and apoptosis. TXNIP is a potential target to ameliorate blinding ocular complications of diabetic retinopathy.

1. Introduction

Diabetic retinopathy (DR) is the most common cause of blindness among the working age group people in the US and around the world. DR has long been considered as a microvascular disease associated with vessel basement membrane thickening, blood retinal barrier breakdown, capillary cell death, acellular capillary, neovascularization, and retinal detachment [1]. However, recent studies have demonstrated that DR is a neurovascular disease that affects both the blood vessel and neuroglia [2, 3]. Chronic hyperglycemia-associated oxidative stress and low-grade inflammation are considered to play critical roles in disease initiation and progression of diabetic complications including DR [4–6]. Yet, the molecular mechanisms underlying hyperglycemic injury and DR pathogenesis are poorly understood. Various glucose metabolic defects and abnormal biochemical pathways are activated in diabetes and under chronic hyperglycemia [7]. Recently, we demonstrated that thioredoxin interacting/inhibiting protein (TXNIP) is significantly increased both in the diabetic rat retina *in vivo* and *in vitro* in retinal endothelial cells in culture and causes pro-inflammatory gene expression for Cox-2, VEGF-A, ICAM1, RAGE, and sclerotic fibronectin (FN) [8–10]. Knockdown of TXNIP

by siRNA in the retina prevents early abnormalities of DR in streptozocin-induced diabetic rats, which include inflammation, fibrosis, gliosis and apoptosis [9, 10].

TXNIP is an early response gene highly induced by diabetes and hyperglycemia [8, 11, 12]. TXNIP was initially identified as one of the proteins that interacts with thioredoxin (TRX) and blocks its thiol reducing function [13, 14]. TRX is a 12-kDa protein, which scavenges ROS and maintains protein cysteine sulfhydryl groups by its redox-active disulfite/dithiol sites. Recent findings further demonstrate a potential role for TXNIP in innate immunity via the NOD-like receptor-NLRP3/caspase-1 inflammasome activation and release of IL-1β in diabetes and oxidative stress [15, 16]. TXNIP interacts with NLRP3 leading to a multiprotein NLRP3 complex assembly and autoactivation of caspase-1. Activated caspase-1 in turn processes pro-IL-1β to its mature and active form leading to other pro-inflammatory gene induction and inflammation [17, 18]. Innate immune receptors TLR4 and RAGE and their endogenous ligands such as high mobility group binding protein HMGB1 and S100 calgranulins are reported to be upregulated in diabetes [19, 20]. Currently, NF-κB is the only pro-inflammatory transcription factor that is implicated in retinal inflammation in diabetes [3, 4, 8, 9] while the role of other factors is undetermined. Innate immune receptors signal through activation of NF-κB and AP1 transcription factors to induce the expression of various pro-inflammatory cytokines and chemokines [4–6].

We recently showed that TXNIP mediates IL-1β expression in primary Schwann cell *in vitro* as well as *in vivo* in partial sciatic nerve injury [10] by the p38 MAPK/NF-κB pathway and transcription factor CREB. Similarly, TXNIP was also shown to be responsible for pro-IL-β expression in adipocytes by high glucose [21] and HG-mediated NLRP3 inflammasome and casapse-1 activation leads to IL-1β maturation [15, 16, 21]. In addition, chronic hyperglycemia may also evoke other innate host defense/survival mechanisms such as ER stress/unfolded protein response (UPR) and hypoxic responses for cell viability in an inflamed and stressful environment [22, 23]. In this regard, chronic hyperglycemia-associated TXNIP expression could potentially lead to thiol oxidation and misfolded protein accumulation in ER lumen and cause ER stress in diabetes. UPR is a highly regulated intracellular signaling pathway that prevents aggregation of misfolded proteins. ER stress is known to be mediated by three UPR branches, namely, the IRE1-XBP1, PERK-eIF-1α, and ATF6 pathways [22, 23]. These UPR signals provide an adaptive mechanism for proper protein folding and processing in the ER and establishment of tissue homeostasis and cell survival. However, when protein misfolding is not resolved, the ER stress and UPR trigger an apoptotic signal to remove demised cells by phagocytes. Therefore, ER stress may play a critical role in deciphering cell survival and premature demise in chronic disease and injury including DR.

Currently, it is recognized that molecular abnormalities of DR begin early before clinically detectable pathologies appear in the retina [2–4, 24]. The pathologies once set in motion are not reversed even after glucose normalization [25, 26]. Therefore, the detection of molecular abnormalities and cellular processes that ultimately lead to later pathologies of DR is of utmost importance in developing therapeutic strategies to prevent blinding ocular complications of diabetes. Retinal Muller glia, blood vessels, and neuronal (ganglion) cells interact closely to maintain retinal tissue homeostasis and cell survival [27]. Muller cells are the primary glial type in the retina and along with microglia and astrocytes are considered as resident innate immune cells. Under stress, they become activated and produce pro-inflammatory cytokines and growth factors to restore tissue homeostasis [6]. However, in chronic diseases such as DR, retinal gliosis is prolonged, causing sustained inflammation, cell injury/death, and worsening disease. Nonetheless, a role for TXNIP in Muller glia reactivity, innate immune response, ER stress, and inflammation under chronic hyperglycemia and diabetes has not been investigated before. In this study, first, we demonstrate that TXNIP is significantly induced in the diabetic rat retina *in vivo* and mediates pro-inflammatory IL-1β expression and the induction of radial glial fibrillary acidic protein (GFAP) indicating Muller cell activation. Secondly, we show that chronic hyperglycemia sustains TXNIP up-regulation in Muller glia in *in vitro* culture and orchestrates a temporal program of innate host defense mechanisms that lead to cellular oxidative stress, ER stress, inflammation, and autophagy/apoptosis. These findings provide important insights as to how retinal Muller glia might respond to chronic hyperglycemia in diabetes and that TXNIP may play a critical role in glial dysfunction and disease progression in DR.

2. Materials and Methods

2.1. Materials. Tissue culture media, serum and antibiotics were purchased from Invitrogen (Carlsbad, CA). Antibodies for TXNIP and beclin 1 were obtained from MBL (Woburn, MA). Anti-S-Nitroso-Cysteine (SNO-Cys) antibody was from Sigma-Aldrich (St. Louis, MO). The LC3B antibody kit for autophagy (Cat number L10382), Mito-Tracker Red CMXROS (M7512), ROS detection reagent CM-H2DCFDA (C6827), and ATP assay kits were from Molecular Probes (Invitrogen). Antibodies for pro-IL-1β, pro-caspase-1, NLRP3 (cryopyrin), and HMGB1 are from Santa Cruz Biotechnology (Santa Cruz, CA), and VDUP1 (TXNIP), tubulin, and Actin were from Abcam (Cambridge, MA). Anti-p65 NF-κB and phospho-p65 NF-κB antibodies were from Millipore (Invitrogen). Electrophoresis Mobility Shift Assay (EMSA) kit for Transcription factors XBP1 and HIF-1α to consensus DNA was custom-made by Signosis Inc. (Sunnyvale, CA). Fluorescent-labeled secondary antibodies anti-rabbit and anti-mouse were obtained from Molecular Probes while those of the anti-goat antibodies were purchased from Abcam (Cambridge, MA). Active caspase-1 (Green FLICA Caspase-1) and Caspase-3 (Green FLICA caspase-3 & 7) were purchased from Immunochemistry Technologies (Bloomington, MN). Predesigned TXNIP siRNAs were purchased from Qiagen (SABiociences). TRIZOL for RNA isolation was from Invitrogen and PCR primers were also synthesized by Invitrogen or Applied Biosystems. First strand cDNA

synthesis kit and SYBR green reagents were purchased from Biorad as well as from Applied Biosystems. Primer sequences are available upon request.

2.2. Diabetes Induction of Rats. Diabetes of adult male Sprague-Dawley rats (~275 g) was induced by intraperitoneal injection of a single dose of streptozotocin, (STZ, 65 mg/kg body weight, Sigma) dissolved in 0.01 M citrate buffer, pH 4.5 as described recently [8, 9]. The rats were treated in accordance with the principles of NIH guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Intravitreal injection of siRNA targeted to rat TXNIP promoter (transcriptional gene silencing, RNAi TGS) has been described before [9] and performed in anaesthetized rats with 40 mg/kg body weight (pentobarbital) on the right eyes of diabetic rats (Treatment) and a similar volume of scramble-RNA on the left eye (Control). The injections were performed twice at day 23 and 27 and they were sacrificed at day 30. An overdose of pentobarbital (200 mg/kg weight) was given to euthanize the rats. The retina were removed, processed for immunohistological analysis or frozen immediately in liquid N_2, and stored at –80°C until used.

2.3. Cell Culture. We used a well-established rat retinal Muller cell line (rMC1) [28]. rMC1 cells were cultured in medium containing DMEM (low glucose, 5.5 mM) and Ham's F-12 (3 : 1 ratio) supplemented with 5.0% fetal calf serum (FCS) and 0.5 mg/mL gentamicin at 37°C in a humidified chamber with a 5% CO2–95% air mixture [9, 29]. Cells at 70–80% confluence were replaced with low serum overnight (0.2% serum medium). For the time course, HG (high glucose, 25 mM) with 24 h treatment was added first; then on the second day HG was added at 4, 2 h, and 0 (no addition) to respective cultures before harvesting. Thus, all cells maintain a similar condition and length of serum-deprivation during the entire experimental procedure. Similarly, for long-term cultures also, HG was added first to the 5-day culture, then after two days for 3 days, 1 day, and 0 before harvesting. Media were changed every 48 h. Cells were harvested by scrapping, snap frozen and stored at −80°C until used.

2.4. SDS-PAGE and Western Blotting. Proteins were extracted in RIPA buffer containing protease inhibitors and their concentrations were determined using a Coomassie Plus (Bradford) Assay Reagent from Pierce (Product number 23238) with BSA as the standard. Absorbance of blue dye of the Coomassie-protein complex was measured at 595 nm using a Gemini Microplate reader (Molecular Devices, Sunnyvale, CA). Thirty micrograms of protein extracts was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDA-PAGE) and Western blot analysis of proteins was performed as described previously [30]. Santa Cruz antibodies were used at 1 : 200 dilutions while antibodies from other sources were diluted at 1 : 1000 dilutions. Secondary antibodies were used at 1 : 3000 dilutions. ECL used to detect the immunoreactive bands.

2.5. Real-Time Quantitative PCR. Total RNAs were isolated by TRIZOL method and first-strand cDNAs (from 1 μg RNA) were synthesized in 20 μL volume using the Bio-Rad iScript cDNA synthesis kit [8, 12]. Messenger RNA expression was analyzed by real-time quantitative PCR using the Bio-Rad Chromo 4 detection system and SYBR Green PCR Master Mix from Bio-Rad or Applied Biosystems (Foster City, CA). Primers were designed using Primer Express v 2.0 (Applied Biosystems) and synthesized by Invitrogen (Carlsbad, CA). Primers for the real-time Q-PCR will be available upon request.

The real-time PCR reaction mixture contained 1X SYBR Green PCR Master Mix, 400 nM forward and reverse primers, and 2 μL cDNA in a final volume of 25 μL. The PCR cycling was programmed as 95°C for 15 s, 55°C for 30 s and 72°C for 30 s 40 cycles followed by the construction of a melting curve through increasing the temperature from 60°C to 95°C at a ramp rate of 2% for 20 min. The real-time PCR samples were evaluated using a single predominant peak as a quality control. Ct values were used to calculate the relative expression level of mRNAs that were normalized to actin [8, 12].

2.6. Determination of Cell Viability. We used MTT assay to measure cell viability at different time periods after HG addition. MTT assay for cell viability was performed in 48 well plates and with 0.5 mg/mL MTT in each well as previously described in our laboratory [31, 32]. rMC1 cells (1×10^4 cells) were grown to 70% confluence and serum-starved overnight and treated with HG. MTT was added for 3 h at the end of the experiment, the media was removed, and cells were kept in 100 μL of DMSO for 10 minutes. The resulting color was diluted with 500 μL of distilled H_2O and detected at 570 nm using a Gemini Microplate reader (Molecular Devices, Sunnyvale, CA).

2.7. Intracellular Reactive Oxygen Species (ROS) Measurement. The formation of intracellular ROS in rMC1 cells was detected as described before [8, 12] by using the fluorescent probe, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA). This dye can enter living cells by passive diffusion it is nonfluorescent until the acetate group is cleaved off by intracellular esterase, and oxidation occurs within the cell. Approximately 1×10^5 cells/mL were cultured in 24 well plates, serum-starved overnight, and glucose was added for the specified time period. Then, CM-H_2DCFDA (10 μM) was incubated for 60 min at 37°C. The medium with the dye is aspirated (to remove the extracellular dye), washed with PBS (3x), and then the PBS is added to cells. The fluorescence was measured in a Gemini Fluorescent Microplate Reader (Molecular Devices) with the bottom read scanning mode at 480 nm excitation and emission at 530 nm.

2.8. Immunohistochemistry (IHC)

(i) Retinal Tissue. Immunohistology of retinal sections was similar to those described recently [9]. The retinas were fixed

in the eyecups with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 min. The retinas were cryoprotected in a sucrose gradient (10%, 20%, and 30% w/v in PB, resp.). Cryostat sections were cut at 16 μm in Tissue Tek (OCT mounting medium). Retinal sections were blocked for 1 h in a PB solution that contained 5% Chemiblocker (Chemicon, Temecula, CA), 0.5% Triton X-100, and 0.05% sodium azide. The primary antibodies were diluted in the same solution (1 : 100 to 1 : 200 dilution depending on the antibody) and applied overnight at 4°C followed by appropriate secondary antibodies (1 : 600 dilutions) conjugated with Alexa Fluor 488 or Alexa Fluor 594 for 2 hours at room temperature. After washing with PB again, rMC1 cells were mounted with an aqueous mounting medium with anti-fade agent with DAPI and sealed with nail polish. The images were captured by an OLYMPUS BX 51 fluorescence microscope, which is fitted with a triple DAPI/FITC/TRITC cube, a DP70 digital camera, and image acquisition software. Some images were also captured with a Zeiss Apotome microscope with Z-section (Zeiss, Oberkochen, Germany). Similar magnification (400x) and exposure time were maintained throughout for comparing images unless otherwise mentioned.

(ii) rMC1 Cells. Cells were grown either in four-chambered tissue culture glass slides (NUNC, Naperville, IL) and exposed to HG for 5 days as described [32]. Cells were fixed with freshly prepared paraformaldehyde (4%) for 2 h in ice or 4°C overnight, washed 10 min each with PB (3 times), and blocked with 5% horse serum in PB for 1 h at room temperature. Following a 30-minute wash with PBS, cells were incubated with primary antibodies (1 : 100 dilutions) overnight at 4°C in a humidified chamber. After washing with PB, cells were further incubated with corresponding secondary antibodies conjugated with Alexa Fluor 488 or Alexa 594 at 1 : 500 dilutions for 1 h at 37°C in a darkened humidified chamber. The cell-associated fluorescence was observed in Olympus BX51 fluorescence microscope.

2.9. Cytochrome c Oxidase and ATP Assays. The mitochondrial oxidative phosphorylation (OxPhos) process consists of the electron transport chain (ETC) and ATP synthase, and it provides more than 90% of cellular energy. The terminal enzyme of ETC is cytochrome *c* oxidase (CcO). CcO transfers electrons from cytochrome *c* to oxygen, which is reduced to water. In intact mammalian cells, this reaction is the proposed rate-limiting step of the ETC under physiological conditions [33]. Therefore, CcO is an ideal marker enzyme of OxPhos and was analysed in this study.

(i) CcO Activity. The oxygen consuming capacity of CcO-analyzed in a closed chamber equipped with a micro-Clark-type oxygen electrode (Oxygraph system, Hansatech, Norfolk, England) as previously described [34, 35]. Briefly, frozen cells were solubilized in 10 mM K-HEPES (pH 7.4), 40 mM KCl, 1% Tween 20, 1 μM oligomycin, 1 mM PMSF, 10 mM KF, 2 mM EGTA, and 1 mM Na vanadate. CcO activity was measured in the presence of 20 mM ascorbate and by addition of increasing amounts of cow heart cytochrome *c*. Oxygen consumption was recorded on a computer and analyzed with the Oxygraph software. Protein concentration was determined with the DC protein assay kit (Bio-rad). CcO-specific activity is defined as consumed O_2 (μM)/min/mg total protein.

(ii) ATP Assay. Determination of ATP levels using the bioluminescent method was performed as described previously [34, 35]. ATP was released from rMC1 cells maintained in 24 well plates using the boiling method. Two hundred fifty μl TE buffer (50 mM Tris-Cl, pH 7.4, 4 mM EDTA) was added to the wells and scrapped off. Cells were immediately transferred to a boiling water bath for 4 min. Samples were put on ice and sonicated briefly and centrifuged. ATP concentration was determined in the supernatant with an ATP bioluminescence assay kit (Invitrogen) according to the manufacturer's manual. Relative fluorescence units (RLUs) were detected in a Luminometer (Promega, Madison, WI). Experiments were performed in triplicates for each condition and data were standardized to the protein concentration using the Biorad protein assay kit.

2.10. Gel-Shift Assay. The gel-shift or electrophoretic mobility-shift assay (EMSA) provides a simple and rapid method for detecting DNA-binding activity of proteins. We investigated the sXBP1 and HIF-1α activity of rMC1 nuclear proteins by using a commercially available gel-shift assay kit from Signosis (Sunnyvale, CA) according to manufacturer's instructions and as described before [32]. After incubation at 16°C for 30 min with biotin-labeled probes and nuclear extracts (5.0 μg protein) in a PCR machine, the protein-DNA complexes were subjected to 6.5% nondenaturing polyacrylamide gels prepared with TBE gel formulation [32]. The gels were run at 100 V for 1 h until the Bromophenol blue dye front is three quarters down the gel, using 0.5x TBE running buffer. For competitive assays, excess cold probes were added to the reaction. Streptavin-HRP and ECL were used to detect and capture the reactive bands using a Cell Bioscience FluorChem E System (Santa Clara, CA).

2.11. Statistical Analysis. Results are expressed as means +/− SE. Student's *t*-test or one-way ANOVA followed by Bonferroni Post Hoc Test was used to compare differences between treatment conditions [8, 9]. A preset P value of <0.05 was considered statistically significant.

3. Results

3.1. Diabetes Induces TXNIP Expression, Muller Glia Reactivity, and Proinflammatory Gene Expression in the Rat Retina. We have recently shown that TXNIP expression is increased in the retina of STZ-induced diabetic rats at both 4 and 8 weeks of diabetes duration [8, 9] and mediates inflammation, gliosis/fibrosis, and apoptosis of retinal cells. In this study, we further support our previous findings by showing that the expressions of TXNIP, pro-inflammatory IL-1β, iNOS, and pattern recognition receptors TLR4 and P2X7R in the retina are elevated at 4 weeks of diabetes (Figure 1(a)).

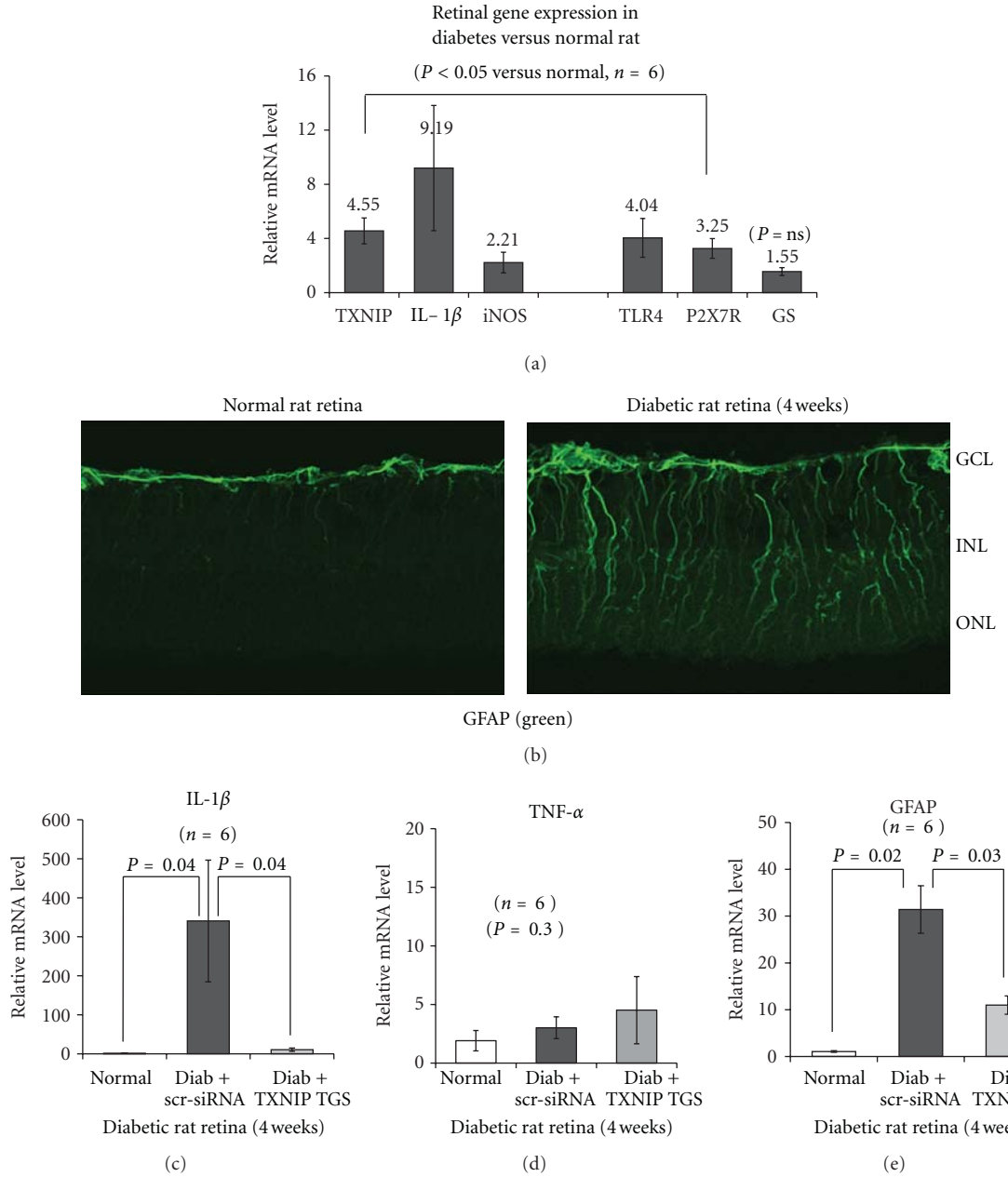

Figure 1: Diabetes induces TXNIP and pro-inflammatory gene expression and glia reactivity in the rat retina. (a) Messenger mRNA levels for TXNIP (4.55-fold ± 0.96) and pro-inflammatory IL-1β (9.19 ± 4.62), iNOS (2.22 ± 0.76), and pattern recognition receptors TLR4 (4.04 ± 1.43) and P2X7R (3.26 ± 0.73) are increased significantly ($P < 0.05$, $n = 6$) in the retina of diabetic rats (4 weeks) when compared with the normal retina. GS is marginally increased (1.55 ± 0.29) but not significant ($P = 0.3$). (b) GFAP staining, a marker of gliosis, is also increased radially throughout the neuroretina in the diabetic rat versus the normal retina, suggesting Muller glia activation. GCL: ganglion cell layer; INL: inner nuclear layer; and ONL: outer nuclear layer. (c–e) TXNIP knockdown by siRNA targeted to the promoter (RNAi TGS, [9]) reduces IL-1β and GFAP mRNA levels in the diabetic retina as compared to the scr-siRNA-treated diabetic rat retina. Under these conditions (4 weeks of diabetes induction in rats), we did not see an increase in retinal TNF-α mRNA level.

On IHC, we observed that the increased pro-inflammatory gene expression is associated with enhanced GFAP staining, a marker for glia activation, in the entire length of the neural retina from diabetic rats when compared with the nondiabetic normal rat retina (Figure 1(b)). Specifically, in the normal rat retina, GFAP staining is restricted to the inner limiting membrane of Muller glia end-feet and ganglion cell layer (GCL), however, in the diabetic rat retina, GFAP filaments extend from GCL to inner nuclear (INL), outer nuclear layer (ONL), and the inner and outer plexiform layers suggesting retinal injury and inflammation. Knockdown of TXNIP by intravitreal injection of promoter-targeted siRNA (RNAi TGS) further reduces IL-1β and GFAP up-regulation significantly ($P < 0.05$, $n = 6$) in

the diabetic rat retina (Figures 1(c) and 1(e)). Under these conditions, we did not observe an enhancement of TNF-α, another pro-inflammatory cytokine, in the diabetic retina (Figure 1(d)). TNF-α has previously been shown to induce at early at 1-2 weeks of STZ-induced diabetic rat retinas and then suppresses until late stages of diabetes duration where it may play a critical role in retinal inflammation and BRB breakdown [26]. Thus, the results demonstrate that there is an early inflammatory response and gliosis in the diabetic retina. Retinal Muller glia forms a close association with neuron and blood vessel and plays a critical function in maintaining retinal homeostasis in health and disease. Therefore, we propose that Muller glia reacts to retinal injury both in retinal vessels and neurons in diabetes and produces pro-inflammatory and survival factors to restore tissue homeostasis.

3.2. HG Induces TXNIP Expression and Innate Immune Responses in rMC1 in Culture. We have shown previously that TXNIP expression and Muller glia activation occur early in the diabetic retina [9]. So far, a role for TXNIP in Muller cell activation and pro-inflammatory gene induction under HG exposure has not been investigated. Therefore, we undertook a series of experiments to examine the temporal response of rMC1 to chronic hyperglycemia in culture. We show that HG exposure in rMC1 induces a significant ($P < 0.05$, $n = 6$) increase in TXNIP mRNA expression (2–24 h) when compared with low glucose (5.5 mM, LG) indicated as time 0 control (Figure 2(a)). We have previously shown that TXNIP mediates pro-IL-1β expression in primary Schwann Cells via the NF-κB-dependent pathway [10]. Therefore, we examined if TXNIP expression correlates with pro-IL-1β induction in rMC1 under HG. As shown in Figure 2(b), HG increases IL-1β mRNA expression at 2 and 4 h ($P < 0.01$) and then reduces at 24 h to the level of the control at 0 time. Furthermore, nuclear NF-κB level is also enhanced by HG in rMC1 from 2 to 24 h (Figure 2(c)), which correlates with activation of caspase-1 in rMC1 (Figure 2(d)). Active caspase 1 is responsible for processing pro-IL-1β (34-kDa) to an active mature form of IL-1β (17-kDa).

We further examine whether the protein levels of TXNIP and pro-IL-1β protein are induced by HG and if the levels of NLRP3 and caspase-1 are important for processing pro-IL-1β to its mature form in a time-dependent manner. TXNIP expression is low in rMC1 under LG while HG upregulates TXNIP (~4-folds) significantly from 2 h to 5 days (Figures 3(a) and 3(c)). Nuclear level of phosphorylated p65 subunit of NF-κB increases at 2 and 4 h but reduces at 24 h (Figure 3(b)); however, it elevates again at day 2 through day 5 (Figure 3(d)). Pro-IL-1β is also increased at 2 and 4 h of HG exposure and reduces at 24 h, and again induces at 3 days of HG (Figures 3(c) and 3(d)), which correlates with NLRP3 expression. Procaspase 1 level is lower at 2 and 4 h and upregulated at 24 h (Figure 3(c)). This is in agreement with the increased level of active caspase-1 at 24 h shown in Figure 2(d). Pro-caspase-1 level rises again at 5 days of HG exposure (Figure 3(d)). The results demonstrate that TXNIP is a glucose-sensitive gene and its expression remains upregulated when hyperglycemia persists. On the other hand, the innate immune response to chronic hyperglycemia by IL-1β, NLRP3 and caspase-1 inflammasome is cyclical.

3.3. Chronic Hyperglycemia Induces ROS Generation and Reduces Cellular ATP in rMC1. We have observed previously that HG sustains TXNIP and induces IL-1β expression. TXNIP is a pro-oxidative stress protein through its interaction/inhibition of the thiol reducing activity and ROS scavenging capacity of TRX. In addition, extracellular ATP release has been shown to involve in NLRP3 inflammasome assembly, and IL-1β processing. Therefore, we examined a time-dependent generation of intracellular ROS and ATP as well as extracellular ATP release in rMC1 upon HG exposure. HG significantly reduces ROS levels early at 4 and 24 h ($P < 0.05$ versus LG, $n = 6$) and then rises at day 2 though not significant (Figure 4(a)). However, at day 3 of HG exposure, rMC1 cells produce significantly higher levels of ROS ($P < 0.05$, $n = 6$) than LG and sustain up to day 5.

Excess nutrient (glucose availability) could enhance glucose metabolic flux through glycolysis and mitochondrial OxPhos via the electron transport chain (ETC) and ATP production. Therefore, we measured temporal changes in intracellular ATP levels in rMC1 after HG exposure. As shown in Figure 4(b), the intracellular ATP concentration is significantly ($P < 0.05$, $n = 4$) enhanced at 4 h and reduces at 24 h when compared with time 0 control. The reduction in ATP persists at day 2 through 5 ($P < 0.01$ versus time 0 control, $n = 3$). At day 3 and 5 of HG, the level of ATP is further reduced to ~65% of the control ATP level at LG (Figure 4(c)). In case of the extracellular ATP level, there is a small but significant increase at 4 and 6 h and then at day 1 onwards their levels are reduced though not significant (unpublished data). These results indicate that an initial increase in ATP generation via the mitochondrial OxPhos may result in ROS generation. Initially, this may induce an antioxidant response leading to a reduction in ROS level. However, as hyperglycemia persists, ROS overwhelms the anti-oxidant capacity.

3.4. Mitochondrial Electron Transport Chain (ETC) Protein Cytochrome c Oxidase Activity (CcO) and MTT Cell Viability Are Maintained under HG. As described previously, ROS generation and ATP reduction occur in rMC1 cells under chronic HG. Therefore, the mitochondrial ETC function, especially the oxygen consumption capacity of CcO activity at Complex IV, may be reduced. We measure the O_2 consuming capacity of CcO in an *in vitro* assay using a closed oxygen chamber. In contrast to our prediction, the CcO activity is unaffected by chronic HG exposure of rMC1 (Figures 5(a) and 5(b)). In addition, we did not observe an initial sigmoidal rate of the CcO activity using different protein concentrations of cytochrome c in the O_2 consuming assay, indicating that, under these experimental conditions, CcO may not be regulated by posttranslational modification as previously described [34].

We next examined whether cell viability is reduced by ROS and ATP in rMC1 cells using MTT assay. An increase in MTT absorbance measures the reductase activity of mitochondrial succinate dehydrogenase at complex II, which

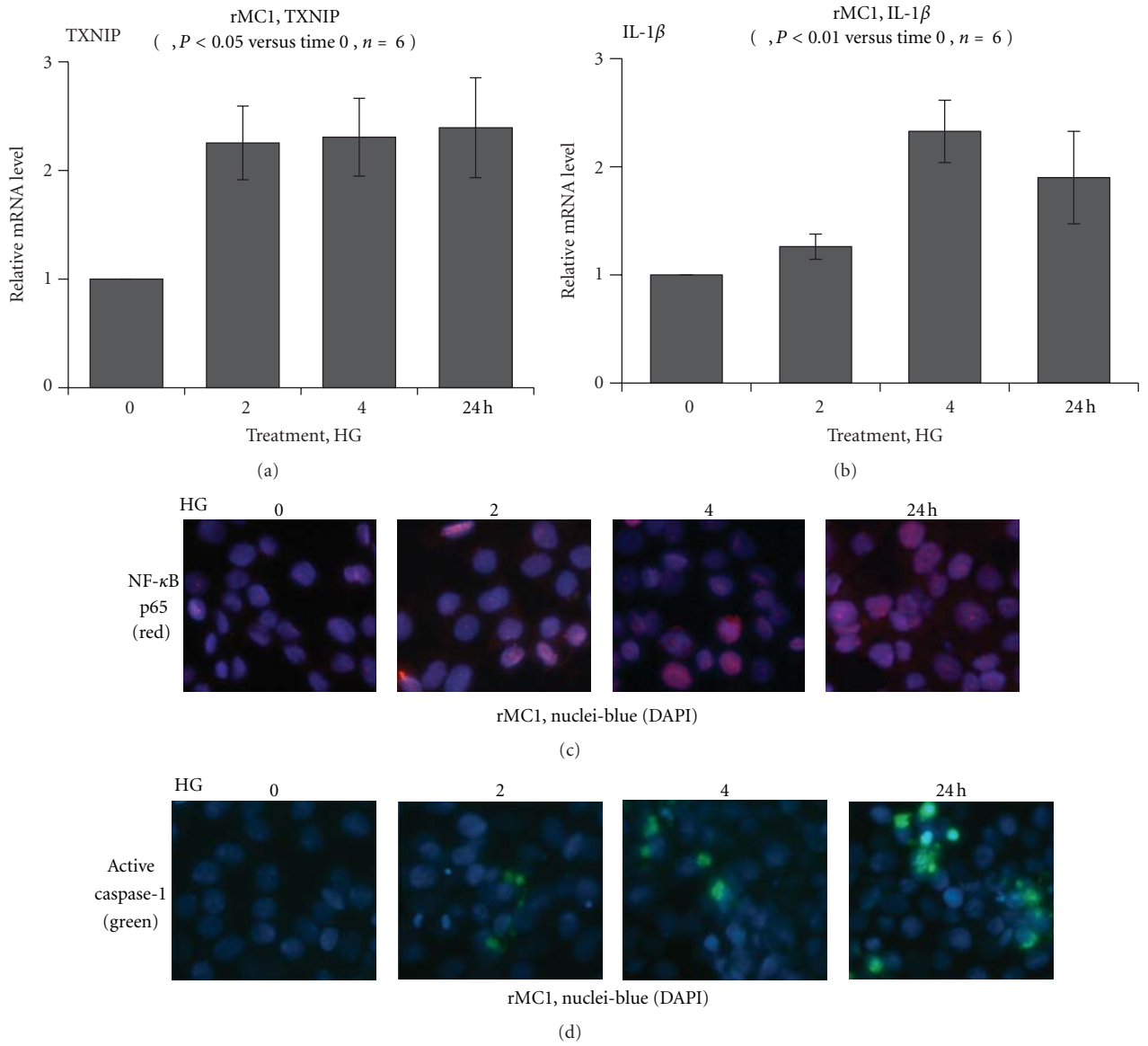

Figure 2: HG induces TXNIP expression and evokes the host innate immune response in rat Muller glia. Quantitative RT-PCR for (a) TXNIP and (b) IL-1β mRNA expression in rMC1 is shown. HG increases TXNIP mRNA at 2–24 h ($P < 0.05$ versus time 0, $n = 6$) while pro-IL-1β mRNA is increased at 2 and 4 h ($P < 0.05$ versus time 0, $n = 6$) and then reduces at 24 h. (c) IHC detects a time-dependent accumulation of the p65 subunit of NF-κB in the nucleus in rMC1 under HG. (d) Active caspase-1 staining is increased in rMC1 using the caspase-1 FLICA probe. A representative of $n = 3$ is shown here.

is frequently used to monitor cell viability. As shown in Figure 5(c), MTT activity is not affected by HG exposure up to 2 days and increases at day 3 through 5 ($P < 0.01$, $n = 6$). These results suggest that rMC1 maintains a functional mitochondria and viability in spite of the ROS generation and ATP depletion. To further ascertain that HG maintains rMC1 viability under increased ROS, ATP release, and IL-1β expression, we treated rMC1 cells with exogenous H_2O_2 (0.2 mM), ATP (0.1 mM), and IL-1β (10 ng/mL) with or without HG. We observed that H_2O_2, ATP, and IL-1β increase ROS levels significantly ($P < 0.05$, $n = 6$) at 4 to 96 h and reduce cell viability as indicated by reduced MTT levels under LG at 48 h (unpublished data). However, when HG is added, the effect of H_2O_2, ATP, and IL-1β on MTT activity is nullified. This occurs even when HG increases the generation ROS further with H_2O_2, ATP, or IL-1β in combination. Therefore, these findings suggest that rMC1 activates a cellular defense and survival mechanism(s) under HG and oxidative stress, and TXNIP may play a role in this process.

3.5. HG Increases Autophagy/Mitophagy, Inflammation, and Apoptosis in rMC1 Cells. We observed that HG increases TXNIP expression and ROS generation and sustained ATP reduction in rMC1 cells. Nonetheless, the mitochondrial CcO enzyme activity and cell viability are maintained.

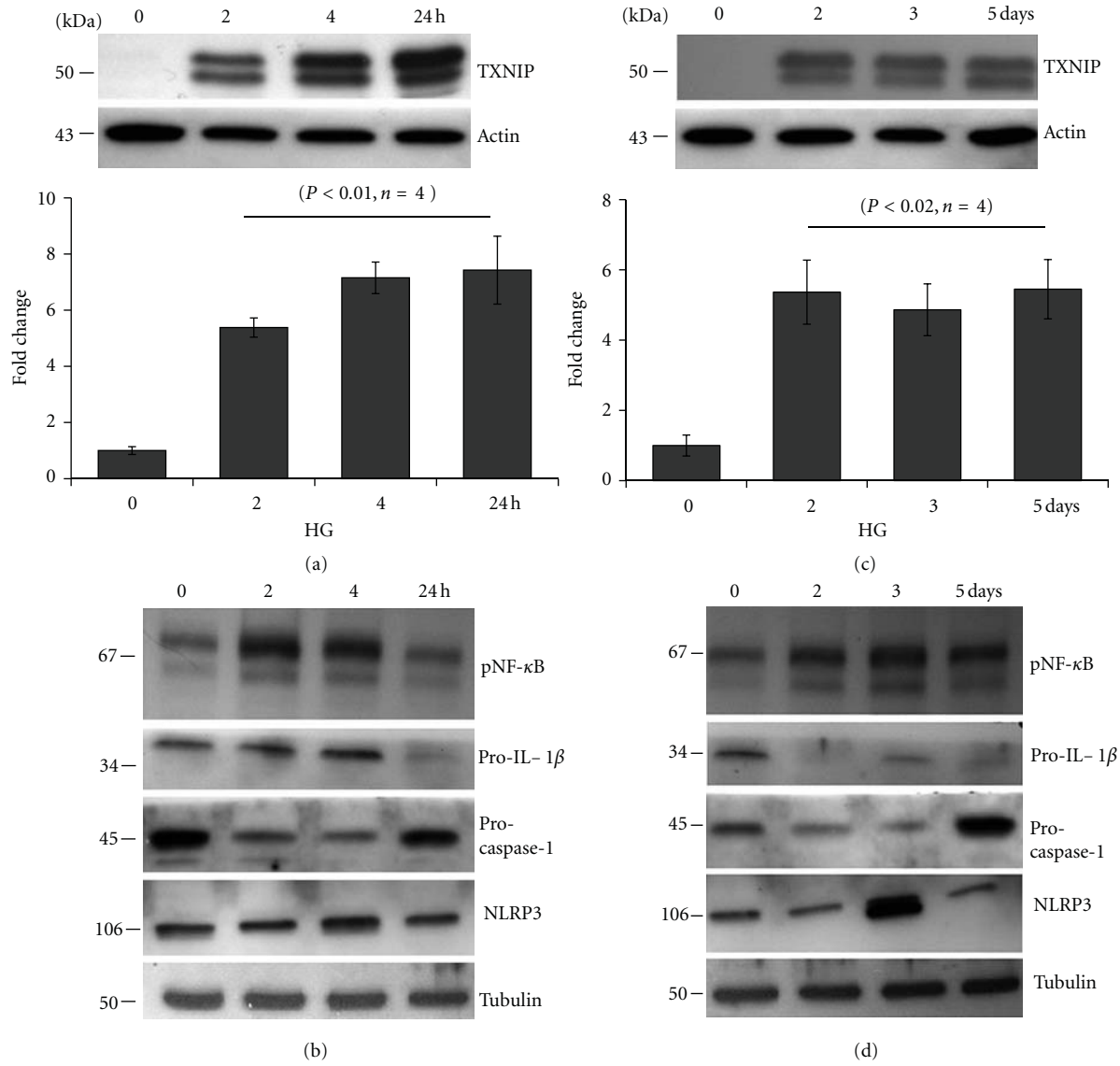

Figure 3: Chronic hyperglycemia persistently upregulates TXNIP expression in rMC1 and induces IL-1β and NLRP3 inflammasome activation. rMC1 cells were cultured under HG for (a and b) 0–24 h or (c and d) 0–5 days and TXNIP proteins were detected by Western blotting. For this, cell extracts were prepared in RIPA buffer and 30 μg protein was analyzed on 12% SDS-PAGE and Western blot for cytosolic TXNIP, Pro-IL-1β, NLRP3, and pro-caspase-1 and the nuclear level of phosphorylated p65 at serine residue 276 (S276) of NF-κB. ECL detected the immunoreactive bands. Actin and tubulin were used as controls for protein loading. A representative blot for each protein is shown here from n = 3-4.

Therefore, we hypothesize that a cell survival program is evoked under chronic hyperglycemia and oxidative stress in rMC1. Excess ROS production causes protein misfolding/aggregation and organelle damage (e.g., mitochondria). Autophagy is a cellular survival mechanism for long-lived fully differentiated cells and for phagocytes under cellular stress to remove damage organelles and their regeneration [36, 37]. Beclin 1 is an autophagy initiating protein that interacts with prosurvival bcl2 and bcl-xL [38]. Under oxidative stress, beclin 1 is released from bcl2 and activates the autophagic process, which further activates the late phage autophagosome marker, light chain LC3B [39]. Therefore, we examined beclin 1 expression in rMC1 under chronic hyperglycemia at day 3 and 5. As shown in Figure 6(a), beclin 1 staining is marginally increased in rMC1 under HG than in LG, however there are more beclin 1 punctae in the cytosol and over the nuclei suggesting activation of an autophagic process. This beclin 1 puncta is not seen up to day 3 (not shown).

Next, we used a mitochondrial staining dye, MitoTracker red, to examine the mitochondrial morphology. We observed an enhanced mitochondrial staining as well as the formation of larger mitotracker puncta (Figure 6(c)), indicating that autophagy/mitophagy may occur in rMC1 under chronic hyperglycemia. Subsequently, using an autophagy assay kit for LC3B staining, we show that HG increases the number

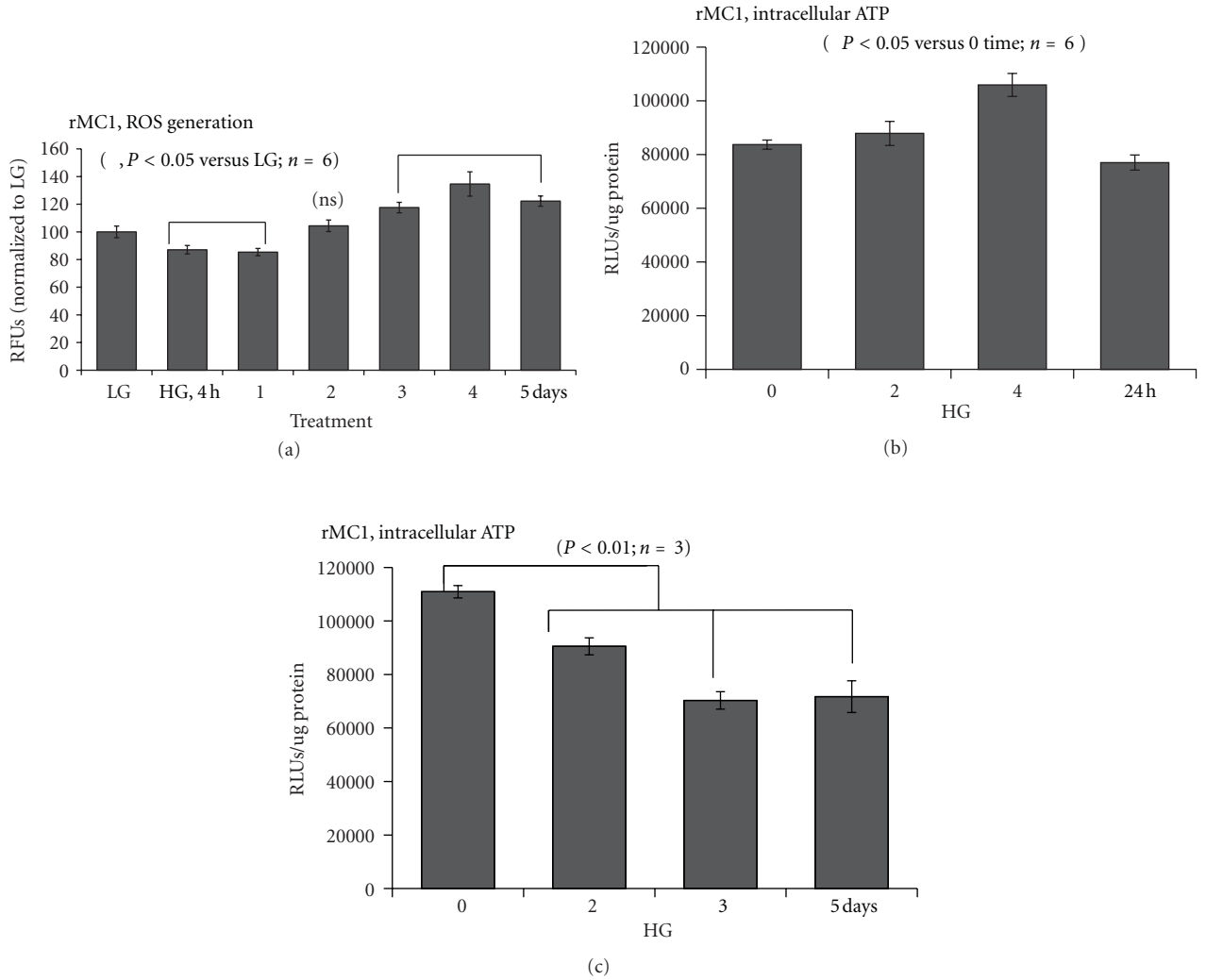

Figure 4: Time-dependent effect of HG on ROS and ATP generation in rMC1. (a) Reactive oxygen species (ROS) generation was measured using a fluorescence probe, CM-H2DFA. ROS level is reduced at both 4 and 24 h significantly ($P < 0.05$, $n = 6$) but rises at day 3 to 5 ($P < 0.05$, $n = 6$). (b-c) ATP level was determined in rMC1 cells by a Renilla-based luminescence assay kit (Invitrogen). Intracellular ATP is increased at 4 h ($P < 0.05$, $n = 6$) and reduces at 24 h as well as at day 2 through 5 ($P < 0.01$, $n = 3$). However, at day 3 and 5, the level of ATP is further reduced to ~65% of the control.

and size of LC3B puncta formation (LC3BII, active form) in rMC1 when compared with LG (Figure 6(d)). Smaller LC3B punctae are also present in rMC1 after 5 days of LG under low serum medium. In addition, we observed that at 8 weeks of diabetes, formation of LG3B puncta occurs in the retina and they colocalize with TXNIP (unpublished data).

The mechanism of autophagy/mitophagy initiation under chronic hyperglycemia, TXNIP, and ROS is not fully understood at the present time. However, the thiol oxidation of the nuclear high mobility group box protein 1 (HMGB1) under oxidative stress has been implicated in its nuclear export and cytosolic interaction with beclin 1 to release from bcl2 and to initiate an autophagic process [39]. Therefore, we examined levels of TXNIP and HMGB1 in the nucleus and cytosol. We observed that TXNIP expression in the nucleus is also increased in rMC1 under HG (unpublished data) in addition to their cytosolic expression as observed in Figure 3. HMGB1 levels, both in the nucleus and in the cytosol, are not altered at 0–24 h but decrease at day 2 and 3 in the cytosol and then increase again at day 5. Furthermore, we observed that protein cysteine (thiol) nitrosylation (SNO) is enhanced in HG as measured by an anti-SNO antibody on IHC, and SNO colocalizes with HMGB1 (unpublished data). In addition, beclin 1 and HMGB1 colocalize in the cytosol. These results show that autophagy/mitophagy is activated in rMC1 under HG and that beclin 1 and oxidized HMGB1 may play a role in initiating the process.

Autophagy/mitophagy is initially a mechanism for cell survival; however excessive and continuous autophagy may remove vital proteins and organelles and induce cell apoptosis [36, 37]. Therefore, we examined whether proapoptotic caspase-3 is activated under chronic HG in rMC1. As shown in Figures 7(a) and 7(b), HG increases TXNIP and caspase-3 staining in rMC1 when compared with LG, suggesting

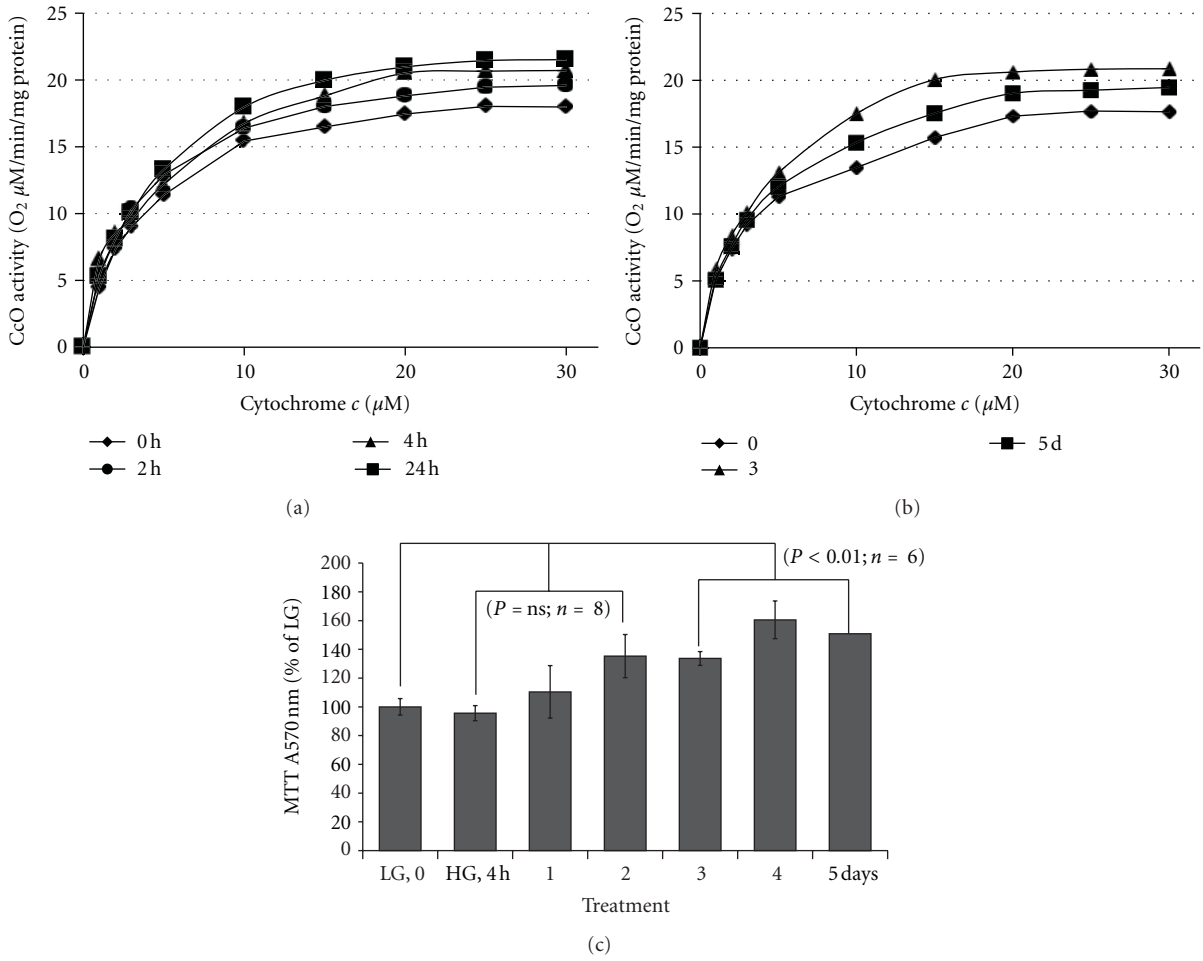

Figure 5: HG effects on mitochondrial electron transport chain (ETC) enzyme activity in rMC1. (a-b) Cytochrome c oxidase (CcO) activity was determined by increasing the amount of substrate cytochrome *c* in a polarographic method as described in Methods. CoO activity is defined as consumed O_2 [μM]/min/protein [mg]. Oxidative metabolism was increased after incubation in high glucose medium as was seen by an incremental increase of CcO activity at 24 h as well as at 3 and 5 day by ~12%. Shown are representative experiments ($n = 3$; standard deviation <4% at maximal turnover). (c) MTT Assay for rMC1 cell viability was measured in 48-well cultures at various time periods of hyperglycemia exposure. MTT activity was not significantly altered up to 2 days; however at day 3 to 5, there is a significant ($P < 0.01$, n = 6–8) increase versus LG (time 0 control).

cells undergoing a path of apoptotic cell death. Furthermore, injured and dying cells induce the expression of pro-inflammatory genes significantly ($P < 0.05$) for TXNIP (~36.55 ± 12.45 at day 3), iNOS (14.47 ± 4.8 at day 3), Cox-2 (4.49 ± 0.88 at day 5) and VEGF-A mRNA (2.15 ± 0.27 day 5) (Figures 7(c)–7(f)). These results suggest that under HG and oxidative stress, rMC1 cells promote an inflammatory and autophagy/mitophagy response to remove defective organelles.

3.6. HG Evokes an Early ER Stress and a Later Hypoxic-Like Response in rMC1. As shown above in Figure 3, TXNIP upregulation is sustained in rMC1 cells under conditions of HG. TXNIP is a pro-oxidant and pro-inflammatory protein [8, 12]. However, we observed at 4 and 24 h after HG exposure in rMC1 that TXNIP up-regulation is associated in fact with a significant decrease in ROS level (Figures 3 and 4). Therefore, we hypothesized that the early induction of ATP generation in rMC1 by HG (Figure 4(b)) might induce a mild increase in mitochondrial ROS (and possibly an increase in protein synthesis) that could potentially cause an ER stress and unfolded protein response, such as the IRE1/XBP1 branch. Spliced form sXBP1 (an active transcription factor) of XBP1 mRNA is translated and translocated to the nucleus where it induces the expression of antioxidant genes (e.g., superoxide dismutases and catalase) [22, 23]. Indeed, expression of SOD1 and catalase effectively increases the cellular antioxidant capacity [40]. Therefore, we examined levels of sXBP1 mRNA. We did not observe a significant alteration in sXBP1 mRNA in HG when compared with LG although there is an increasing trend at 4 h (Figures 8(a) and 8(b)). However, the DNA-binding activity of the nuclear sXBP1 is increased in EMSA (Figure 8(c)) at 4 and 24 h by HG and reduces at day 3 and 5. These results suggest that there is an early ER stress response in rMC1 under HG, which may effectively scavenge ROS (Figure 4(a)). However, as the ROS

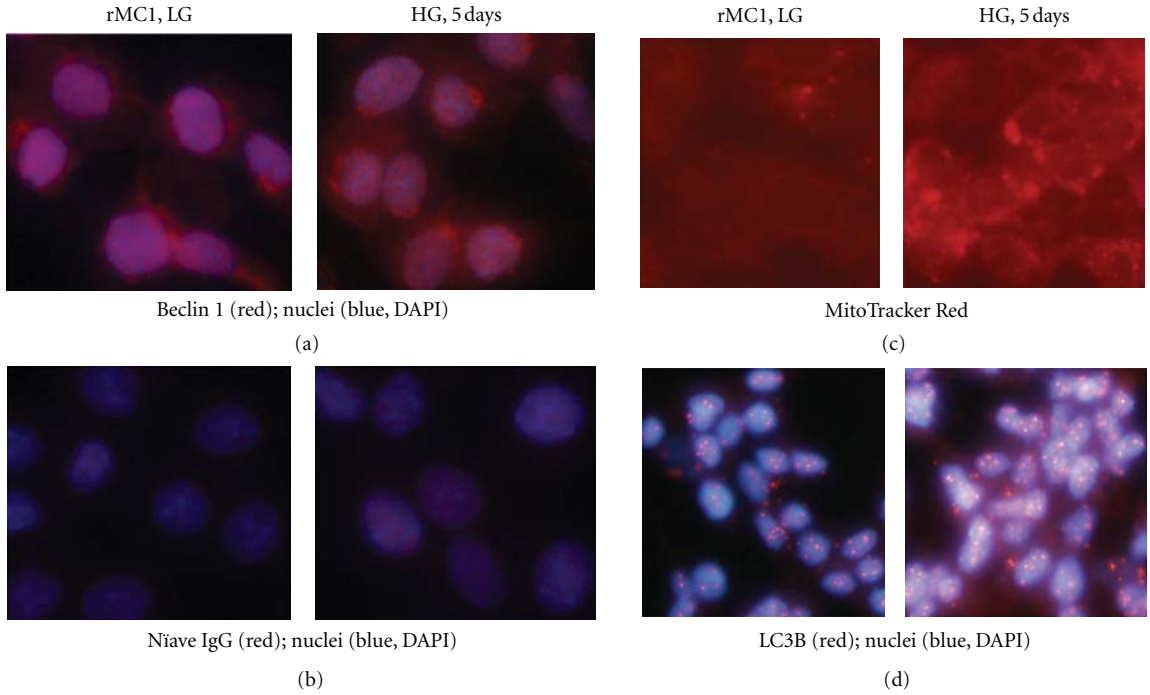

Figure 6: HG activates autophagy/mitophagy in rMC1. Cells were grown in HG for 5 days under low serum conditions in a four-well slide chamber with media changing for every 48 h and 24 h for the final day. Cells were fixed and stained for (a) beclin 1 antibody and (b) a naïve IgG for control antibody reaction. There are more beclin 1 puncta in HG than in LG. (c) MitoTracker Red staining also reveals more mitochondrial puncta in HG than in LG. (d) LC3B antibody staining shows an increased LC3B level and autophagic puncta formation in rMC1 under HG than in LG. The pictures are representative of 3 separate experiments.

continues to accumulate under sustained hyperglycemia, the anti-oxidant capacity is overwhelmed and an oxidative stress occurs at day 3 to 5 when XBP1 activity is reduced. Activation of other branches of UPR/ER stress (e.g., PERK-eIF-2 and ATF6) is yet to be investigated. Nonetheless, sustained ROS/RNS generation will cause excessive organelle damage and protein aggregation leading to apoptotic signal induction (Figure 7(b)), which may involve ER stress mediated apoptotic signals, CHOP (transcription factor CEBP homologous protein), and Bim (Bcl-2 interacting mediator of cell death) expression [41]. Further studies are being pursued to test this hypothesis.

Under persistent hyperglycemia, ROS, and sustained ATP depletion (Figure 4(b)), a hypoxic-like response in rMC1 may evoke and induce pro-inflammatory gene expression for iNOS, VEGF-A, and Cox-2 as seen in Figures 7(d)–7(f). Therefore, we measured the activity of hypoxia inducible transcription factor (HIF) 1α in EMSA and the result is shown in Figure 8(d). There is no increase in DNA-binding activity of HIF-1α in rMC1 up to day 3 by HG. However, at day 5 the HIF-1α DNA-binding activity is enhanced, which correlates positively with the expression of its down stream gene target, vascular permeability, and proangiogenic VEGF-A (Figure 7(f)). These results suggest strongly that chronic HG evokes an initially time-dependent early innate immune and ER stress response in rMC1, followed by ATP reduction, ROS accumulation, and HIF-1α activation leading, at least in part, to pro-inflammatory and proangiogenic gene expression.

3.7. Knockdown of TXNIP by siRNA Blocks ATP Reduction, ROS Generation, and LC3B Activation by HG in rMC1. Finally, we asked to what extent the sustained induction of TXNIP in rMC1 under HG is responsible for metabolic dysregulation in rMC1. We used a transient transfection method of siRNA to knock down TXNIP and assess its effect on ATP and ROS levels and LC3B expression in rMC1 under HG. First, we showed that HG-induced TXNIP protein level is reduced by two different TXNIP siRNAs (siTXNIP1 and siTXNIP3). As shown in Figure 9(a), siTXNIP3 gave a consistent suppression of TXNIP. Conversely, HG is still able to induce TXNIP expression in scrRNA-transfected cells. Therefore, we used the scrRNA (control) and siTXNIP3 in further studies. We observe that HG reduces ATP level significantly ($P = 5.2E - 05$, $n = 6$) in scrRNA-transfected cells, which is absent in siTXNIP3-transfected rMC1 cells (Figure 9(b)). In addition, HG-induced ROS generation in scrRNA-transfected cells is also blocked by siTXNIP3 (Figure 9(c)). Furthermore, the staining of autophagic marker LC3B is increased by HG in scrRNA-treated cells, which is reduced by siTXNIP3 (Figure 9(d)). The results demonstrate that TXNIP mediates, at least in part, cellular oxidative stress, ATP reduction, and an autophagic response in retinal Muller glia under chronic hyperglycemia.

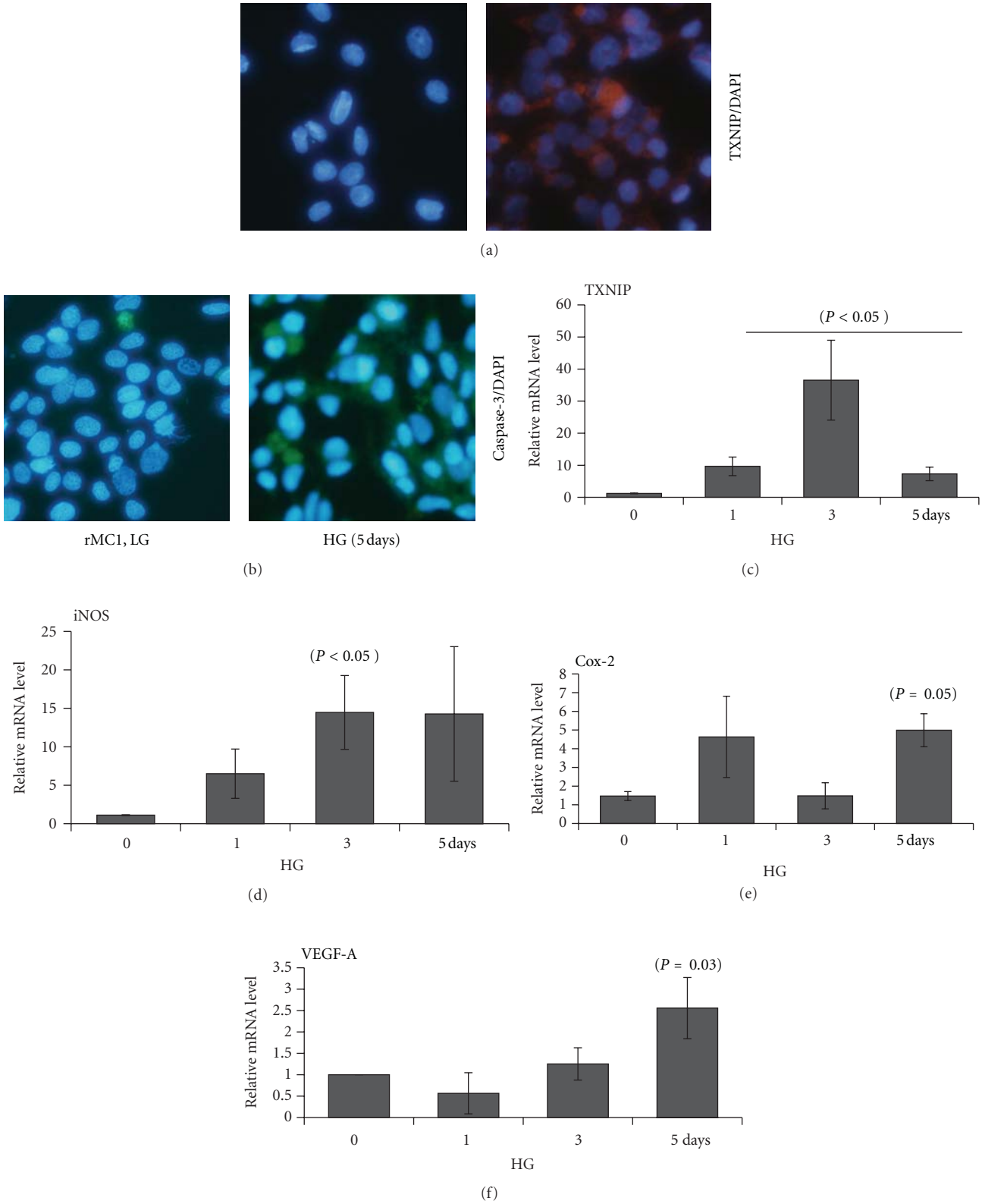

Figure 7: HG-induced TXNIP expression correlates with proapoptotic caspase-3 expression in rMC1. (a) IHC of TXNIP. Txnip staining is enhanced in rMC1 under HG exposure for 5 days, which correlates with increased (b) proapoptotic caspase 3 staining in similar duration of HG using a caspase-3 FLICA staining kit. A representative of $n = 3$ is shown here. (c–f) HG induces TXNIP and pro-inflammatory gene expression in rMC1. Total RNA was isolated with TRIZOL and mRNA levels for (c) TXNIP, (d) iNOS, (e) Cox-2, and (f) VEGF-A were measured by qRT-PCR at various time periods of HG exposure ($n = 3$-4). P values were compared against respective controls at time 0.

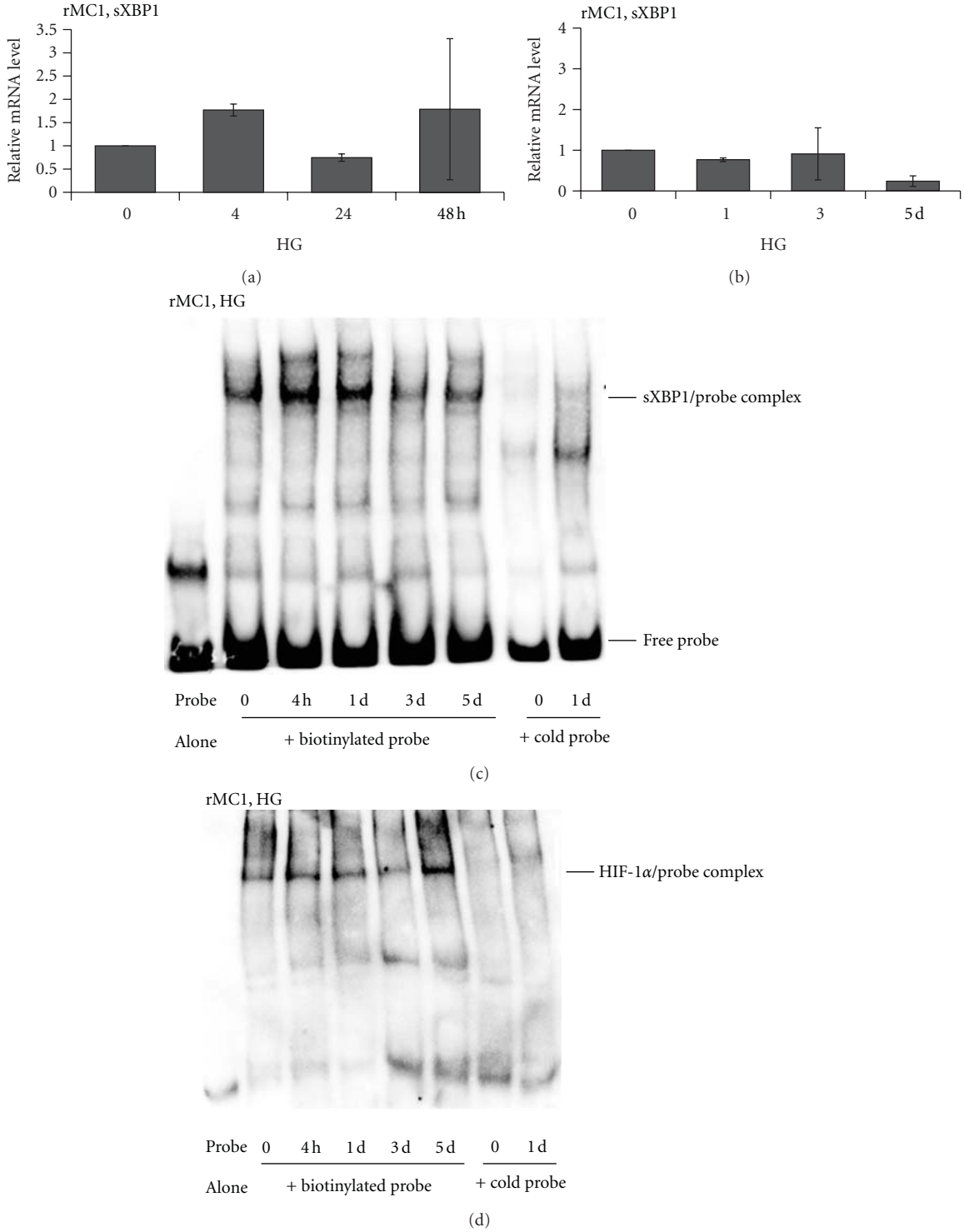

Figure 8: HG induces an early ER stress and a later hypoxia-like response in rMC1. Messenger RNA for the spliced form of ER stress marker XBP1 (sXBP1) was measured in rMC1 at various time periods (a) 0–24 h and (b) 0–5 days after HG exposure by qRT-PCR There is no significant change in sXBP1 mRNA ($p = ns$, $n = 3$) when compared with respective controls at time 0. (c) The DNA-binding activity of sXBP1 in rMC1 was further measured by EMSA using nuclear extracts (5 μg) and biotin-labeled DNA probes with or without a competitive cold DNA probe as described in Methods. Protein-DNA complexes were detected by streptavin-HPR and ECL. In the presence of competitive cold probe, the sXBP1 reactive band is abolished indicating the specificity of the sXBP1-DNA binding (last two lanes). Probe alone without the nuclear extract was also run as a control (first lane). The sXBP1 activity is increased at 4 h and 1 day in rMC1 by HG and then returns to the control level observed at day 0. (d) The DNA-binding activity of the hypoxic response factor HIF-1α in nuclear extracts was also measured using a biotin-labeled HIF-1α binding consensus DNA probe. HIF-1α activity is not increased up to day 3 in rMC1 by HG but enhances at day 5. Competitive cold probes block the DNA-binding of HIF-1α, indicating the specificity of the binding assay. The images are representative of $n = 3$ in both (c) and (d).

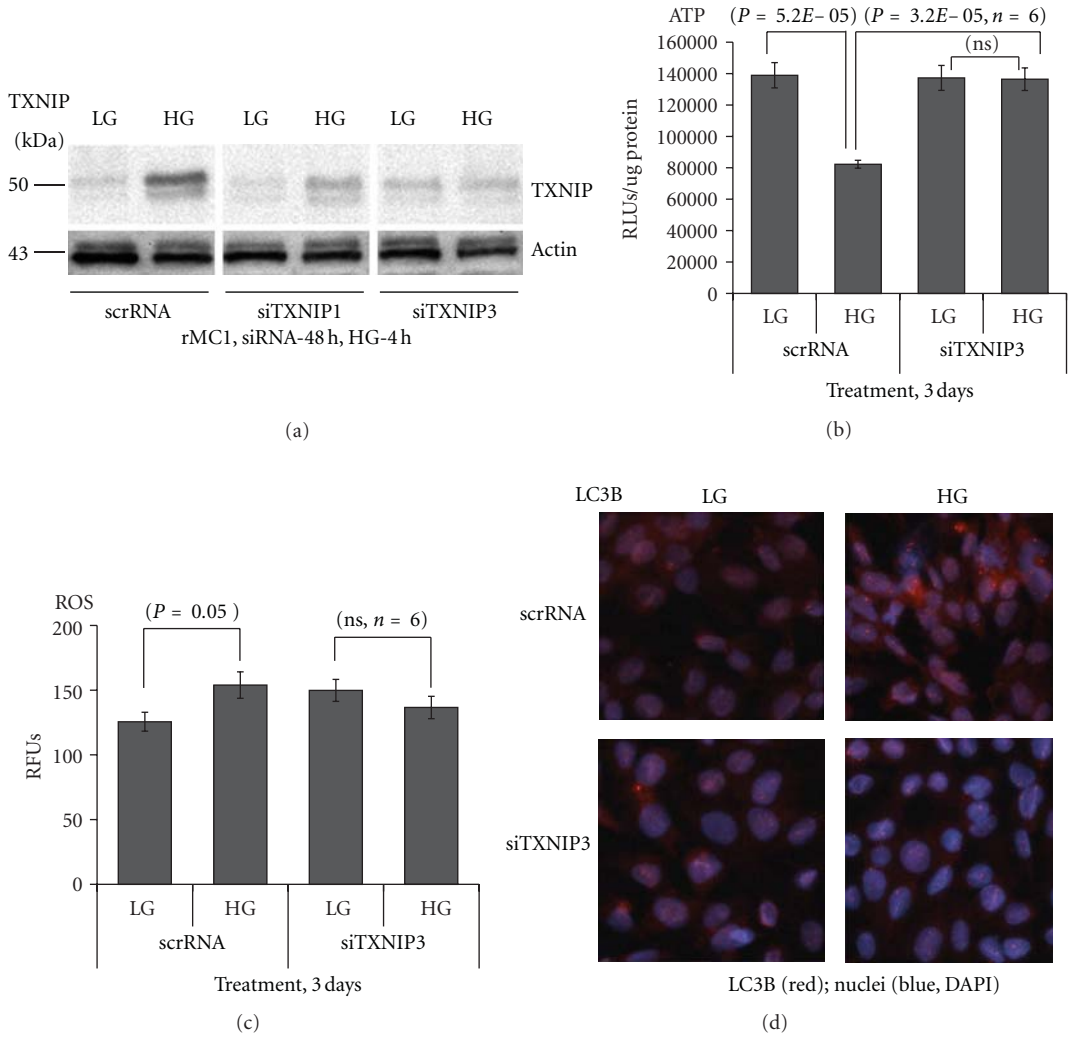

Figure 9: TXNIP knockdown by siRNA blocks HG-mediated ATP reduction, ROS generation, and LC3B expression in rMC1. (a) Seventy to eighty percent confluent rMC1 cells were transfected transiently with an scramble siRNA (scrRNA, control) or with TXNIP mRNA-targeted siRNAs-siTXNIP1 and siTXNIP3 (20 nM each) using HiPerfect transfection reagent in six-well plates or 60 mm culture plates in duplicates. After 6 h, complete media containing 5% serum were replaced and kept for 24 h. Media was subsequently changed to low serum media (0.2% serum) for 48 h. Afterwards, HG was added for 4 h and TXNIP amount was measured by Western blotting. We observe that siTXNIP3 gives a consistent suppression of TXNIP (~70%) and used in further studies. A representative blot of $n = 4$ is shown here. Actin was used as a control for protein loading and siRNA specificity. (b) rMC1 cells were transiently transfected in 24-well plates with siTXNIP3 for 48 h and then HG was added for 72 h in low serum media. Intracellular ATP concentration was measured and normalized to protein concentration (RLUs/μg protein). HG reduces ATP level in scrRNA-treated rMC1 cells while siTXNIP3 transfection blunted HG effects. (c) Similar to ATP determination, we also measured ROS levels after 72 h of HG exposure in both scrRNA and siTXNIP3-transfected rMC1 with CM-H2DCFDA. HG increases ROS levels in scrRNA cells ($P < 0.05$, $n = 6$) versus LG but this effect was not observed in siTXNIP3 cells. (d) LC3B staining under HG exposure for 5 days is also reduced in siTXNIP3-transfected cells when compared with the scrRNA cells under similar duration of HG exposure (right panels). Under LG, LC3B staining is minimal in both scrRNA and siTXNIP3-treated rMC1 cells after 5 days. A representative of $n = 3$ is shown here.

4. Discussion

Muller glia plays an important function in retinal tissue homeostasis by taking up glucose and nutrients from the blood. It supplies metabolic products to neurons for proper functioning and participates in uptake and detoxification of neurotransmitters [5, 42]. Therefore, an injury to Muller glia itself and sustained gliosis will produce proinflammatory cytokines and proangiogenic factors that may further affect the retinal vasculature and neurons leading to ocular complications of diabetes [43, 44]. TXNIP plays a critical role in retinal inflammation and apoptosis [9, 26, 45]. Here, we provide evidences that HG activates TXNIP in retinal Muller cells *in vitro* as well as *in vivo* in the diabetic rat retina (this study, [9]). We demonstrate a temporal response of Muller glia to chronic HG exposure and programming of a series of innate host defense mechanisms which involve (i) sustained TXNIP up-regulation, (ii) innate immune/ER stress responses—(iii)

ATP reduction, (iv) oxidative/nitrosative stress, (v) hypoxic-like response, (vi) autophagy/mitophagy, (vii) inflammation and, (viii) an apoptotic signal. Thus, a temporal and spatial survival response of Muller cells to chronic hyperglycemia emerges and TXNIP may orchestrate some of these events. Definitely, these observations will have important implications toward understanding the molecular basis for disease development and progression of DR and in designing stage-specific therapeutic strategies to reduce retinal inflammation and neurovascular dysfunction in diabetes.

TXNIP is considered as a pro-oxidative stress, pro-inflammatory, and proapoptotic protein under chronic hyperglycemia, diabetes, and cellular stress [9, 11, 13, 26, 46]. However, these deleterious effects of HG in cells are observed at later stages of sustained hyperglycemic exposure while TXNIP is an early response gene to HG and tissue injury. Therefore, TXNIP may be considered as an early sensor of metabolic and cellular danger and a significant component of the innate host defense mechanism(s) and wound healing processes [10, 15, 16]. In support of this hypothesis, we recently showed that TXNIP is responsible for S100B-RAGE axis-induced expression of the innate immune response mediator, pro-IL-1β, and secretion of mature IL-1β in primary Schwann cells in culture and partial sciatic nerve injury recovery [10]. Under these conditions, an increase in ROS generation was not observed [8, 10]. This finding is in agreement with our current data showing that HG induces TXNIP and pro-IL-1β expression and activation of NLRP3 inflammasome and caspase-1 at 4–24 h without ROS generation (Figures 2 and 3). In fact, this is also similar to the condition of chronic Granulomatous Disease where impaired ROS production due to a genetic defect does not affect IL-1β secretion [47, 48].

In acute infection models using circulating monocytes and stromal macrophages, however the NLRP3 inflammasome assembly, caspase-1 activation, and pro-IL-1β processing and secretion require ROS generation and ATP release [15, 49–51]. According to this model, during oxidative stress, ROS dissociates TXNIP bound to TRX. TXNIP then binds to NLRP3 to mediate NLRP3 inflammasome assembly containing ASC (Apoptotic Speck Protein Containing a Caspase Recruitment Domain (CARD)) and procaspase-1 [15, 16]. This subsequently leads to caspase-1 autocleavage and activation. Activated caspase-1 then processes pro-IL-1β to its mature form and is then secreted extracellularly. IL-1β is a potent pro-inflammatory cytokine and, upon release, induces several pro-inflammatory cytokines and chemokines. Therefore, IL-1β expression is highly regulated at multiple steps including at least two signals—a priming and a processing/maturation—while the mechanism of extracellular secretion is yet to be determined [52].

In our study, pro-IL-1β, NLRP3 inflammasome and procaspase-1 levels oscillate at 4 h and day 3 of HG exposure. The first response at 4 h occurs in the absence of ROS release as discussed previously while the second response at day 3 occurs under ROS/oxidative stress. We and others [10, 21] showed that TXNIP is responsible for pro-IL-1β priming/expression but the role of TXNIP in NLRP3 inflammasome assembly and caspase-1 activation is yet to be resolved as conflicting data exist at present [15, 21, 53]. Therefore, further studies will focus on the mechanism(s) of NLRP3 inflammasome activation and pro-IL-1β processing and the role of TXNIP in this process in the diabetic retina and under chronic hyperglycemia.

Nutrient excess (glucose availability) leads to increase rates of glycolysis and energy (ATP) production, which is evident from our data showing elevated intracellular ATP levels at 4 h. Mitochondrial ATP synthesis produces ROS during electron transfer and OxPhos at ETC in the mitochondrial inner membrane. Furthermore, glucose and ATP induce increased protein synthesis in the ER lumen and ER stress leading to a UPR response to enhance protein folding and anti-oxidant capacity via an induction of mitochondrial and cytosolic superoxide dismutases and catalase [40]. Indeed, we observed an activation of the IRE1/XBP1 pathway in rMC1 by HG, which is demonstrated by the enhanced DNA-binding activity of nuclear sXBP1 in EMSA (Figure 8(c)). sXBP1 is known to activate the transcription of anti-oxidant genes including those stated above [40]. This may partly explain why we observed a decrease in ROS level at 4 and 24 h of HG treatment in spite of TXNIP up-regulation and IL-1β expression. Nonetheless, as hyperglycemia persists, the sXBP1 activity reduces and the defensive program is switched to a reduction in cellular ATP content, potentially in an attempt to minimize mitochondrial ROS generation through the aerobic respiration [54]. This may activate the cytosolic anaerobic glycolysis via an inhibition of pyruvate kinase at TCA cycle and lactate production, which is a survival response for phagocytic and cancer cells in a hypoxic environment during inflammation and tumor progression [55, 56]. Even then, the ETC in mitochondrial inner membrane continues to leak electrons and produces ROS at complexes I and III [57]. This eventually will lead to accumulation of ROS since TXNIP up-regulation can reduce the ROS scavenging capacity of TRX. Such an event seems to occur at day 3 of HG exposure in rMC1s, where there is a second surge in IL-1β and NLRP3 expression is observed (Figure 3(d)). The surge may be a second attempt to correct cellular metabolic defects and induce cell survival. However, these responses cause a further decline in cellular ATP level evoking a hypoxic-like response (HIF-1α activation) under hyperglycemia and normoxia (Figure 8(d)).

Sustained TXNIP up-regulation may establish an oxidative stress environment in rMC1 at later stages of HG exposure (Figure 4(a)) after an initial innate immune response, ER stress, and ATP reduction. As ROS and iNOS expressions increase at day 3, nitrosative stress and protein thiol S-nitrosylation (SNO) as well as tyrosine nitration activities will be enhanced [58–60]. Initially, SNO modification of pro-apoptotic caspase-3 prevents its release from mitochondria [60]; however continued ROS/RNS will damage proteins, nucleic acids, and organelles including ER and mitochondria. Therefore, another ancient survival program of autophagy (mostly activated under nutrient starvation to recycle protein aggregates and organelles) and mitophagy (removal of damaged mitochondria by autophagic processes) is activated [36–39]. This is supported by our observation that in spite of the significant increase in ROS levels (day 3 to 5

of HG), activity of mitochondrial membrane enzyme cytochrome c oxidase at complex IV is sustained and cell viability is preserved. Preservation of cell viability is indicated by increased MTT that measures the reductase activity of succinate dehydrogenase at complex II. These findings suggest an active regeneration of mitochondria by removing the oxidatively damaged ones and provide cell viability. Such an assumption is supported by the observation that autophagic LC3B punctae are increased in rMC1 (Figure 6) and MTT activity is enhanced (Figure 5).

The mechanism of autophagy initiation is complex and recent studies have pointed to a role of the nuclear DNA-binding protein HMGB1 under oxidative stress [60]. Nuclear TXNIP may cause HMGB1 oxidation (S-nitrosylation) and nuclear export [61, 62]), which participates in beclin 1 dissociation from antiapoptotic bcl2 and initiation of autophagy [38, 39]. Oxidation of HMGB1 at cysteine residues 23, 45, and 106, probably by inhibition of TRX by TXNIP in the nucleus, causes its translocation to the cytosol and interaction with beclin 1 [38, 39]. HMGB1 is an abundant nuclear protein considered to have a dual function: it acts (i) as a transcriptional activator in the nucleus by binding to linker DNA between nucleosomes inducing DNA bending and enhances transcription factor accessibility, and (ii) as a pro-inflammatory cytokine (passively or actively secreted) by injured and dying cells as a danger signal and damage-associated molecular pattern (DAMP) molecule, which binds to innate pattern recognition receptors (PRRs) such as TLR2, TLR4 and RAGE [20, 61]. (iii) In addition, HMGB1 may also have a role in autophagy in rMC1 (this study, [60]). Previously, we showed that RAGE activation by its endogenous ligand S100B activates TXNIP expression and induces pro-inflammatory genes for IL-1β, Cox-2, VEGF-A, ICAM1, and fibrotic FN [8–10]. While IL-1β is an early mediator of innate immunity and inflammation, HMGB1 is regarded as a late mediator in chronic inflammatory diseases [63, 64]. Thus, the HMGB1-TLR4 and/or HMGB1-RAGE axis may further activate TXNIP and IL-1β expression thereby potentiating a vicious cycle of innate immunity and sterile inflammation under chronic hyperglycemia. The fact that knockdown of TXNIP by siRNA blocks IL-1β expression in the retina *in vivo* (Figure 1(c)) and ATP depletion, ROS generation, and LC3B induction in rMC1 *in vitro* (Figure 9) suggest that TXNIP is critical for Muller glia activation, autophagy, and inflammation. TXNIP therefore may represent a potent gene and therapeutic target to prevent DR pathogenesis [8, 9].

Sustained ROS/RNS generation and activation of autophagy/mitophagy will eventually trigger an apoptotic signal and premature cell death due to excessive removal of essential organelles and proteins in the cell while damaged mitochondria leaks electrons [57, 58]. Mitochondrial oxidative/nitrosative stress causes ER stress, which in turn induces mitochondrial damage thus generating a vicious cycle of organelle damage and tissue injury [41]. Here, we show that increases in both TXNIP and proapoptotic caspase-3 occur at later periods of HG exposure for example, at day 5. Nonetheless, we propose that the ER stress and autophagic and apoptotic signal will be shifted towards a caspase-1/7-dependent pro-inflammatory cell death (pyroptosis) [65]. This is because apoptosis is an ATP-dependent (consuming) process and requires active ATP synthesis [65]. In the current study, we observe ATP depletion under sustained HG and a late surge in caspase-1 level at day 5. Furthermore, the caspase-3 probe, FLICA, used in this study may also recognize caspase-7, which is downstream of caspase-1 and closely related to caspase-3. In agreement with the idea of pyroptosis under HG, the dying and oxidatively stressed rMC1 produces pro-inflammatory genes such as TXNIP, iNOS, Cox-2, and proangiogenic VEGF-A (Figures 7(c)–7(f); [66]). The induction of VEGF-A is particularly interesting because of its association with ATP depletion and HIF-1α activation. VEGF-A is a potent vascular permeability and proangiogenic factor that is regulated by HIF-1α and implicated in diabetic vasculopathy [56, 67]. Protein S-nitrosylation also consumes molecular oxygen [58]; therefore, it may result in reduced mitochondrial ATP production and generation of an intracellular hypoxic-like environment inducing HIF-1α activation under normoxia. An understanding of the TXNIP-HIF-1α-VEGF-A axis and its temporal response under chronic hyperglycemia in Muller cells that enhances VEGF-A expression in diabetes will be important. It is generally accepted that Muller glia is the main source of VEGF-A in the neural retina [68, 69].

An accumulation of HIF-1α in diabetic retinas of STZ-mice was recently reported [67], which is also associated with an increase in VEGF-A expression. Furthermore, a disruption of the Hif-1α gene in Müller cells alleviates retinal inflammation and vascular leakage in diabetic Hif-1α KO mice, further indicating an important role of Müller glia-derived HIF-1α-VEGF-A axis in DR [67]. In addition, attenuation of ER stress by chemical chaperones such as 4-phenyl butaric acid reduces VEGF-A expression in retinal endothelial cells in culture induced by hypoxia as well as in a mouse model of oxygen-induced retinopathy (OIR) [23, 70, 71]. Thus, several reports have pointed to an important role of ER stress/UPR signaling in retinal inflammation, hypoxia, and perhaps autophagy. Nonetheless, a critical function of TXNIP in ER stress, autophagy, and apoptosis/pyroptosis in animal models of DR remains to be investigated, which will form the basis for a separate manuscript. Sustained and excessive induction of each of these cellular defensive/survival processes, including ER stress and UPR, will evoke chronic inflammation and DR pathogenesis. ER-stress, autophagy, and innate immune responses are survival mechanisms; however prolonged induction of these cellular processes will ultimately lead to premature cell death and disease progression of microvascular complications of diabetes. Cellular DAMPs (ATP, uric acid, HMGB1, mitochondrial DNA, etc.) released by injured and dying cells are recognized by innate PRRs such as TLR4, TLR9, RAGE, P2X7R, and NLRP3 inflammasome and together the DAMPs and PRRs may play causative roles in retinal ER-stress, autophagy, inflammation, and DR pathogenesis.

In conclusion, we show for the first time that HG sustains TXNIP expression in rat Muller glia and orchestrates a duration-dependent cellular program of innate host defense and survival mechanisms that culminate in oxidative stress,

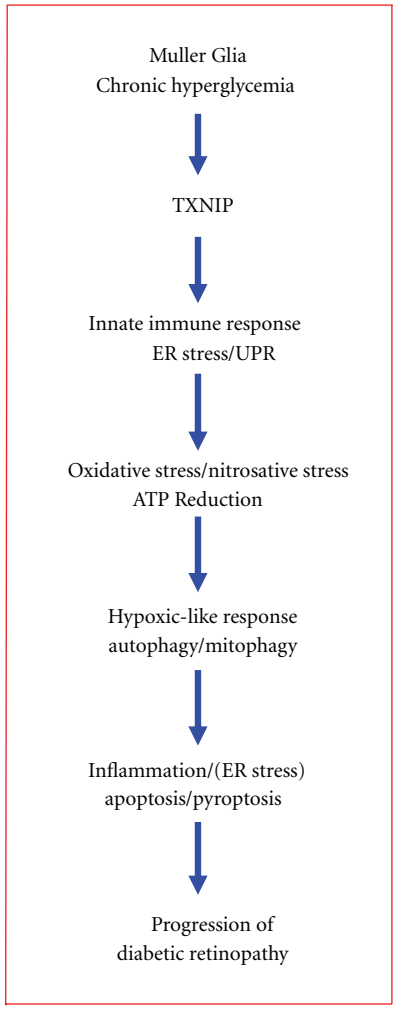

Figure 10: A schematic summary of potential cellular responses by retinal Muller glia in chronic hyperglycemia and diabetes. The sequence of molecular events that retinal Muller cells react to chronic hyperglycemia include (i) sustained upregulation of TXNIP, (ii) an initial innate immune and UPR response to excess glucose metabolism and oxidative phosphorylation (ATP generation), (iii) oxidative stress (ROS/RNS generation) and a hypoxia-like response through ATP reduction, (iv) an induction of an autophagic-mitophagic pathway, and (v) ER-stress and inflammation. These cellular responses constitute intrinsic cell survival/defense mechanisms, which, under chronic cell stress and injury, may promote premature cell death and disease progression of DR.

ER stress, autophagy, inflammation, and cell death. While detailed mechanisms are yet to be worked out, a temporal pattern emerges here as to how retinal Muller glia might respond to chronic hyperglycemia in diabetes. These include (i) sustained TXNIP up-regulation, (ii) an initial innate immune and ER stress response to excess glucose metabolism (ATP generation), (iii) oxidative stress and a hypoxia-like response through ATP reduction, (iv) induction of an autophagic-apoptosis pathway, and (v) inflammation. A temporal response of Muller glia to chronic hyperglycemia and potential molecular events are summarized in Figure 10. These findings clearly point to a crucial role of TXNIP in Muller glia activation, oxidative/nitrosative, and ER stress, and sterile inflammation under chronic hyperglycemia and suggest a potential gene and drug target for preventing neurovascular injury/cell death and pathogenesis of DR. Lastly, while our manuscript is in submission, an article came out to demonstrate purinergic and glycemic induction of TXNIP in rMC1 in culture [72], which also supports our findings in the present study.

Conflict of Interests

All authors declare that they have no conflict of interests for this study.

Acknowledgments

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Research Article

Modulation of Apoptosis Pathways by Oxidative Stress and Autophagy in β Cells

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Human islets isolated for transplantation are exposed to multiple stresses including oxidative stress and hypoxia resulting in significant loss of functional β cell mass. In this study we examined the modulation of apoptosis pathway genes in islets exposed to hydrogen peroxide, peroxynitrite, hypoxia, and cytokines. We observed parallel induction of pro- and antiapoptotic pathways and identified several novel genes including BFAR, CARD8, BNIP3, and CIDE-A. As BNIP3 is an inducer of autophagy, we examined this pathway in MIN6 cells, a mouse beta cell line and in human islets. Culture of MIN6 cells under low serum conditions increased the levels of several proteins in autophagy pathway, including ATG4, Beclin 1, LAMP-2, and UVRAG. Amino acid deprivation led to induction of autophagy in human islets. Preconditioning of islets with inducers of autophagy protected them from hypoxia-induced apoptosis. However, induction of autophagy during hypoxia exacerbated apoptotic cell death. ER stress led to induction of autophagy and apoptosis in β cells. Overexpression of MnSOD, an enzyme that scavenges free radicals, resulted in protection of MIN6 cells from cytokine-induced apoptosis. Ceramide, a mediator of cytokine-induced injury, reduced the active phosphorylated form of Akt and downregulated the promoter activity of the antiapoptotic gene bcl-2. Furthermore, cytokine-stimulated JNK pathway downregulated the bcl-2 promoter activity which was reversed by preincubation with SP600125, a JNK inhibitor. Our findings suggest that β cell apoptosis by multiple stresses in islets isolated for transplantation is the result of orchestrated gene expression in apoptosis pathway.

1. Introduction

The major pathways of apoptosis are the extrinsic pathway, initiated by Fas and other death receptors resulting in the activation of caspase-8, and the intrinsic mitochondrial pathway, regulated by Bcl-2 family of proteins leading to the activation of caspase-9 [1, 2]. These two pathways converge with the activation of caspase-3. Both apoptotic pathways are involved in β cell death in type 1 and type 2 diabetes [3]. Fas (CD95/APO-1) is a 36-kD death receptor protein that initiates apoptosis in many cell types when cross-linked to Fas ligand (FasL/CD95L) [4]. A histological study of human diabetic pancreas biopsies has demonstrated Fas expression on β cells and FasL expression on the infiltrating cells [5]. However, the intrinsic mitochondrial pathway, regulated by the Bcl-2 family of proteins, consisting of proapoptotic (Bax and Bak1) and antiapoptotic (Bcl-2, Bcl-xL, Mcl-1, etc.) proteins [6], has been shown to play a predominant role in the loss of isolated islets [7]. Imbalance between these two groups of proteins results in the release of cytochrome c, which activates caspase-9 [2]. BH3-only proteins, a subset of proapoptotic proteins, act as sensors of cellular stress [8, 9]. Members of this family include Bad, Bid, Bmf, Hrk, Bim, Bik, Noxa, and Puma. They induce apoptosis by activating proapoptotic proteins or by neutralizing antiapoptotic proteins. Bid cleaved by caspase-8 translocates to mitochondria and causes cytochrome c release, thus linking the two pathways of apoptosis [10].

Autophagy is a lysosomal degradative pathway that provides energy through self-digestion under conditions of starvation. During oxidative stress, autophagy serves as a defense mechanism to clear oxidatively damaged proteins

and organelles [11]. There are three major pathways of autophagy: (1) chaperone-mediated autophagy (CMA), which is found in mammalian cells alone and degrades cytosolic proteins selectively; (2) microautophagy, whereby lysosomes directly engulf cytosolic constituents through invaginations of the lysosomal membrane; and (3) macroautophagy (referred to as autophagy), in which cytosolic contents including organelles and proteins are sequestered within double-membrane structures called autophagosomes that fuse with lysosomes and lead to degradation. A series of maturation steps involving the ATG family of proteins are involved in the formation of autophagosomes. Among the 30 ATG genes identified in yeast, 11 (ATG1, 3–10, 12, and 16) have orthologs in mammalian cells. Autophagic vesicles contain multiple proteins, including Type (or Class) III PI3 kinase (vps34), Beclin 1 (ATG6), UVRAG, and Ambra. An important step in the formation of autophagosomes is the conjugation of LC3 (ATG8) with phosphatidylethoanlamine to form LC3-PE (LC3-II) which is a standard marker for autophagy. Extensive autophagy could lead to type 2 cell death, a second mode of programmed cell death [12]. Aged insulin-secreting granules in β cells are degraded by crinophagy [13]. An imbalance between insulin production and secretion, which is likely to occur in type 2 diabetes, induces autophagy to degrade accumulated insulin granules [14]. Ubiquitinated protein aggregates that accumulate in β cells of islets in obese Zucker rats have been shown to stimulate autophagy [15]. Increased autophagic activity has been observed in the islets of Rab3A$^{-/-}$ mice which display a defect in insulin secretion [13].

Islets are clusters of different cell types including α, β, δ, and PP cells, with insulin-producing β cells being the major component (70–80%). The blood vessels inside islets are essential for the supply of oxygen, nutrients, and secretion of hormones. These vessels are disrupted during the islet isolation process. Thus, islets are vulnerable to injury in the early stages after transplantation due to the delay in revascularization [16]. Even after revascularization, the vascular density is considerably less compared to endogenous islets [17]. The expression of genes associated with angiogenesis is decreased in diabetic transplant recipients, further delaying the revascularization process [18]. The molecular mechanism of apoptosis in β cells of islets in the transplantation setting is not clearly understood. The objective of the present study was to profile the expression of apoptosis pathway genes in human islets exposed to stresses associated with islet isolation and transplantation and to determine the role of stress-signaling pathways.

2. Experimental Procedures

2.1. Culture of Human Islets and MIN6 Cells. Human islets isolated from cadaveric donors were provided by Integrated Islet Distribution Program (IIDP). Islets with purity of 70% to 95% and the viability of 70%–95% were used. Islets were cultured in CMRL 1066 medium (Mediatech Inc., Hendon, VA) supplemented with human serum albumin (0.5%; Octapharma, Vienna, Austria) and nicotinamide (10 mM) and referred to as Miami medium. To induce hypoxia in islets, culture dishes were placed inside a modular incubation chamber (Billups Rothenberg, Del Mar, CA) and flushed with a gas supply of 1% oxygen, 5% CO_2, and 94% N_2 for 20 min. The chamber was then placed inside the cell culture incubator. MIN6 cells, a mouse pancreatic β cell line [19], obtained from Dr. Miyazaki (Kyoto University, Japan) were cultured in RPMI medium containing 10% FBS (Gemini Bioproducts, Sacramento, CA), 100 μg/mL streptomycin (Gemini Bioproducts), 100 U/mL penicillin (Gemini Bioproducts), and 50 μM β-mercaptoethanol (BME; Sigma Aldrich, St. Louis, MO) at 37°C in a humidified atmosphere of 5% CO_2. Autophagy was induced in MIN6 cells by culturing them in low serum (0.1%) serum-containing medium and in human islets by amino acid deprivation.

2.2. Gene Expression Profiling of Apoptosis Pathways. Human islets isolated from donor pancreas were exposed to Hydrogen peroxide, peroxynitrite, hypoxia, or a combination of cytokines (IL-1β, TNF-α, and IFN-γ). A pathway-specific, PCR-based array (SABioscience, Frederick, MD) was performed to determine the expression of a panel of genes in apoptosis pathway (listed in Table 1). RNA samples following DNase treatment were converted to cDNA by the following procedure: 1 μg of RNA was reverse transcribed in a 50 μL reaction mixture with 125 U of Moloney Murine leukemia virus reverse transcriptase, 20 U RNasin ribonuclease inhibitor, 4 mM deoxyribonucleoside triphosphates, 5 mM $MgCl_2$, 1X PCR Buffer II, and 0.5 μg of random hexanucleotide primers (Applied Biosystems, Carlsbad, CA). The reaction mixture was sequentially incubated at 65°C for 5 min, 42°C for 60 min, and the reverse transcription reaction was stopped by heating to 95°C for 5 min and cooled to 4°C. The experimental cocktail was prepared by adding 102 μL of the diluted cDNA to 1278 μL of the RT^2 qPCR master mix containing SYBR Green (SA Biosciences, Frederick, MD) and 1173 μL H_2O. 25 μL of this cocktail was added to each well of the 96-well PCR array plate containing primers for the 84 genes in apoptotic pathway, five housekeeping control genes, and three RNA and PCR quality controls. Real-time PCR was performed in an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). The thermal cycling conditions were 1 cycle of 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and of 1 minute at 60°C. After amplification, real-time data acquisition and analysis were performed through the Data Analysis Web Portal (SA Biosciences). Data analysis is based on the delta-delta Ct method with normalization of the raw data to GAPDH as described in the manufacturer's manual.

2.3. RNA Isolation and Real-Time Quantitative RT-PCR. Total RNA was isolated from treated islets. After DNAse treatment, RNA integrity was determined by capillary electrophoresis using RNA 6000 Nano LabChip. The mRNA levels of selected genes (BFAR, CARD8, CIDE-A, and BNIP3) identified in the expression profiling described previously were examined by real-time quantitative RT-PCR using Taqman probes. Assay on Demand (Applied Biosystems) was

used for BNIP3. The sequence for primers and probes for other targets is as follows:

BFAR

Forward Primer: GGAGGACATCGTCACCAAGC

Reverse Primer: AGTATTTGACCAGGAACTCTCT CCA

TaqMan Probe: 5′-6FAM-TCTGGATCTTAAGGAG CCTACGTGGAAGCA-TAMRA-3′

CARD8

Forward Primer: GACTTCCGGTCGCCATGAT

Reverse Primer: ACCATTGAAGATGGCCCAGA

TaqMan Probe: 5′-6FAM-TGGGCGGTAAACGCG GTTAGTGC-TAMRA-3′

CIDE-A

Forward Primer: TCTTTCAGACCTTGGGAGACA AC

Reverse Primer: TGGCTGCCCGGCATC

TaqMan Probe: 5′-6FAM-CGCATTTCATGATCTT GGAAAAAGGACAGA-TAMRA-3′.

The PCR reactions were monitored in real time in an ABI Prism 7700 sequence detector (Perkin Elmer Corp./Applied Biosystems). After amplification, real-time data acquisition and analysis were performed.

2.4. Western Blot Analysis. MIN6 cells and human islets exposed to stress conditions were washed with ice-cold PBS and lysed with mammalian protein extraction reagent (M-PER, Pierce, Rockford, Illinois, USA) supplemented with phosphatase inhibitors (20 mM of sodium fluoride, 1 mM of sodium orthovanadate and 500 nM of okadaic acid) and protease inhibitors (Sigma P8340). Lysates were centrifuged at 20,800 ×g, and the protein concentration was determined in the supernatant samples by a dye-binding method [20]. Diluted samples containing equal amounts of protein were mixed with 2 X Laemmli sample buffer. The proteins were resolved on a 12% SDS-polyacrylamide gels. Following transfer to PVDF membranes (Millipore, Bedford, MA), the blots were blocked with TBST (20 mM Tris-HCl (pH 7.9), 8.5% NaCl, and 0.1% Tween 20) containing 5% non-fat dry milk at room temperature (RT) for 1 h and exposed to primary antibodies (1 : 1000; Cell Signaling, Danvers, MA) in TBST containing 5.0% BSA at 4°C overnight. The blots were washed with TBST and anti-rabbit IgG conjugated to alkaline phosphatase (Cell Signaling) was added for one hour at RT. After incubation in the presence of alkaline buffer (10 mM Tris-HCl (pH 9.5), 10 mM, NaCl and 1 mM $MgCl_2$), signals were developed with CDP-Star reagent (New England Biolabs, Beverly, MA) and exposed to X-ray film. The intensity of bands was measured using Fluor-S MultiImager and Quantity One software from Bio-Rad.

2.5. Transfection Procedure. The activity of bcl-2 promoter (truncated with CRE site, −1640–1287) linked to firefly luciferase reporter gene (provided by Linda Boxer, Stanford University School of Medicine) was measured in cultured MIN6 cells by a transient transfection assay using the procedure described earlier [21]. Plasmids (2 μg) and LipofectAMINE reagent 2000 (Invitrogen-Life Technologies, Carlsbad, CA) (4 μL) were diluted separately in 100 μL of Opti-MEM I, mixed, and incubated at room temperature for 20 min. A constitutively active renilla luciferase (pRL-TK-luc) was included to correct for transfection efficiency and for nonspecific actions of treatments on luciferase activity. The plasmid mixture were added to MIN6 cells cultured in 12 well dishes to about 70% confluence. The transfected MIN6 cells were cultured in low serum (0.1%) medium with appropriate treatment. The treated cells were washed with cold PBS and lysed with 100 μL of reporter lysis buffer. The lysate was centrifuged (10,600 ×g; 20 min) to collect the supernatant. The activities of firefly luciferase and renilla luciferase were measured using a dual luciferase assay kit (Promega, Madison, WI). The ratios of these two luciferase activities were taken as the measure of bcl-2 promoter induction.

2.6. Immunocytochemistry. MIN6 cells were cultured in a Lab-Tek II Chamber Slide system; following transfection and treatment, they were fixed in 4% paraformaldehyde for 30 minutes at RT. After washing with PBS, fixed cells were permeabilized in PBS containing 0.2% Triton X-100 and 5% BSA for 90 minutes. The cells were incubated in the presence of antibodies for active caspase-9 or active caspase-3 (1 : 250) in 3% BSA at 4°C overnight, washed in PBS, and exposed to secondary antibodies linked to Cy3 (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) in 3% BSA for 90 minutes at RT. Cells were then washed in PBS, sealed with mounting medium and examined by digital deconvolution microscopy using a Zeiss Axioplan 2 microscope fitted with Cooke SensiCam^{QE} high performance CCD camera. Immunocytochemistry with islets was carried out by a similar procedure but with islets in suspension. Primary antibodies were at the dilution of 1 : 2000 for insulin and 1 : 500 for LC3-II and phosphorylated Akt. Appropriate secondary antibodies linked to Cy3 or FITC in 3% BSA were used. Washing steps were performed by centrifugation at 500 RPM for 5 min. After the final step of washing the fluorescent labeled islets in PBS, they were suspended in mounting medium and placed inside secure seal hybridization chambers for fluorescent microscopy. Images were taken in multiple z-planes and assembled together by digital deconvolution microscopy.

Statistical analysis was performed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test.

3. Results

3.1. Gene Expression Profiling of Apoptosis Pathways in Human Islets under Stress. We performed an apoptosis pathway-specific gene expression array with RNA isolated from

Table 1: Functional grouping of apoptosis pathway genes.

Bcl-2 family	BAD, BAG1, BAG3, BAG4, BAK1, BAX, BCL2, BCL2A1, BCL2L1, BCL2L10, BCL2L11, BCL2L2, BCLAF1, BID, BIK, BNIP1, BNIP2, BNIP3, BNIP3L, HRK, MCL1
Caspase family	CASP1, CASP10, CASP14, CASP2, CASP3, CASP4, CASP5, CASP6, CASP7, CASP8, CASP9
IAP family	BIRC1, BIRC2, BIRC3, BIRC4, BIRC6, BIRC8
TRAF family	TRAF2, TRAF3, TRAF4
CARD family	APAF1, BCL10, BIRC2, BIRC3, CARD4, CARD6, CARD8, CASP1, CASP2, CASP4, CASP5, CASP9, CRADD, NOL3, PYCARD, RIPK2
Death domain family	CRADD, DAPK1, FADD, FAS (TNFRSF6), TNFRSF10A, TNFRSF10B, TNFRSF11B, TNFRSF1A, TNFRSF21, TNFRSF25, TRADD
Death effector domain family	CASP8, CASP10, CFLAR, FADD
CIDE domain family	CIDE-A, CIDE-B, DFFA
p53 and DNA damage response	ABL1, AKT1, APAF1, BAD, BAX, BCL2, BCL2L1, BID, CASP3, CASP6, CASP7, CASP9, GADD45A, TP53, TP53BP2, TP73
Anti-apoptosis	AKT1, BAG1, BAG3, BCL2, BCL2A1, BCL2L1, BCL2L10, BCL2L2, BFAR, BIRC1, BIRC2, BIRC3, BIRC4, BIRC6, BIRC8, BNIP1, BNIP2, BNIP3, BRAF, CFLAR, IGF1R, MCL1, TNFRSF7
TNF ligand family	CD40LG (TNFSF5), FASLG (TNFSF6), LTA, TNF, TNFSF10, TNFSF7, TNFSF8
TNF receptor family	CD40 (TNFRSF5), FAS (TNFRSF6), LTBR, TNFRSF10A, TNFRSF10B, TNFRSF11B, TNFRSF1A, TNFRSF1B, TNFRSF21, TNFRSF25, TNFRSF7, TNFRSF9

Table 2: Mean fold (>2.0) changes in apoptosis pathway gene expression in islets after exposure to oxidative stress (H_2O_2 and peroxynitrite), hypoxia, and cytokines.

Genes	Role in apoptosis	Peroxynitrite	H_2O_2	Hypoxia	Cytokines
Bcl2A1	Anti-apoptotic; Bcl2 family	3.2	2.1	2.4	11.4
Bid	Proapoptotic; BH3 only	3.7	3.5	2.5	3.4
Fas	Extrinsic; receptor	4.3	3.1	3.4	7.4
Fas ligand	Extrinsic; receptor ligand	2.4	2.1	NS	3.4
TRAIL	Extrinsic; receptor ligand	4.6	3.6	NS	4.2
A20	Extrinsic; anti-apoptotic	2.3	NS	2.5	7.4
c-Flip	Extrinsic; anti-apoptotic	2.6	NS	NS	4.2
Caspase-3	Marker for apoptosis	4.5	3.2	3.2	2.3
BIRC3	Caspase inhibitor	3.2	2.4	NS	8.7
CARD8	Caspase-9 inhibitor	−2.3	−2.4	−2.1	−2.3
BRAF	Signaling kinase	−3.7	−3.5	−2.5	NS
BFAR	Anti-apoptotic; links both pathways	−4.8	−3.5	−4.2	NS
CIDE-A	Causes DNA fragmentation	3.2	2.4	3.8	NS
BNIP3	Autophagy Inducer	2.6	NS	5.6	NS

Human islets (2000 IEQ) were exposed to 200 μM of peroxynitrite, 200 μM of H_2O_2 or a mixture of cytokines (2 ng/mL of IL-1β, 10 ng/mL of TNF-α and 10 ng/mL of IFN-γ) for 24 h or cultured under hypoxic conditions (1% oxygen) for 8 h. The cDNA synthesized from isolated RNA was mixed with Master Mix containing SYBR Green and distributed into 96 wells containing primers for the 84 genes associated with the apoptotic pathway. Five housekeeping control genes and three RNA and PCR quality controls were also included. PCR analysis was carried out and the fold changes between control and treated were calculated based on ΔC_t and corrected for GAPDH expression. Results are the mean obtained from four different batches of human islets. NS: not significant.

human islets exposed to oxidative stress, hypoxia and proinflammatory cytokines. Genes in intrinsic and extrinsic pathways were induced in stressed islets (Table 2). For example, Fas, fas ligand, and TRAIL, important regulators of the extrinsic pathway, were induced (150–360%) by oxidative stress and cytokines. Bid that links both pathways was induced by 150–270%. The expression of CARD8, an inhibitor of caspase-9, a marker for the intrinsic pathway of apoptosis, was downregulated ~50% by oxidants, hypoxia, and cytokines. Downregulation of BRAF (60–73%), a critical signaling kinase, suggests that these islets are likely to be less responsive to growth factor-mediated cell survival pathways. Caspase-3, a marker for apoptosis, is activated by proteolytic cleavage. In this study, we also observed increased expression of caspase-3 by all the stresses tested. CIDE-A which causes DNA fragmentation was induced (140–280%)

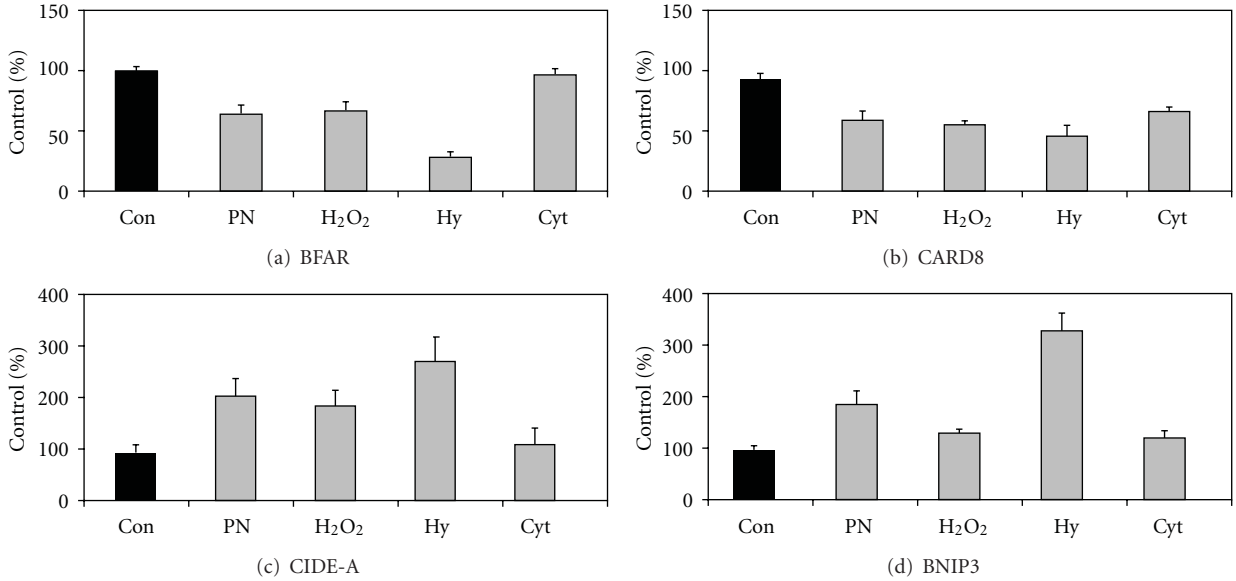

Figure 1: Stress-induced modulation of genes in apoptosis pathway. Human islets (2000 IEQ) cultured in Miami medium were exposed to multiple stresses including peroxynitrite (PN; 200 μM), H_2O_2 (200 μM), or a mixture of cytokines (Cyt; 2 ng/mL of IL-1β, 10 ng/mL of TNF-α, and 10 ng/mL of IFN-γ) for 24 h or cultured under hypoxic conditions (1% oxygen) for 8 h. RNA was isolated from treated islets for the RT-PCR analysis of BFAR, CARD8, CIDE-A, and BNIP3 using Taqman probes. Results are M ± SE of experiments with four independent batches of human islets. $^*P < 0.01$; $^{**}P < 0.001$ when compared to untreated control (Con).

by oxidative stress and hypoxia. Interestingly, activation of a parallel cytoprotective pathway in response to stress was evident by the induction of several genes in the antiapoptotic pathway including, Bcl2A1, c-IAP2 (BIRC3), A20, and c-flip (CFLAR). BNIP3, an inducer of autophagy was induced by 160% and 460% by peroxynitrite and hypoxia, respectively. Our findings from gene expression profiling points to the complex nature of cell death pathway in islets exposed to stresses associated with islet isolation and transplantation.

3.2. Modulation of Apoptosis Pathway Genes in Islets. Some of the genes including BFAR, CARD8, CIDE-A, and BNIP3 listed before have not been reported previously in the context of apoptosis in islets. Therefore, their induction was further confirmed by a more sensitive RT-PCR analysis using Taqman probes. BFAR (bifunctional apoptosis regulator) is a multidomain protein that interacts with members of the extrinsic and intrinsic apoptosis pathways and inhibits apoptosis. CARD8, a member of the caspase-associated recruitment domain (CARD) family, inhibits the activation of caspase-9. Levels of BFAR and CARD8 decreased by 35–40% when exposed to oxidative stress and by 50–70% and when incubated under hypoxic conditions (Figure 1). Cytokines decreased (30%) the expression of CARD8 but not BFAR. The mRNA levels of CIDE-A, a gene that acts downstream of caspases to mediate DNA fragmentation, increased by 95–180% in islets exposed to oxidative stress while cytokines did not have any effect on the expression of this gene. The expression of BNIP3 which plays an important role in mitochondrial autophagy increased in islets exposed to peroxynitrite (92%) and hypoxia (240%).

3.3. Induction of Autophagy in MIN6 Cells. Autophagy is a self-digestive process by which cells generate energy during starvation and degrade damaged proteins and organelles. We examined autophagic pathways in MIN6 cells cultured under low serum conditions by Western blot analysis. A key step in the formation of autophagosomes is the conjugation of LC3 (LC3-I-18 kD) with phosphatidylethanolamine to form LC3-PE (LC3-II-16 kD), an important marker of autophagy. As shown in Figure 2(a), the levels of LC3-II increased with increasing concentrations of trehalose, particularly in low-serum medium. The levels of ATG4, LAMP-2, and UVRAG also increased in a similar manner. An increase in Beclin 1 was seen only at 100 mM of trehalose in low-serum medium. Exposure of β cells to the autophagy inhibitor 3-methyladenine (3MA) reduced the formation of LC3-II in the presence of trehalose (Figure 2(b)) whereas another inhibitor bafilomycin A1 (Bf) increased the accumulation of LC3-II both under basal conditions as well as following exposure to trehalose (Figure 2(b)) because it interferes at the late lysosomal degradation step leading to accumulation of autophagosomes. Furthermore, induction of ER stress with thapsigargin and tunicamycin (Calbiochem, La Jolla, CA) led to formation LC3-II (Figure 2(c)) suggesting a crosstalk between these two pathways.

3.4. Autophagic Preconditioning Protects Human Islets from Hypoxia-Induced Apoptosis. Amino acid (AA) starvation of human islets by incubation in Hank's balanced salt solution induced autophagy in β cells as shown (arrows) by the punctated staining of LC3-II in insulin-positive cells (Figure 3(a)). Autophagy can be protective under conditions

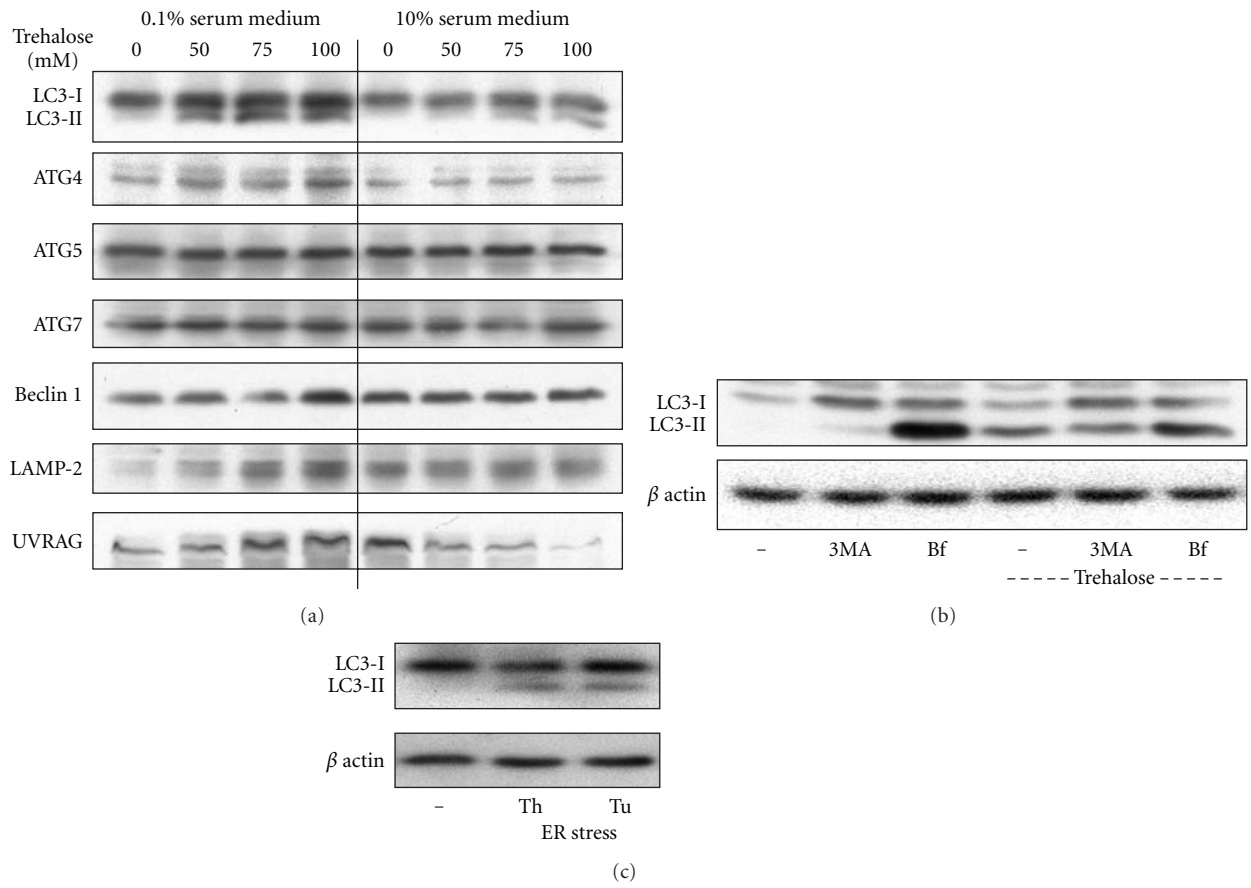

Figure 2: Induction of autophagy in MIN6 cells. (a) MIN6 cells grown to 60% confluence were incubated in low (0.1%) serum or regular (10%) serum medium in the presence of increasing concentrations of trehalose, an inducer of autophagy, for 24 h. (b) MIN6 cells were preincubated in the presence of 10 mM of 3-methyladenine (3MA) or 100 nM of bafilomycin A1 (Bf) for 20 min followed by exposure to 100 mM of trehalose for 24 h. (c) ER stress was induced in MIN6 cells by culturing them in the presence of thapsigargin (Th; 100 nM) or tunicamycin (Tu; 1 μg/mL) for 24 h. Lysates of the treated cells ((a)–(c)) were analyzed by Western blotting for markers of autophagy. Representative images from three independent experiments are presented.

of starvation in transplanted islets till vascularization takes place. However, excessive autophagy is known to result in cell death through crosstalk with apoptosis. Therefore it becomes a dilemma from a therapeutic angle whether to inhibit or activate autophagy in transplanted islets. To test the strategies for modulation autophagy during hypoxia, we used trehalose, rapamycin, and amino acid starvation as inducers during hypoxia. When islets were exposed to hypoxia in the presence of autophagy inducers, there was exacerbation of apoptosis. Activation of caspase-3 during hypoxia increased further in the presence of trehalose (40–65%; $P < 0.01$), rapamycin (35%; $P < 0.05$), and salt solution (115%; $P < 0.001$) (Figure 3(b)) suggesting the need for moderate autophagy during hypoxia. Next we attempted autophagic preconditioning before exposure to hypoxia as an alternate strategy. A high-throughput screen of small molecules [22] has identified among inducers of autophagy, several FDF-approved drugs including Niguldipine, Penitrem A and trifluoperazine. Therefore we screened a number of compounds at different concentrations using MIN6 cells cultured under hypoxia conditions. Based on those results, we tested some of them in human islets by preincubation followed by culture under hypoxic conditions. Significant protection ($P < 0.01$) from hypoxia-induced apoptosis was seen with all the compounds tested (Figure 3(c)). Niguldipine showed that 75% decreases in active cleaved form of caspase-3 followed by penitrem (55%), trifluoperazine (40%), and trehalose (35%).

3.5. Antiapoptotic Actions of MnSOD and Exendin-4 in MIN6 Cells. MnSOD is an antioxidant enzyme that plays an important role in scavenging free radicals generated by oxidative stress. To determine the role of oxidative stress in cytokine-mediated β cell apoptosis, a cDNA encoding MnSOD-GFP chimeric protein was overexpressed in MIN6 cells followed by exposure to a mixture of cytokines (IL-1β, TNF-α, and IFN-γ) for 48 h. Activation of caspase-9, a marker for the intrinsic pathway of apoptosis, and activation of caspase 3, a general marker for apoptosis, were examined in treated cells by immunofluorescent staining. Cytokines induced apoptosis by the intrinsic pathway in MIN6 cells as shown by the staining for the active forms of

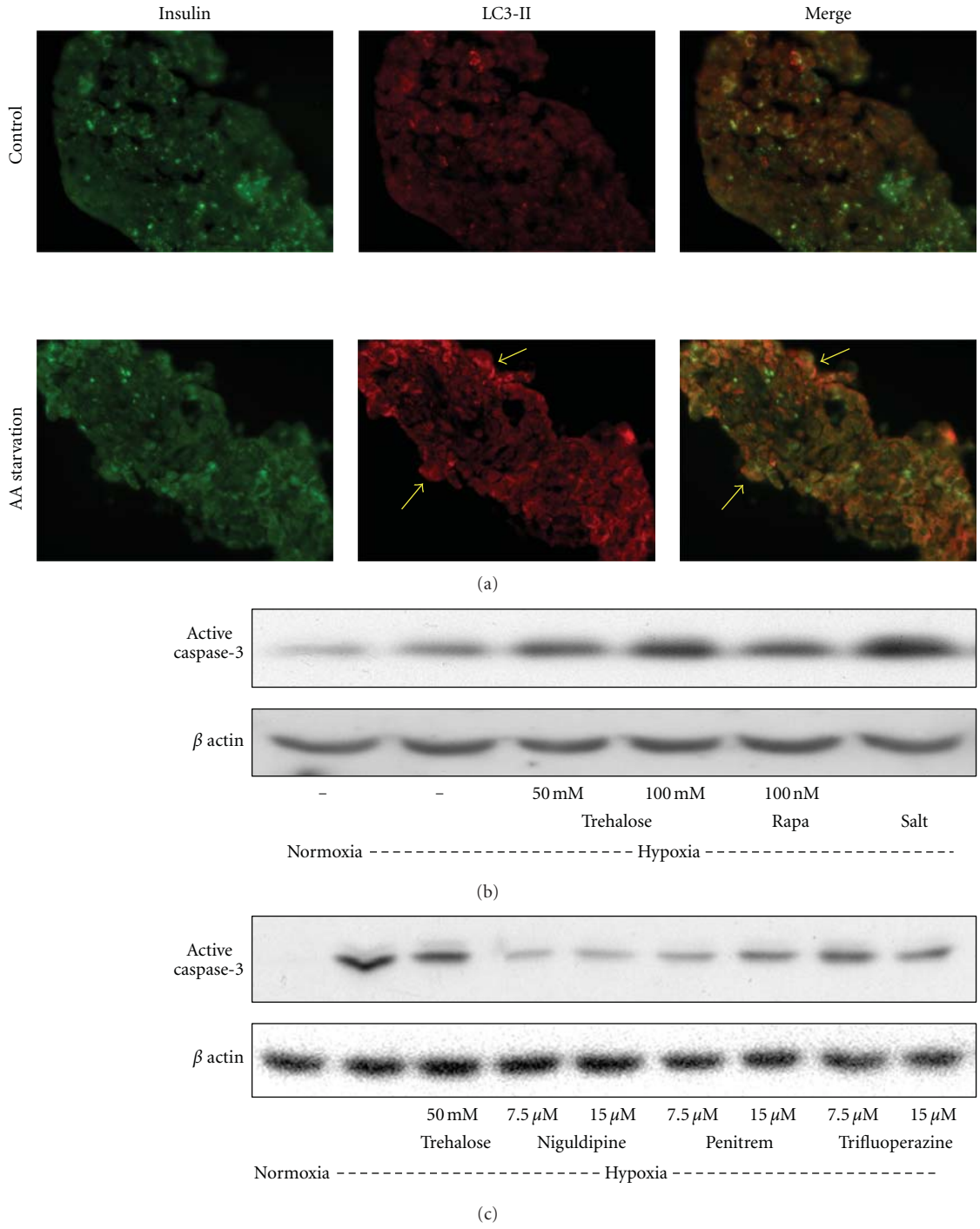

Figure 3: Induction of autophagy in human islets. (a) Human islets were incubated for 8 h in Miami medium (control) or in Hank's balanced salt solution for amino acid (AA) starvation. Treated islets were fixed in paraformaldehyde and embedded in OCT. Frozen sections (7 μm thickness) were immunostained for insulin with FITC (Green) and for LC3-II with cy3 (red). Formation of autophagosomes was visualized by the punctated staining of LC3-II in β cells (arrows). (b) Human islets were cultured under normoxic or hypoxic conditions in the absence and presence of inducers of autophagy, trehalose, rapamycin (Rapa) or salt solution (Salt) for 8 h. Cell lysates were processed for Western blot analysis of the active form of caspase-3. Induction of autophagy during hypoxia exacerbated apoptosis in islets. (c) Human islets were preincubated with inducers of autophagy for 4 h. Following change of medium, the cells were cultured under normoxic or hypoxic conditions for 12 h, lysed and processed for the Western blot analysis of active caspase-3. Autophagic preconditioning protected islets from hypoxia-induced apoptosis. Representative images from experiments with three independent batches of human islets are presented.

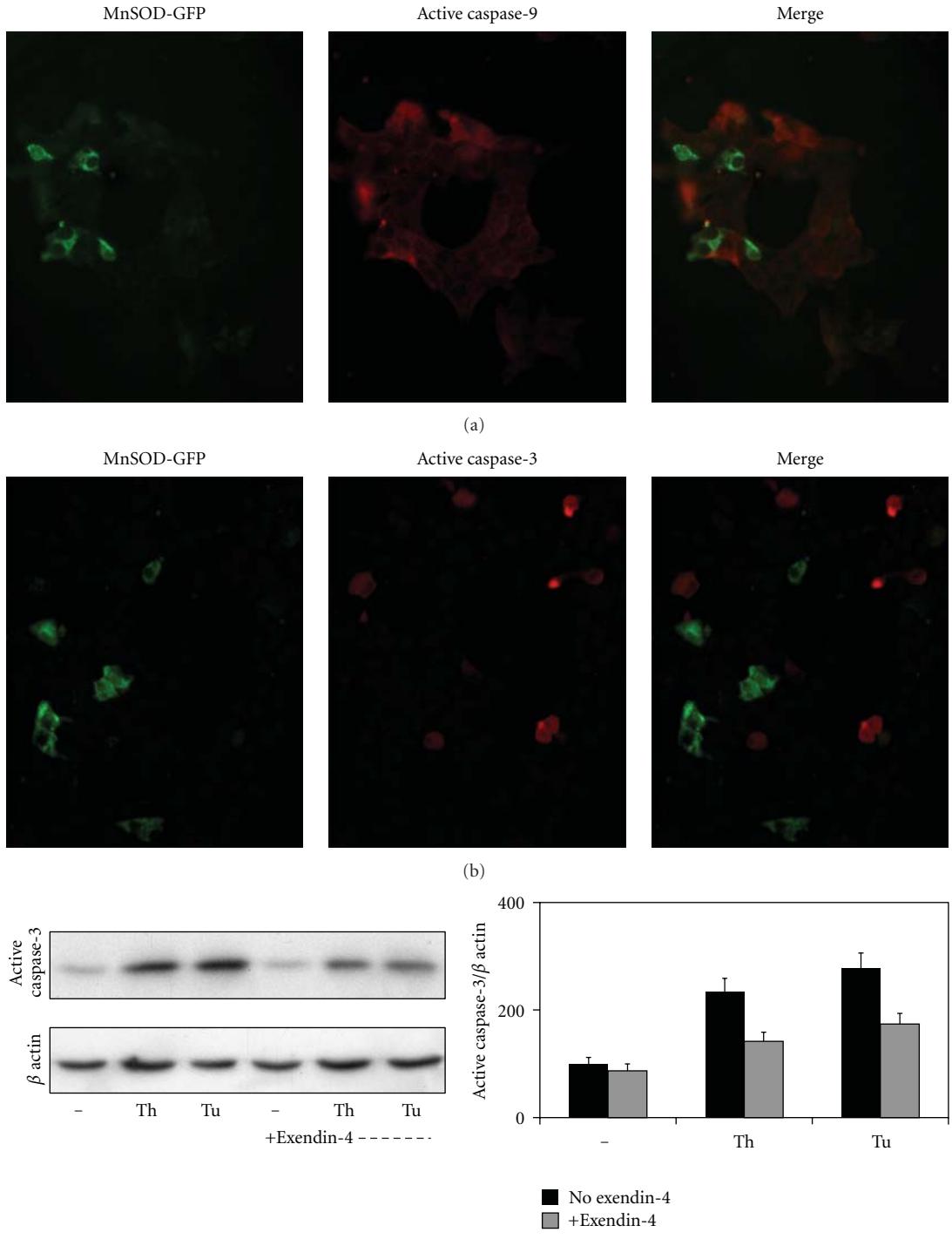

Figure 4: Antiapoptotic actions of MnSOD and exendin-4 in MIN6 cells: MIN6 cells cultured on chamber slides were transfected with cDNA encoding MnSOD-GFP chimeric protein. The transfected cells were exposed to a mixture of cytokines (2 ng of IL-1β, 10 ng of TNF-α and 10 ng of IFN-γ) for 24 h. The cells were fixed, permeabilized and probed with antibodies specific for the active cleaved fragment of caspase-9 (a) and caspase-3 (b). This was followed by probing with secondary antibodies linked to Cy3. The images were analyzed by digital deconvolution microscopy. The cells expressing MnSOD (green) are protected from cytokine-induced apoptosis. Images from three independent experiments are presented. (c) MIN6 cells were cultured in the absence and presence of exendin-4 (50 nM). ER stress was induced by exposing the cells to thapsigargin (Th; 100 nM) or tunicamycin (Tu; 1 μg/mL) for 24 h. Lysates of the treated cells were analyzed by Western blotting for active caspase-3 and β actin. Band intensities were quantitated by scanning. Results are M $\pm$ SE of three independent experiments. $^{**}P < 0.001$ when compared to untreated control. $^{*}P < 0.01$ versus respective ER stress control in the absence of exendin-4.

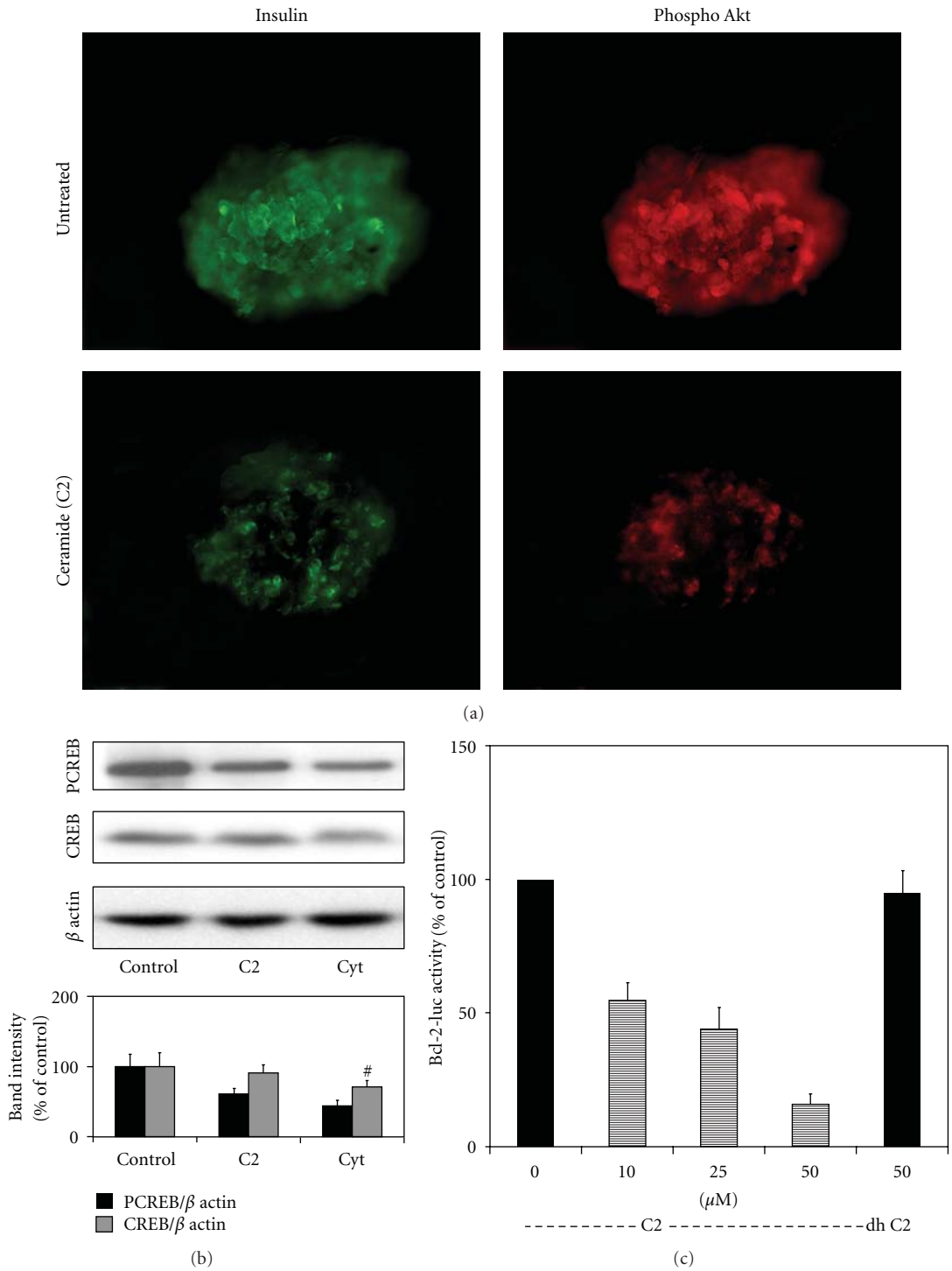

Figure 5: Ceramide is a mediator of cytokine-mediated CREB downregulation. (a) Human islets were exposed to C2 (10 μM), a synthetic ceramide, for 24 h. The islets were fixed and immunostained for phospho Akt (Thr 308). The images were analyzed by digital deconvolution microscopy. Decrease in the active form of Akt was observed in ceramide-treated islets. (b) Islets were incubated in the absence and presence of C2 and a combination of cytokines for 24 h and processed for the Western blot analysis of phosphorylated form of CREB, total CREB and β actin. Band intensities were quantitated by scanning. The results are M $\pm$ SE of 3 independent experiments. $^{*}P < 0.01$; $^{**}P < 0.001$; $^{\#}P < 0.05$ compared to untreated control. Active form of CREB was decreased by both C2 and cytokines. (c) MIN6 cells were transfected with a CRE site-containing bcl-2 promoter linked to a firefly luciferase reporter and a constitutively active renilla luciferase (for transfection efficiency). The transfected cells were exposed to increasing concentrations of C2 and 50 μM of an inactive analogue (dh) for 24 h. The cells were processed for luciferase activities using a dual luciferase assay kit. The ratio of firefly and renilla luciferase activities was taken as a measure of bcl-2 promoter activity. Bcl-2 promoter activity was inhibited by the ceramide. The results are M $\pm$ SE of 4 independent experiments. $^{*}P < 0.01$; $^{**}P < 0.001$ compared to untreated control.

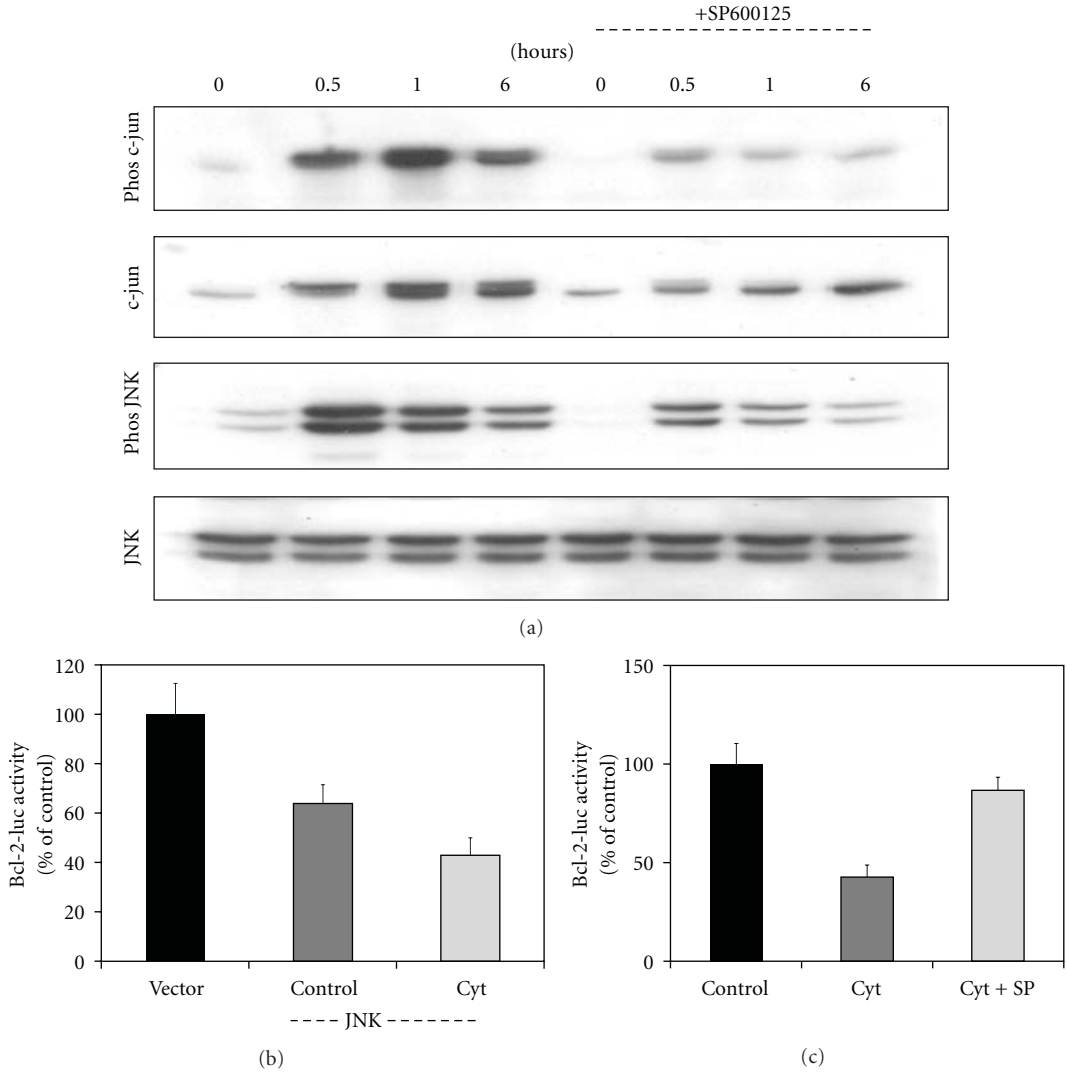

Figure 6: Activation of JNK by cytokines. (a) MIN6 cells were incubated in the absence and presence of 20 μM JNK inhibitor, SP600125 and/or a combination of cytokines, IL-1β (2 ng/mL) TNF-α (10 ng/mL) and IFN-γ (10 ng/mL) for the indicated time periods. The treated cells were processed for the Western blot analysis of phosphorylated (Phos) c-jun, total c-jun, phosphorylated (Phos) JNK and total JNK. ((b) and (c)) MIN6 cells were transfected with a CRE site-containing bcl-2 promoter linked to a firefly luciferase reporter and a constitutively active renilla luciferase. A combination of plasmid mixture encoding the JNK isozymes or their vector was also included for one experiment (b). Transfected cells were incubated in the absence and presence of a combination of cytokines and JNK inhibitor (SP600125) as indicated for 24 h followed by the assay for luciferases. Bcl-2 promoter activity was inhibited by cytokines and JNK isozymes. Cytokine action on bcl-2 promoter activity was blocked by the JNK inhibitor. The results are M ± SE of 4 independent experiments. $^{*}P < 0.01$; $^{**}P < 0.001$ versus untreated control.

caspase-9 (Figure 4(a)) and caspase-3 (Figure 4(b)) with cy3 (red). MnSOD-expressing cells were identified by the green fluorescence. Staining for active caspase-9 and -3 did not colocalize with MnSOD-GFP-expressing cells suggesting that these cells are protected from cytokine-induced apoptosis. At least 1000 GFP-expressing cells were examined for each experiment. We also tested the cytoprotective action of exendin-4, a glucagon-like peptide- (GLP-1) analogue, in MIN6 cells following induction of ER stress. Exposure of MIN6 cells to thapsigargin and tunicamycin, inducers of ER stress, caused apoptosis as shown by 135–180% ($P < 0.001$) increases in the levels of active caspase-3 (Figure 4(c)). Preincubation of MIN6 cells with exendin-4 resulted in significant ($P < 0.01$) protection with 40% decrease in the activation of caspase-3.

3.6. Cytokine-Generated Ceramides Interfere with Activation of Akt and CREB. Ceramides generated by cytokines are known to decrease Akt phosphorylation [23]. Akt plays a significant role in CREB phosphorylation and activation, leading to improved cell survival [24]. Therefore, human islets were exposed to 10 μM of synthetic ceramide (C2) and the active phosphorylated form of Akt was immunofluorescently stained with Cy3 (red) along with staining for

insulin with FITC (green). Treatment with C2 resulted in decrease in the levels of active form of Akt (Figure 5(a)). Western blot analysis of human islets treated with C2 or a combination of IL-1β, TNF-α, and IFN-γ showed 40–55% decrease in PCREB, the active phosphorylated form of CREB (Figure 5(b)). There was also a modest decrease ($P < 0.05$) in CREB levels in cytokine-treated islets. The activity of CRE-site containing bcl-2 promoter was also inhibited in a dose-dependent manner by C2 whereas the inactive analogue (dh C-2) did not affect the reporter activity (Figure 5(c)). We have previously reported similar downregulation of bcl-2 promoter by cytokines [25]. Thus a synthetic ceramide mimics the action of cytokines on β cell apoptosis.

3.7. Activation of JNK Pathway by Cytokines Plays a Role in Downregulation of Bcl-2 Promoter Activity. Cytokines strongly stimulate the signaling pathways leading to JNK activation. JNK-mediated activation of the transcription factor c-jun plays a critical role in inducing stress-associated genes in β cells [26]. A combination of cytokines strongly increased the active phosphorylated forms of JNK isozymes with the peak activation ($P < 0.001$) being observed at 30 min (Figure 6(a)). The levels of total JNK remained the same during the 6 h incubation period. Activation of JNK led to increases in the active phosphorylated form of c-jun. Maximum activation (370%) was seen at 1 h. The levels of c-jun also increased (110–235%) during 6 h exposure to cytokines as it is an autoregulated gene. Preincubation with the JNK inhibitor resulted in significant ($P < 0.01$) decreases in the phosphorylation of c-jun and in the levels of c-jun. As SP600125 also acts on kinases upstream of JNK, the phosphorylation of JNK itself was reduced. The bcl-2 promoter activity was inhibited when a combination of JNK isozymes was also expressed (Figure 6(b)). Cytokines further enhanced the downregulation of bcl-2 promoter activity by JNK isozymes whereas JNK inhibitor blocked cytokine action on bcl-2 promoter (Figure 6(c)).

4. Discussion

Oxidative stress is known to play an important role in β cell death in diabetes and in transplanted islets. Autophagy is a physiological mechanism that protects β cells under conditions of starvation and hypoxia. However, excessive autophagy resulting from oxidative stress can lead to β cell death by crosstalk with apoptosis pathway. In this study, we report the complex modulation of apoptosis pathway genes in human islets exposed to oxidative stress, hypoxia, and cytokines suggesting interactions between pathways involved in cell death as well as cell survival. We demonstrate that autophagic preconditioning of human islets leads to protection against hypoxia-induced apoptosis. Inducers of ER stress were also found to activate autophagy and apoptosis in β cells.

Pathway-specific, PCR-based arrays represent a novel technique to determine the expression of a family of genes associated with a specific pathway. We examined the expression of apoptosis pathway genes in human islets exposed to multiple stresses associated with diabetes, islet isolation, and transplantation. We observed significant increases in the expression of genes that are involved in opposing pathways of cell survival and cell death. It is also important to mention four genes, namely BFAR, CARD8, CIDE-A, and BNIP3, as their role in β cell apoptosis has not been reported previously. BFAR is a multidomain protein that improves cell survival by interacting with members of extrinsic and intrinsic pathways of apoptosis. Caspase-associated recruitment domain (CARD) family of proteins participates in the activation or inhibition of caspases. CARD8 inhibits the activation of caspase-9. The levels of both BFAR and CARD8 decreased significantly in islets exposed to oxidative stress and hypoxia, suggesting a decrease in antiapoptotic defense in islets. Levels of CIDE-A, a gene that acts downstream of caspases to mediate DNA fragmentation, increased 2- to 3-folds in islets exposed to stress, suggesting late stages of apoptosis. The expression of BNIP3 which plays an important role in autophagy by disrupting the interaction between Beclin 1 and Bcl-2 was induced by peroxynitrite and hypoxia. BNIP3 promotes mitochondrial autophagy as an adaptive response to hypoxia [27].

Several studies have reported significant interactions between the pathways of apoptosis and autophagy [12, 28, 29]. Apoptosis and autophagy could act as partners to induce cell death. For example, death-associated protein kinase (DAPK) family members, activated by cytokines, are involved in cell death by apoptosis as well as autophagy [12]. Conjugation of ATG5 with ATG12 is an important step in the formation of autophagosomes during basal autophagy. However, when there is excessive autophagy, ATG5 has been reported to interact with components of pathways of apoptosis. Calpain cleaves ATG5 to generate a truncated form of this protein which translocates to mitochondria and induces the release of cytochrome c from mitochondria to activate the intrinsic pathway. The antiapoptotic proteins Bcl-2 and Bcl-xL bind and inhibit Beclin 1-mediated autophagy [30]. This interaction results in the inhibition of autophagy because Beclin 1 will not be able to bind to class III PI 3-kinase (VPS34) and UVRAG, an important step in the initiation of autophagosome formation. Essentially, Bcl-2 keeps autophagy under control (Figure 7). We have demonstrated previously that Bcl-2 levels are regulated by CREB [25]. Therefore, under conditions of CREB downregulation, the pathway of autophagy can be dysregulated. In addition, JNK1-mediated phosphorylation of Bcl-2 also results in the disruption of its interaction with Beclin 1, leading to activation of autophagy [31]. Thus physiological autophagy which improves cell survival can become uncontrolled when β cells are exposed to oxidative stress and cytokines.

Autophagy is beneficial to islets in the transplantation setting because it clears organelles damaged by oxidative stress generated during islet isolation and provides energy during starvation (hypoxia). Immediately after transplantation, islets are exposed to hypoxia due to delayed revascularization, leading to a decreased supply of nutrients to β cells at the islet core. However, we did not observe protection of islets when autophagy was induced by trehalose,

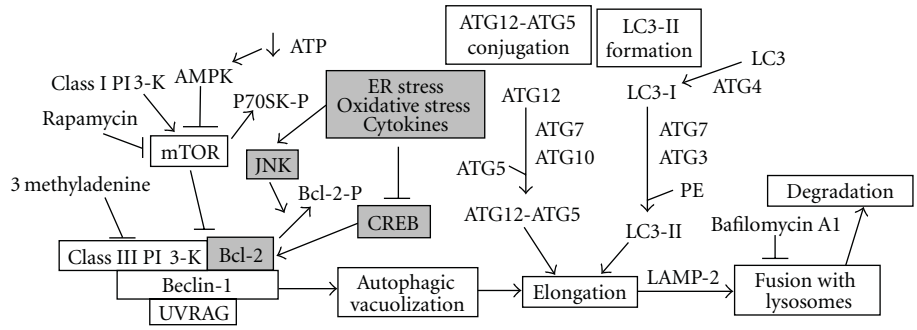

Figure 7: Interactions of signaling by ER stress, oxidative stress, and cytokines with the pathways of autophagy: Induction of autophagy requires the assembly of Beclin-1 with class III PI 3 kinase (Vps34) and UVRAG. Rapamycin is an activator of autophagy as it is negatively regulated by class I PI 3 kinase through mammalin target of rapamycin (mTOR). The elongation step involves two ubiquitin-like conjugation system. ATG12 is activated by E1-like ATG7, transferred to E2-like ATG10 and conjugated to ATG5. Similarly, LC3-I is activated by E1-like ATG7, transferred to E2-like ATG3, and conjugated to phosphatidylethanolamine (PE). Inhibitors of autophagy include 3 methyl adenine which inhibits class III PI 3-K and bafilomycin A1 which inhibits the formation of autophagolysosome. Interaction of Bcl-2 with Beclin-1 keeps the autophagic pathway under control. CREB induces the expression of Bcl-2 which is downregulated by ER stress, oxidative stress and cytokines through JNK.

rapamycin and salt solution during hypoxia (Figure 3(b)). Next, we attempted an autophagic preconditioning experiment with the inclusion of other new inducers. There was indeed significant protection in these cells when exposed to hypoxia as shown by decrease in the activation of caspase-3 (Figure 3(c)). Especially, FDA-approved drugs including niguldipine and penitrem A showed significant protection. Islets immediately following isolation are likely to display defects in the autophagic machinery and are likely to succumb to hypoxia-induced cell death after transplantation. Thus, there is a need to restore the normal autophagic pathway in cultured islets before transplantation. Current understanding in the islet transplantation field is that it is desirable to culture islets for 24–72 h before transplantation since islets can recover from isolation-induced stress and immunogenicity of the islets is reduced. This culture period could also be used to assess and manipulate autophagic pathway to improve transplantation outcome.

The pancreatic β cells are particularly vulnerable to oxidative stress-induced injury due to low-level expression of antioxidant enzymes [32, 33]. Markers of oxidative stress and cellular fragility (RBC) have been shown to be elevated in nondiabetic relatives of type 1 diabetic patients [34]. It has been reported that β cell apoptosis could be significantly reduced in NOD mice, an autoimmune diabetic animal model, after administration of an MnSOD mimetic that is known to scavenge free radicals [35]. Proinflammatory cytokines induce the expression of iNOS through NF-κB and GAS sites in iNOS promoter leading to the generation of nitric oxide [36]. When nitric oxide combines with superoxide generated by macrophages or cytokines, highly toxic peroxynitrite is generated. Several studies have demonstrated that peroxynitrite is an important mediator of cytokine-induced β cell death in type 1 diabetes [37–39]. In addition, ceramide is known to be one of the mediators of cytokine action. The decrease of active form of Akt by ceramides can interfere with growth factor action on CREB. We observed that peroxynitrite and C2, a synthetic ceramide, mimic cytokine-induced apoptosis pathway (Table 1 and Figure 5).

Although CREB elicits antiapoptotic pathways in β cells, its function can be impaired by other transcription factors in diabetes. For example, JNK and its nuclear target, c-jun, have been shown to be mediators of β cell-death. JNK-activated c-jun induces the expression of stress-associated genes [40]. Cytokines strongly stimulate the signaling pathways leading to JNK activation [26]. Cell-permeable peptide inhibitors of JNK block cytokine-induced β cell death [41]. Inhibition of JNK is being considered as an important strategy to improve β cell survival. We have reported that SP600125, a JNK inhibitor, activates CREB [42].

ER stress is known to interact with the pathways of apoptosis and autophagy. For example, Islet amyloid polypeptide (IAPP) which is colocalized and secreted with insulin from β cell granules [43] induces β cell apoptosis in cultured islets and in type 2 diabetes [44–46]. ER stress is the main mechanism through which IAPP aggregates cause β cell apoptosis [47, 48]. Silencing of IAPP with siRNA improves the survival of cultured human islets [49]. ER stress is also a potent inducer of autophagy [50, 51]. It has been suggested that autophagy might represent a protective cellular response under conditions of ER stress [52]. We observed formation of LC3-II, a marker for autophagy in MIN6 cells following induction of ER stress with thapsigargin and tunicamycin (Figure 2(c)). ER stress inducers also activated caspase-3 which was significantly reduced by exendin-4, a GLP-1 analogue (Figure 4(c)).

It this study, we demonstrate that multiple stresses, generated during islet isolation and transplantation, induce β cell death by orchestration of gene expression patterns in

apoptosis pathway. Although a parallel cell survival pathway is also activated, it is overwhelmed by stress-induced proapoptotic genes. Autophagy, a physiological cytoprotective pathway, interacts with apoptosis when induced in excess in the islet transplantation setting. Therefore, multiple approaches are needed to improve islet transplantation outcome.

Acknowledgments

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Review Article

Endoplasmic Reticulum Stress and Insulin Biosynthesis: A Review

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Insulin resistance and pancreatic beta cell dysfunction are major contributors to the pathogenesis of diabetes. Various conditions play a role in the pathogenesis of pancreatic beta cell dysfunction and are correlated with endoplasmic reticulum (ER) stress. Pancreatic beta cells are susceptible to ER stress. Many studies have shown that increased ER stress induces pancreatic beta cell dysfunction and diabetes mellitus using genetic models of ER stress and by various stimuli. There are many reports indicating that ER stress plays an important role in the impairment of insulin biosynthesis, suggesting that reduction of ER stress could be a therapeutic target for diabetes. In this paper, we reviewed the relationship between ER stress and diabetes and how ER stress controls insulin biosynthesis.

1. Introduction

1.1. Diabetes and ER Stress. The endoplasmic reticulum (ER), a membrane compartment located near the nucleus, is the organelle where polypeptides, which will become secretory proteins or membrane proteins, are synthesized from mRNA and become mature proteins after undergoing folding, assembly, glycosylation, disulfide bonding, and posttranslational modifications [1]. The ER is well developed in endocrine cells such as pancreatic beta cells in which secretory proteins are synthesized. Proper functioning of the ER is essential to cell survival. ER stress is defined as an imbalance between client protein load and folding capacity and can be caused by multiple mechanisms including increases in improperly folded proteins, impairment of protein transport from the ER to the Golgi, inhibition of protein glycosylation, reduced disulfide bond formation, and calcium depletion of the ER lumen. When ER stress occurs, cellular defense mechanisms related to the ER stress response are activated. The ER stress response is comprised of (1) activation of the protein-kinase-RNA-(PKR-) like ER kinase (PERK) and reduction of protein translation by phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2α); (2) activation of the inositol-requiring 1 (IRE1)/X-box- binding-protein- 1 (XBP-1) protein and the activating transcription factor 6 (ATF6) through the increased expression of ER chaperones and subsequent increase in ER folding capacity; (3) ER stress-associated protein degradation, which degrades unfolded or improperly folded proteins; (4) apoptosis by the activation of CCAAT/enhancer-binding homologous protein (CHOP) [2–4].

Type 2 diabetes is characterized by insulin resistance and pancreatic beta cell dysfunction. Pancreatic beta cells compensate for insulin resistance by hypersecretion of insulin; however, at some point, pancreatic beta cells fail to secrete sufficient insulin, resulting in diabetes [5]. The pancreatic beta cells are susceptible to ER stress not only because of physiological variations in glucose levels that potently stimulate insulin translation but also because of other conditions that can cause ER stress such as metabolic dysregulation associated with obesity, including excess nutrients [6–10] and inflammatory cytokines [11–13]. Obesity can also contribute to insulin resistance via ER stress [14, 15]. In addition, many studies have shown that increased ER stress induces pancreatic beta cell dysfunction and diabetes mellitus using genetic models of ER stress [16–24].

1.2. Glucotoxicity or Lipotoxicity. For type 2 diabetic patients, a common clinical course is to respond to therapy initially by normalizing their fasting glucose levels, but then to undergo

gradual deterioration in glycemic control, despite optimal medical management using a variety of drugs [25]. Although optimal management of type 2 diabetes regulates fasting glucose levels, most patients continue to have abnormally elevated postprandial glucose levels, which results in glucotoxicity [26]. Glucotoxicity is defined as nonphysiological and potentially irreversible pancreatic beta cell damage caused by chronic exposure to supraphysiological glucose concentrations [26]. Supraphysiological glucose concentrations also have adverse effects on cellular structure and function in tissues throughout the body. Chronic exposure to high glucose concentrations in HIT-T15 cells over 50 weeks caused a decrease in insulin gene transcription factors such as pancreatic-duodenal-homeobox-factor-1 (PDX-1) and MafA, insulin promoter activity, insulin mRNA, insulin content, and insulin secretion [27–31]. Glucotoxicity implies damage and irreversibility and should be differentiated from desensitization and exhaustion, which are reversible phenomena [32].

Obesity is a major predisposing factor for type 2 diabetes. The increase in free fatty acids (FFAs) due to obesity causes insulin resistance and, as a consequence, pancreatic beta cells secrete more insulin to compensate for insulin resistance and maintain normoglycemia. However, failure to compensate for insulin resistance by the pancreatic beta cells results in beta cell dysfunction and diabetes [5]. This phenomenon, which is a lipid-induced pancreatic beta cell dysfunction, is lipotoxicity. Chronic exposure to FFAs results in decreased insulin gene expression and proinsulin processing [33] and increased pancreatic beta cell death [34]. Recently, the concept of "glucolipotoxicity" has been introduced because lipotoxicity is dependent on elevated glucose levels and elevated glucose and FFAs have a synergistic effect on impairing pancreatic beta cell function [35]. Recently, increasing evidence has implicated ER stress in glucotoxicity and glucolipotoxicity in pancreatic beta cells [6, 9, 10].

1.3. Insulin Biosynthesis. Pancreatic-beta-cell-specific expression of the insulin gene requires both ubiquitous and cell-specific activators, which have target sequences within the enhancer located between −340 and −90 nucleotides relative to the transcription start site [36, 37]. Transcription of the insulin gene is regulated primarily by transcription factors such as PDX-1, BETA/NeuroD, and RIPE3b1/MafA. Under physiological conditions, MafA and PDX-1 bind to the C elements and the A boxes, respectively. Glucotoxicity greatly diminishes protein levels of PDX-1 through a posttranscriptional mechanism, and of MafA through a posttranslational mechanism. These abnormalities lead to decreases in insulin mRNA, insulin content, and glucose-induced insulin secretion [28, 30].

The ER plays an important role in the biosynthesis of insulin since the early steps of insulin biosynthesis occur in the ER [38]. The insulin gene encodes the preproinsulin polypeptide. Insulin is the posttranslational product of preproinsulin and is a globular protein containing two chains, A (21 residues) and B (30 residues). As preproinsulin is synthesized in the cytoplasm with a signal peptide, it is cotranslationally translocated into the lumen of the ER through the interaction between the signal peptide and the signal recognition particle on the ER membrane. The signal peptide of preproinsulin is cleaved in the ER and proinsulin is produced. In the lumen of the ER, proinsulin undergoes protein folding whereby three disulfide bonds are formed, which are essential for stability and bioactivity. Properly folded proinsulin is then delivered to the Golgi apparatus and packaged into secretory granules [39]. The conversion of proinsulin to insulin takes place in the secretory granules. Mature insulin is then released by exocytosis [40]. Therefore, ER stress due to increased misfolded proinsulin may induce beta cell dysfunction and diabetes.

Various conditions that are associated with diabetes mellitus, such as glucotoxicity and lipotoxicity, have been implicated in ER stress in pancreatic beta cells [41–43]. This paper focuses on the relationship between ER stress and insulin gene biosynthesis.

2. PERK/ATF4

When unfolded proteins accumulate in the ER lumen, the first response is to attenuate further translation of mRNAs, which reduces the ER load of new protein, preventing further accumulation of unfolded protein. This translational attenuation is mediated by PERK/eIF2α [19, 44]. In response to ER stress, eIF2α is phosphorylated by PERK. EIF2α is a heterotrimeric protein that is required to bring the initiator methionyl-transfer RNA (Met-tRNA) to the ribosome. PERK is a type I transmembrane serine/threonine kinase localized in the ER membrane. Under unstressed conditions, the ER chaperone Bip binds to the ER luminal domain of PERK and maintains this protein in an inactive form. Upon induction of ER stress, Bip binds to unfolded proteins and is thus competitively dissociated from PERK, leading to the activation of PERK by oligomerization and *trans*-autophosphorylation. Consequently, activated PERK phosphorylates eIF2α and inhibits translation [3, 44]. Phosphorylated eIF2α promotes expression of stress-induced genes, such as the transcription factors ATF4 and CHOP [22]. Moreover, in response to long-term adaptation to stress conditions, phosphorylation of eIF2α induces the expression of the growth arrest and DNA damage gene, GADD34. GADD34 is a stress-inducible regulatory subunit of a holophosphatase complex that dephosphorylates eIF2α together with protein phosphatase 1c (PP1c), and is an important component of translational recovery during the ER stress response [45, 46].

Many studies have reported the relationship between PERK and diabetes [18–20, 47–49]. Wolcott-Rallison syndrome indicates that the PERK gene is correlated with diabetes. Wolcott-Rallison syndrome is a rare human autosomal recessive genetic disorder characterized by early infancy type 1 diabetes resulting from mutations in the PERK gene [18, 47]. A similar phenotype has been described in PERK-/- mice [19]. The exocrine and endocrine pancreases develop normally in Perk-/- mice, but there is a progressive loss of insulin-producing pancreatic beta cells in the islets of Langerhans of Perk-/- mice postnatally, resulting in hyperglycemia and reduced serum insulin levels. Moreover, ER distention and activation of the ER stress transducer IRE1α,

accompanied by increased cell death, leads to progressive diabetes mellitus and exocrine pancreatic insufficiency [19]. Zhang et al. reported that PERK-deficient mice exhibit severe defects in fetal/neonatal pancreatic beta cell proliferation and differentiation, resulting in low pancreatic beta cell mass, defects in proinsulin trafficking, and abrogation of insulin secretion, which together culminate in permanent neonatal diabetes [20]. Yusta B et al. reported that exendin-4, a GLP-1 receptor agonist, reduced the downregulation of insulin translation and improved cell survival under ER stress conditions. Exendin-4 increased ATF4 and CHOP expression and also potentiated the induction of GADD34 and PP1c activity, resulting in decreased phosphorylation of eIF2α and a faster recovery from translational repression. These findings show that GLP-1 receptor signaling modulates the ER stress response, leading to enhanced pancreatic beta cell survival [48].

3. IRE/XBP

The second response to ER stress is an increase in proteinfolding activity via the induction of ER chaperones such as Bip. This response is mediated by IRE1 and ATF6. IRE1 is a type I transmembrane endonuclease localized in the ER membrane. Similar to PERK, activation of IRE1 is triggered by dissociation of Bip from the IRE1 ER luminal domain, which leads to oligomerization and *trans*-autophosphorylation. Subsequently, IRE1 induces splicing of XBP-1, which upregulates unfolded protein response (UPR) target genes [2–4, 50]. IRE-XBP-1 signaling is important in secretory cells, such as exocrine pancreatic cells [51]. Lipson et al. reported that chronic hyperglycemia induces ER stress and activates IRE1, resulting in suppression of insulin expression at the transcriptional level [7]. Transient high glucose conditions induced the activation of IRE1α in pancreatic islet cells. Inactivation of IRE1α signaling by siRNA or inhibition of IRE1α phosphorylation decreases insulin biosynthesis under the transient high glucose conditions. However, IRE1 activation by high glucose concentrations was not accompanied by Bip dissociation and XBP-1 splicing, but IRE1 target genes were upregulated. These findings suggest that under transient high glucose conditions like postprandial hyperglycemia, IRE1α is activated and enhances proinsulin biosynthesis. By contrast, sustained activation of IRE1 signaling by chronic high glucose exposure causes ER stress, leading to the suppression of insulin mRNA expression. These findings suggested that sustained activation of IRE1α may decrease insulin biosynthesis at the transcriptional level. Overall, physiological IRE1α activation by transient high glucose conditions has a beneficial effect, but pathological IRE1α activation by chronic high glucose exposure is harmful to cells. In addition, under transient hyperglycemic conditions, activation of IRE1α was not accompanied by XBP-1 splicing, but long-term exposure to high glucose induced IRE1α activation and XBP-1 splicing, suggesting that XBP-1 splicing could be a marker of chronic hyperglycemic conditions [7]. Pirot et al. reported that IRE/XBP-1 increases degradation of insulin mRNA. Cyclopiazonic acid (CPA) is a sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) blocker, which depletes ER calcium stores and induces ER stress. When INS-1 cells were treated with CPA, insulin mRNA levels decreased. Treatment with CPA did not affect insulin promoter activity, indicating that the decrease in insulin mRNA was not caused by a decrease in insulin transcription. By contrast, when cells were pretreated with actinomycin D, which arrests transcription, treatment with CPA induced a decrease in insulin expression that was due to mRNA degradation. Moreover, this study showed that insulin mRNA degradation in response to CPA was paralleled by an increase in IRE1 activation [52]. Another study has suggested that sustained production of spliced XBP-1 (XBP-1s) induces beta cell dysfunction by decreasing insulin gene expression, leading to apoptosis [53]. Adenoviral-mediated overproduction of XBP-1s resulted in increased XBP-1 activity and increased expression of XBP-1 target genes. XBP-1s overexpression impaired glucose-stimulated insulin secretion, increased beta cell apoptosis, and decreased levels of insulin, Pdx1, and Mafa mRNA. XBP-1s knockdown partially restored cytokine/ER-stress-driven insulin and Pdx1 inhibition. These data suggest that prolonged XBP-1s production induces beta cell dysfunction through inhibition of Pdx1, MafA, and insulin expression leading to beta cell apoptosis [53].

4. ATF6

ATF 6 is another mediator of ER- stress-induced transcription. ATF6 is a member of the ATF, cAMP response element-binding protein, basic-leucine zipper (bZIP) DNA-binding protein family of transcriptional activators [54]. ATF6 is a 90 kDa protein (p90ATF6) of 670 amino acids [55] and contains a transmembrane domain (amino acids 378–398) with the N terminus facing the cytoplasm [56]. In the unstressed state, p90ATF6 is localized to the ER [56]. In response to ER stress, ATF6 translocates from the ER to the Golgi [56, 57], where it is cleaved by site 1 and site 2 proteases [58]. Proteolytic cleavage of ATF6 removes the N-terminal cytosolic domain, which is transported into the nucleus and directly induces transcriptional activation of chaperone molecules such as BIP/GRP78 and other enzymes that are essential for protein folding [56, 58–60]. Moreover, activated ATF6 directly binds to the ER stress response element (ERSE) and induces XBP-1 expression.

The spliced form of XBP-1 is produced from the upregulated XBP-1 mRNA by activation of IRE1α and binds to ERSE sequences directly. When ER stress occurs, activation of ATF6 is rapid because it is produced from a preexisting precursor protein, whereas activation of XBP-1 is slow because XBP-1 mRNA must be induced, spliced, and translated to produce an active form of XBP-1. This observation suggests that ATF6 is activated early in response to ER stress and XBP-1 functions in more sustained ER stress [61, 62]. A prolonged increase in the demand for insulin often leads to defects in insulin secretion, resulting in sustained hyperglycemia [63]. Chronic hyperglycemia has deleterious effects on beta cell function, as shown in primary cultured rat and human islet cells and in beta cell lines [64].

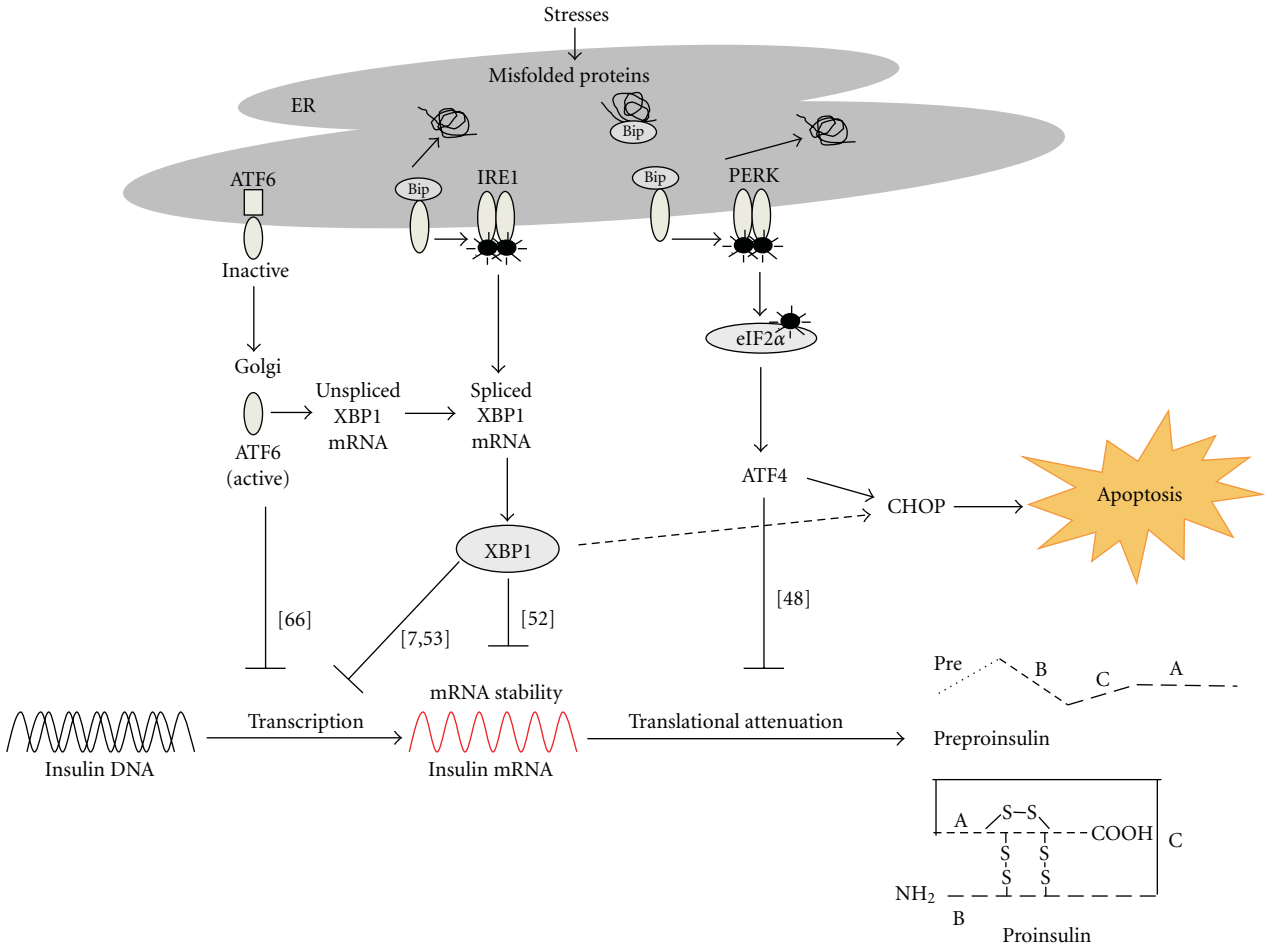

FIGURE 1: Possible mechanisms of insulin biosynthesis by endoplasmic reticulum (ER) stress. Insulin biosynthesis is affected by ER stress through different mechanisms, such as activation of the following pathways: transcription factor 6 (ATF6), inositol-requiring 1 (IRE1)/X-box-binding-protein-1 (XBP-1), and protein-kinase-RNA (PKR-) like ER kinase (PERK). The exposure of beta cells to high glucose chronically induces ER stress, resulting in the activation of ATF6. Activation of ATF6 impairs insulin gene expression. Long-term exposure to high glucose induces IRE1α activation and XBP-1 splicing, leading to the suppression of insulin mRNA expression and to increases in the degradation of insulin mRNA. In addition, downstream of PERK, ATF4, and CHOP inhibit proinsulin synthesis via translational attenuation mediated by PP1c and GADD34. Numbers in parentheses are the references cited in this paper.

Previously, our studies confirmed that chronic high glucose induces beta cell dysfunction. Under basal glucose concentrations (5 mmol/l glucose), insulin mRNA is expressed abundantly in INS-1 cells, but, in the presence of 30 mmol/l glucose, insulin mRNA expression was decreased in a time-dependent manner until only minimal levels were detected by 96 h. The expression of PDX-1 and MafA, which are the main transcription factors associated with insulin gene expression, also decreased over time in the 30 mmol/l glucose [65]. In addition, chronic hyperglycemia induced ER- stress in cultured INS-1 cells and ER stress-induced activation of ATF6 impaired insulin gene expression via upregulation of the orphan nuclear receptor small heterodimer partner (SHP). Prolonged exposure of INS-1 cells to a high concentration of glucose increased ER stress in the cells, and ER stress induced by the chemical ER stressors tunicamycin, thapsigargin, and DTT impaired insulin gene expression. Among the three different signaling pathways of the ER stress response (ATF6, IRE1, and PERK), ATF6 inhibited insulin promoter activity, but IRE1-XBP1 and PERK-eIF2α-ATF4 did not. Adenovirus-mediated overexpression of the active form of ATF6 (Ad-ATF6) in INS-1 cells down-regulated PDX-1 and MafA gene expression and repressed the cooperative action of PDX-1, BETA2, and MafA in stimulating insulin transcription [66].

Several *in vitro* studies have shown that SHP negatively regulates insulin biosynthesis and secretion in pancreatic beta cells [67]. SHP also directly represses the transcriptional activity of the basic helix-loop-helix transcription factor BETA2, a positive regulator of insulin gene expression [68], and indirectly represses p300-enhanced BETA2/NeuroD transcriptional activity through inhibition of the BETA2-p300 interaction [69]. Previously, we reported that glucotoxicity in INS-1 cells is mediated by SHP. Culture of INS-1 cells and rat pancreatic islets in the continuous presence of high glucose concentrations increased SHP mRNA

expression, followed by a decrease in insulin gene expression. Furthermore, adenovirus-mediated overexpression of SHP in INS-1 cells impaired glucose-stimulated insulin secretion as well as insulin gene expression [65]. Interestingly, Ad-ATF6 increased SHP gene expression, and downregulation of endogenous SHP expression by siRNA-SHP blocked ATF6-induced suppression of insulin gene expression. These data suggest that ER-stress-induced beta cell dysfunction is mediated, at least in part, by ATF6-induced transcriptional activation of SHP. Collectively, chronic high glucose exposure induced ER stress, and ER stress-induced activation of ATF6 resulted in beta cell dysfunction mediated, in part, by the upregulation of SHP expression [66].

5. Conclusions

Many studies have reported that ER stress plays an important role in the pathogenesis of diabetes and that the UPR has an important role in regulating pancreatic beta cell functions. These studies have suggested that resolving ER stress could be a therapeutic target for diabetes. Moreover, it has been suggested from experimental evidence that ER stress mediates glucolipotoxicity-induced suppression of insulin biosynthesis (Figure 1). In addition to impaired insulin biosynthesis, understanding impaired insulin secretion and beta cell failure, including apoptosis in glucolipotoxicity-induced ER stress conditions, could have important implications for the development of therapeutic strategies for type 2 diabetes mellitus. However, we do not know completely how ER stress affects the pathogenesis of human diabetes. To clarify the role of ER stress in insulin biosynthesis, investigations to determine whether ER stress is implicated in the development of human diabetes and the interaction of the three arms of ER stress are needed. Besides, it will be important for future studies to address the relationship between the ER stress response gene and human disease by performing a genetic study.

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Review Article

The Role of Glucosamine-Induced ER Stress in Diabetic Atherogenesis

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Cardiovascular disease (CVD) is the major cause of mortality in individuals with diabetes mellitus. However the molecular and cellular mechanisms that predispose individuals with diabetes to the development and progression of atherosclerosis, the underlying cause of most CVD, are not understood. This paper summarizes the current state of our knowledge of pathways and mechanisms that may link diabetes and hyperglycemia to atherogenesis. We highlight recent work from our lab, and others', that supports a role for ER stress in these processes. The continued investigation of existing pathways, linking hyperglycemia and diabetes mellitus to atherosclerosis, and the identification of novel mechanisms and targets will be important to the development of new and effective antiatherosclerotic therapies tailored to individuals with diabetes.

1. Introduction

Worldwide, cardiovascular disease (CVD) is the leading cause of premature death in both men and women. Risk factors for CVD include abnormal lipid levels, smoking, hypertension, abdominal obesity, stress, sedentary lifestyle, and diabetes mellitus [1]. While the incidence of CVD has declined in many developed countries, this trend is expected to reverse in the near future [2]. This is largely due to the dramatic, worldwide increase in the incidence of diabetes mellitus. Driven by changes in lifestyle and an escalating rate of obesity, the number of individuals with diabetes may already be as high as 350 million [3, 4]. Diabetes mellitus is a major, independent risk factor for cardiovascular disease (CVD), and individuals with diabetes are 2 to 3 times more likely to die from CV causes than people with no history of diabetes, even after controlling for other CV risk factors [5–9]. These individuals are also at increased risk of diseases that are associated with CVD and atherosclerosis including hypertension and renal failure. Ultimately, this translates to a CV mortality rate in diabetic patients of approximately 75% [6, 7]. The increasing incidence of diabetes means that the global burden of this chronic disease on health care resources will continue to rise for the foreseeable future.

It is not clear why individuals with diabetes are predisposed to CVD. Recent reports from clinical trials examining the effects of intensive blood glucose lowering on CV risk, including ACCORD (Action to Control Cardiovascular Risk in Diabetes) [10], ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation) [11], UKPDS (United Kingdom Prospective Diabetes Study) [12], and VADT (Veterans Affairs Diabetes Trial) [13], suggest that the relationship between hyperglycemia and CVD is complex. Despite a vast amount of research, currently available treatments show only limited CV benefit and CVD continues to be the major cause of mortality.

There is a strong correlation between hyperglycemia and both micro- and macrovascular disease [14–18]. The negative effects of elevated glucose levels on vascular function can include decreased proliferation of endothelial cells, the impairment of some parameters of vascular responsiveness, and increased endothelial programmed cell death [19–21]. It is well established that aggressive blood glucose

lowering significantly decreases the incidence and severity of microvascular disease including retinopathy, renal failure and peripheral nerve dysfunction [14, 15]. Recent evidence suggests that increased glycemic control also correlates with a reduction in macrovascular disease; however, the relationship between glucose lowering and a decrease in CVD has been much more difficult to demonstrate [15]. Several explanations have been put forth to rationalize the inability of clinical trials to demonstrate a strong improvement in cardiovascular outcomes through glycemic control including the possibilities that the trials were underpowered, were too short in duration, or were too focused upon fasting glucose rather than postprandial glucose levels. Alternatively, these findings may indicate that the quality of glycemic control presently achievable is insufficient to be effective in protecting against macrovascular disease. Therefore, even short-term deviations in the control of blood glucose may promote vascular dysfunction.

The pathophysiology of T2D-associated CVD is further complicated by multiple risk factors, collectively known as the metabolic syndrome, that commonly accompany chronic hyperglycemia. The metabolic syndrome is clinically defined as a combination of abdominal obesity, insulin resistance (prediabetes), atherogenic dyslipidemia, and hypertension [22]. The metabolic syndrome is a major cause of morbidity and mortality with cardiovascular disease being the primary clinical outcome [22]. Other complications can include respiratory difficulties, chronic skeletal muscle problems, gall bladder disease, infertility, hepatic steatosis, circulatory problems, and certain cancers [23, 24].

Therefore, while a role for hyperglycemia in the development and progression of atherosclerosis is supported by a great deal of basic research, the clinical role of elevated glucose levels in macrovascular disease is less clear. Furthermore, despite a great deal of research, the mechanisms that may link high glucose concentrations to the molecular and cellular pathways of disease development are not fully understood. This paper will focus on potential direct proatherogenic consequences of hyperglycemia.

2. Mechanisms and Pathways Linking Diabetes and Hyperglycemia to CVD and Accelerated Atherosclerosis

Several mechanisms have been proposed to explain the proatherogenic effects of diabetes and hyperglycemia. In general these have focused upon the intracellular effects of elevated levels of glucose, and the increased availability of glucose metabolites, in cells of the vascular wall. There is evidence that hyperglycemia is associated with increased aldose reductase activity that can lead to increased consumption of NADPH and depletion of GSH levels resulting in elevated levels of reactive oxygen species (ROS) and subsequent cellular damage [25, 26]. Glucose-induced PKC activation has been implicated in decreased endothelial vasodilation [27] and increased production of ROS [28] that could contribute to endothelial dysfunction. It has also been proposed that the conversion of sorbitol to 3-deoxyglucosone can feed into the production of advanced glycation endproducts (AGEs). AGES are formed through a nonenzymatic process, known as the Maillard reaction, involving the reaction of the aldehyde groups of reducing sugars with the amino groups of proteins [29, 30]. There are several potential pathways where AGE-modified proteins could be damaging; the formation of AGEs may alter protein function [31], disrupt the extracellular matrix [31, 32], and/or lead to the modification of lipoprotein particles thereby increasing their atherogenicity. However the predominant vascular effect of AGEs appears to occur through their interaction with RAGE (receptor for AGE) found on macrophages and endothelial and smooth muscle cells [33–35]. The AGE-RAGE interaction triggers a signal transduction cascade that results in the production of intracellular ROS that can initiate an inflammatory response [36, 37].

While preclinical evidence supports a causative role for oxidative stress in atherogenesis [38–41], virtually every well-controlled clinical trial has failed to show a cardiovascular benefit in diabetic patients receiving antioxidant supplements [42–46]. There are several ways to rationalize this apparent paradox by questioning: the specific antioxidants tested, the doses prescribed, and/or the power and duration of the trials themselves. However, these clinical observations may be indicative of the existence of other important molecular mechanisms or pathways that play a causative role in diabetic atherogenesis in addition to oxidative stress.

2.1. The Hexosamine Pathway. Conditions of hyperglycemia also result in the shunting of excess intracellular glucose through the hexosamine biosynthetic pathway (HBP). In a typical cell, under normoglycemic conditions, 1 to 3% of total glucose will be converted to glucosamine-6 phosphate by the enzyme glutamine:fructose-6 phosphate amidotransferase (GFAT) [47]. When intracellular glucose levels rise, flux through this pathway increases. Furthermore, increased GFAT expression and activity have been reported in tissues from humans with diabetes [48]. The net result is an elevated intracellular concentration of glucosamine. This effect has been observed in cultured cells challenged with elevated concentrations of glucose as well as in vascular and hepatic tissues of hyperglycemic animals [49–52].

Increased hexosamine pathway flux has been implicated in several diabetes-associated complications including insulin resistance [47, 53] and pancreatic β cell death [54], as well as atherosclerosis [55]. The molecule mechanisms that underlie the pathogenic effects of increased HBP flux are not fully understood. Most research has focused upon the role of UDP-N-acetylglucosamine (UDP-GlcNAc), the end-product of the HBP pathway and a substrate for both O- and N-linked protein glycation, as a causative agent. It is well established that elevated glucosamine concentrations drive the O-linked glycosylation of proteins including transcription factors [56] and nuclear pore proteins [57], as well as signaling factors [58] which potentially alters their function, stability, and/or activity. Specifically, studies have suggested that O-glycosylation may regulate transcription, plasma lipids, and gluconeogenesis by modulating the activation

of RNA polymerase II [59], angiopoietin-like protein 3 [60], FoxO1, and CRTC2 [61, 62], respectively (further reviewed in [63]). Glucosamine has been shown to desensitize insulin-stimulated glucose uptake in both adipocytes [47] and skeletal muscle cells [64], probably by inhibiting the translocation of the glucose transporter, GLUT4, to the cell surface [65]. In addition, increased hexosamine pathway activity can promote the transcription of proinflammatory and prothrombotic factors including TGF-α, TGF-β, and PAI-1 [66–68]. Therefore, the hyperglycemia-induced O-GlcNAc modification of proteins may change gene expression patterns and alter the function of specific factors that contribute to a proatherogenic, prothrombophilic vascular environment. More studies are required to test this theory and to precisely determine the factors and downstream pathways that may be involved in the acceleration of vascular disease.

UDP-GlcNAc is also a substrate for N-linked protein glycation that occurs in the endoplasmic reticulum (ER). N-glycosylation is an important posttranslational modification of nascent proteins that is critical for proper protein folding [69]. Mutations in asparagine residues of specific proteins, which are critical for N-glycosylation, result in disrupted folding, secretion and/or activity [70–72]. Tunicamycin, a UDP-GlcNAc antagonist, has been shown to inhibit N-glycosylation and activate the cell's quality control mechanism: the unfolded protein response [73–75]. Ultimately, disruptions in the N-glycation process can lead to an accumulation of unfolded/misfolded proteins in the ER that perturb the ER homeostatic balance; this is known as "ER stress." An additional intracellular effect of glucosamine, which has not been investigated in the context of diabetes and atherosclerosis, is its ability to promote the accumulation of unfolded proteins in the ER, thereby leading to ER dysfunction and cell injury [75–78].

3. The Endoplasmic Reticulum and the Unfolded Protein Response

In a typical eukaryotic cell, the ER is responsible for the proper modifying, folding, and trafficking of approximately one-third of all proteins. ER-localized modifications of nascent proteins include disulfide bond formation and N-linked glycosylation, which are critical to protein folding [69]. Unfolded/misfolded proteins are directed to undergo ER-associated degradation (ERAD), and, under physiological conditions, the ER is able to maintain a homeostatic balance between folded and misfolded proteins [79]. When the ER processing capacity is overwhelmed, unfolded/misfolded proteins accumulate and disrupt the ER homeostatic balance; this is known as ER stress.

Traditional ER-stress-inducing agents are known to disrupt protein folding by interfering with disulphide bond formation (dithiothreitol) [80], ER Ca^{2+} balance (A23187, thapsigargin) [81], ER membrane structure (palmitate, unesterified cholesterol) [82, 83] or by blocking protein N-glycosylation (tunicamycin) [84]. Conditions of ER stress activate the unfolded protein response (UPR) which functions to restore ER homeostasis (Figure 1). The UPR is a three pronged signaling cascade that is initiated by transmembrane ER proteins known as inositol-requiring enzyme (IRE)-1, activating transcription factor (ATF)-6, and PKR-like ER kinase (PERK) [85]. Initiation of these pathways alleviates ER stress by decreasing protein synthesis, increasing ER chaperone levels, and facilitating degradation of irreversibly misfolded proteins. Under conditions of chronic ER stress, upregulation of pathways involved in lipid accumulation (SREBP) and inflammation (NF-κB) can occur [49, 86–88]. If the UPR is unable to restore ER homeostasis, proapoptotic signaling factors (i.e., GADD153/CHOP) are upregulated to initiate programmed cell death [89].

3.1. ER Stress and Atherogenesis. There is increasing experimental evidence in support of a direct and causative role for ER stress in the development and/or progression of atherosclerosis. First, several independent risk factors for CVD, including hyperglycemia [49], hyperhomocysteinemia [7, 88], obesity [90], and elevated levels of palmitate [91] and unesterified cholesterol [92], have been associated with ER stress, suggesting that this pathway may represent a common or unifying mechanism of accelerated atherogenesis [93, 94]. Secondly, activation of the UPR has been noted at all stages of atherosclerotic development, from a fatty streak to an advanced occlusive plaque [95]. Third, conditions of ER stress can activate/dysregulate metabolic pathways that are directly involved in the development of atherosclerotic lesions. ER-stress-inducing agents promote lipid accumulation by activating the sterol regulatory element binding proteins (SREBPs), which are transcription factors that control lipid biosynthesis and uptake [88, 96, 97]. ER-stress-inducing agents also activate NF-κB, the transcription factor responsible for promoting inflammatory gene expression [98, 99]. Finally, ER stress has been shown to activate caspases and promote apoptosis of human aortic endothelial cells and other cell types [100, 101]. Together, lipid accumulation, inflammation, and endothelial apoptosis are the hallmark features of atherosclerosis [102, 103].

3.2. Glucosamine-Induced ER Stress. Our lab has recently overexpressed the HBP rate limiting enzyme, GFAT, using an adenoviral expression system in cell culture and measured a significant increase in UPR gene expression and downstream effects of ER stress including lipid accumulation, inflammatory gene expression, and apoptotic signaling under hyperglycemic conditions [86]. We have shown that addition of exogenous glucosamine, or increased endogenous production of glucosamine, can disrupt the capacity of the ER to process nascent proteins and initiate an ER stress response. Furthermore, this effect has been observed in cell types that are relevant to the development of atherosclerosis, including human aortic smooth muscle cells, monocyte-derived macrophages, and HepG2 cells [49, 50, 100, 101]. Thus, elevated levels of glucosamine may play an important role in ER and cellular dysfunction associated with atherogenesis.

It is not known how increased concentrations of glucosamine (but not mannose) disrupt protein folding in

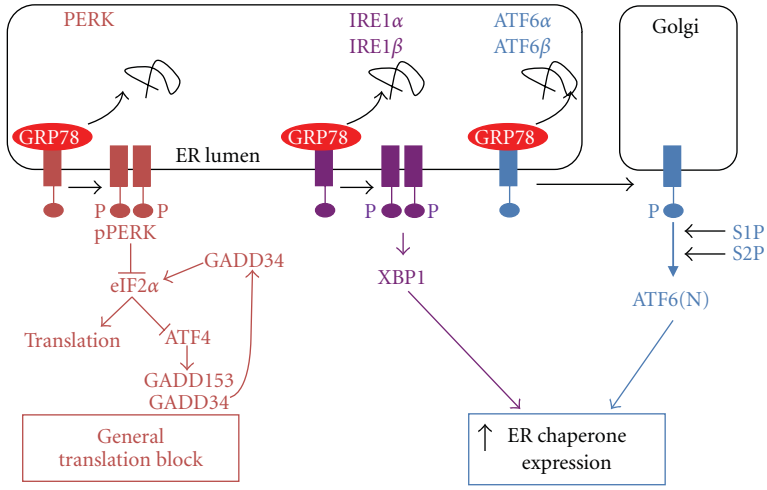

Figure 1: The unfolded protein response to endoplasmic reticulum stress. ER stress occurs when the capacity of the ER to process/fold proteins is exceeded by the load of nascent proteins entering the ER. The function of the UPR is to reestablish ER homeostasis by decreasing protein flux into the ER (translation block) while increasing the folding capacity of the ER (increased chaperone expression). Conditions of ER stress lead to the dissociation of ER chaperone GRP78 from the trans-ER-membrane signaling factors PERK, IRE1, and ATF6, resulting in their activation. Activated PERK phosphorylates and inhibits the activity of eIF2α, an essential factor in general protein translation. PERK is also involved with the downstream activation of transcription factors including ATF4 and GADD153. Activated IRE1 assists in the alternative splicing of XBP-1 resulting in the translation of a transcription factor, XBP-1, which is involved in upregulation of the expression of ER chaperones. Activated ATF6 translocates to the Golgi where proteases S1P and S2P release an N-terminal transcription activation domain that works in concert with XBP-1 to upregulate ER chaperone expression.

the ER. UDP-N-acetylglucosamine is an essential substrate for both O- and N-linked protein glycosylation, and protein glycosylation is an important step in the proper folding of many proteins [69]. It is known that elevated concentrations of glucosamine increase levels of O-linked protein glycosylation [49] and alter N-linked glycosylation patterns of specific proteins including ApoB100 [104]. It is possible that either of these effects could promote ER stress. In cultured HepG2 cells, our lab has shown that PUGNAc, an inhibitor of O-GlcNAcase, increases protein-O-GlcNAc levels but does not promote ER stress [49]. This may suggest that glucosamine-induced ER stress is caused by free and not protein O-linked glucosamine. We hypothesize that increased levels of glucosamine, or a derivative of glucosamine, may interfere with a step in the N-linked glycation of proteins resulting in the production of misfolded proteins and the activation of the UPR.

Elevated levels of glucosamine and glucosamine-induced ER stress have been previously implicated in acquired insulin resistance [47, 53, 105, 106]; however, there is some controversy to whether this effect is physiologically relevant in humans. Incubation of relatively high concentrations of glucosamine (1–10 mmol/L) in adipose, muscle, or endothelial cell cultures has been implicated in impaired insulin action [106–109]. Furthermore, high levels of intravenously injected glucosamine (plasma concentrations of 0.5–1.8 mmol/L) in both animals and humans have also been shown to cause insulin resistance [110, 111]. The recommended daily dose of oral glucosamine supplements, commonly taken to treat joint pain, are far lower (plasma concentrations of ~3 μmol/L), and data suggest that these supplements have no effect on insulin sensitivity [112, 113]. Additional studies will be required to determine the effects of chronic hyperglycemia on endogenous, intracellular levels of glucosamine and possible effects on insulin resistance.

4. Hyperglycemia, ER Stress, and Accelerated Atherosclerosis

To investigate the molecular mechanisms that link hyperglycemia to atherosclerosis, we have established a model in which we inject ApoE$^{-/-}$ mice with multiple low doses of streptozotocin (STZ) [49, 50, 114]. Using this model we have observed a correlation between hyperglycemia, the accumulation of glucosamine in the artery wall, vascular ER stress, and accelerated atherogenesis [49] (Figure 2). Significantly, ER stress levels in the endothelium of hyperglycemic mice increase prior to the development of the atherosclerotic lesions, a result that is consistent with ER stress playing a causative role in lesion development [50]. In addition, accelerated lesion development is observed in these diabetic mice before the onset of dyslipidemia, suggesting that hyperglycemia is sufficient to independently promote the activation of proatherogenic processes [49].

In a direct test of the atherogenic potential of glucosamine, we have recently found that ApoE$^{-/-}$ mice given drinking water containing 5% glucosamine (w/v) for 7 weeks have significantly increased vascular and hepatic ER stress levels in addition to larger atherosclerotic lesions than mice given regular water or water containing 5% mannitol (w/v) [115]. This is consistent with a report from Tannock et al. who found that 5 weeks of glucosamine supplementation increased lesion size in LDL-receptor-deficient mice [116].

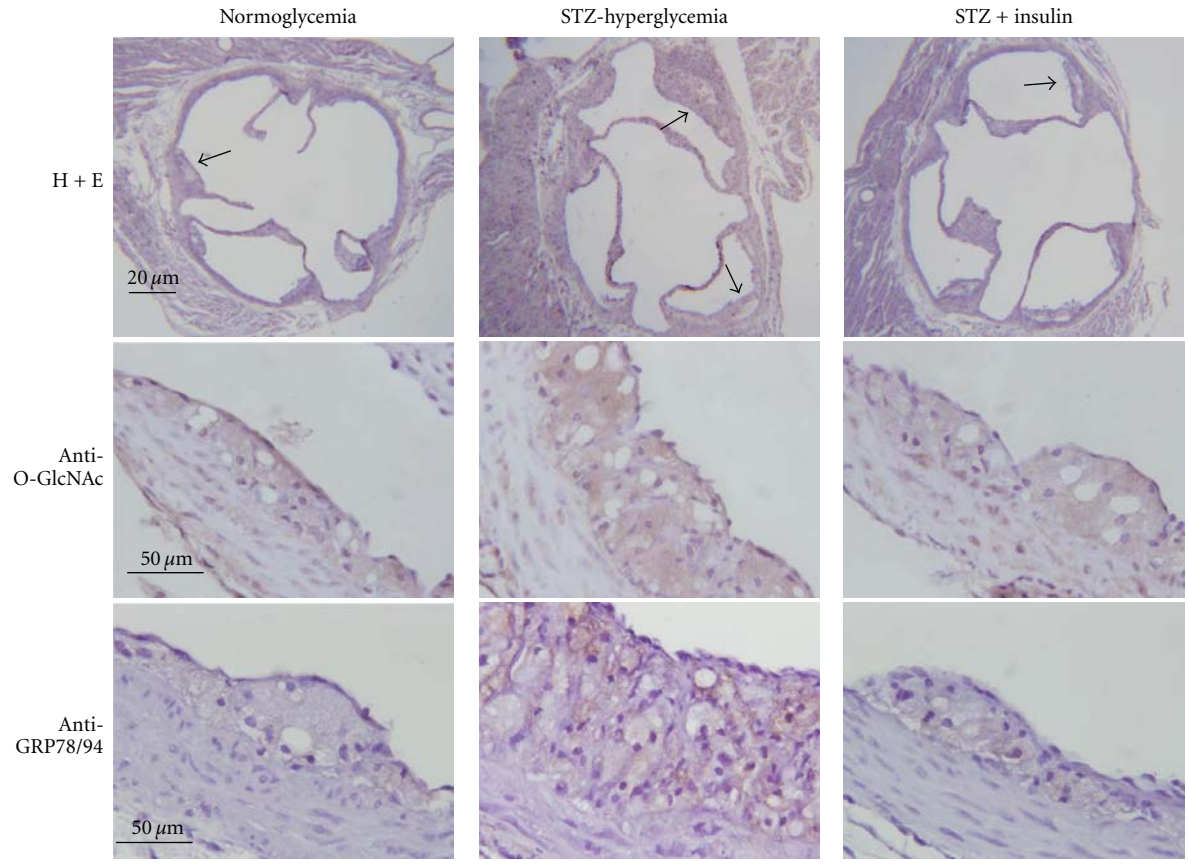

Figure 2: Analysis of aortic root from normoglycemic, STZ-injected hyperglycemic, and STZ-injected insulin-treated ApoE$^{-/-}$ mice. Hyperglycemic mice show increased vascular O-linked GlcNAc, elevated levels of ER stress markers (GRP78/94), and significantly accelerated atherosclerotic lesion development, relative to normoglycemic controls. Normalization of glucose levels with insulin attenuates O-GlcNAc accumulation, ER stress, and atherogenesis.

Our data suggests that glucosamine-induced ER stress plays a direct and causative role in accelerated atherogenesis.

5. ER Stress in Patients with Metabolic Syndrome

There is ample evidence *in vitro* and in animal models to support a role for ER stress in the development and complications of diabetes. Recently, small clinically relevant studies involving humans with metabolic syndrome have been carried out. Patients with diabetic nephropathy have been shown to have increased GFAT expression in glomerular epithelial and mesangial cells and that GFAT is expressed in most tissues involved in diabetic complications [48, 117]. Pancreatic beta cells isolated from type 2 diabetics have been shown to have marked expression of ER stress markers [118] and increased susceptibility to ER stress compared to nondiabetic controls [119] and that ER stress may contribute to IL-1β production, mild islet inflammation [120], and β-cell failure [118]. Our lab has recently shown that isolated leukocytes from human subjects with metabolic syndrome, compared to healthy subjects, have elevated levels of ER stress markers and that there is an association between acute and chronic dysglycemia and ER stress in humans [86]. Each of these trials is consistent with diabetes-associated ER stress playing a clinically relevant role in the pathogenesis of diabetic complications.

6. Targets for Potential Therapeutic Intervention

The identification of a role for ER stress and/or the UPR in the development and progression of diabetes-associated atherosclerosis is significant, not only because it gives us insight on an important disease process, but also because it illuminates novel potential targets for therapeutic intervention (Figure 3). Efforts to develop strategies to manipulate the UPR have already begun, especially with respect to other diseases and disorders in which ER stress is thought to play a role. At least three general approaches have been used to address this problem. The first involves reducing the levels of ER stress directly by relieving the load of misfolded proteins though the addition of chemical chaperones such as 4-phenylbutyric acid (4-PBA), taurine-conjugated ursodeoxycholic acid (TUDCA), or dimethyl sulfoxide (DMSO) [121–123]. The mechanisms by which these small molecules function to reduce ER stress levels are not well defined. However, such strategies have been shown to be effective

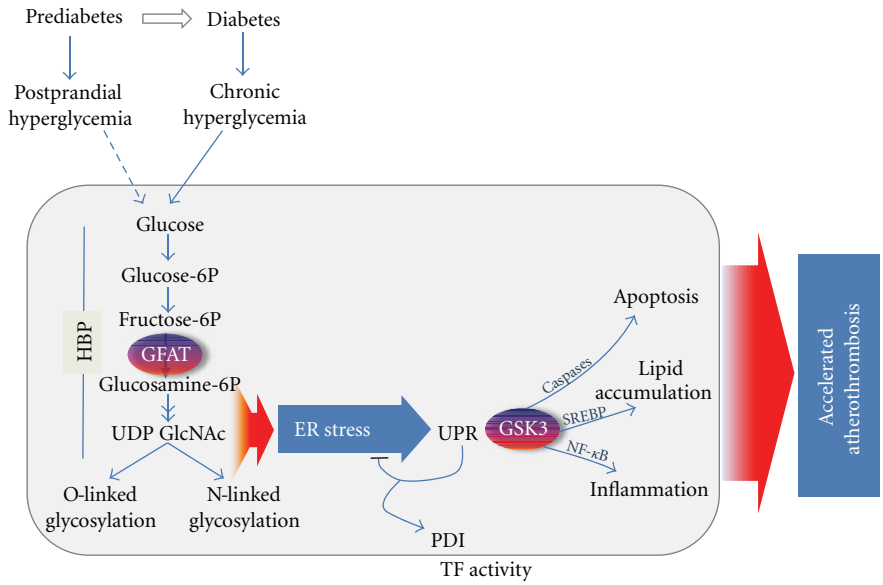

Figure 3: Working model of diabetes-associated accelerated atherothrombosis. Chronic hyperglycemia leads to increased flux through the hexosamine biosynthesis pathway (HBP) resulting in accumulation of UDP-N-acetylglucosamine (UDP-GlcNAc), a substrate for both O- and N-linked protein glycosylation, as well as increased levels of ER stress. Disruptions in ER homeostasis lead to activation of the unfolded protein response (UPR) and downstream effects including activation of glycogen synthase kinase (GSK)-3. Our results suggest that ER-stress-induced GSK-3 induces proatherogenic processes leading to the accelerated development of atherothrombosis.

in vitro and *in vivo*, and 4-phenylbutyric acid has been shown to attenuate atherosclerosis in an ApoE$^{-/-}$ mouse model [124]. A second strategy is to augment the protective aspects of the endogenous UPR. This has previously been accomplished through the over-expression of ER-resident protein chaperones including GRP78. The third approach is to target some of the detrimental downstream effects of ER stress. Examples of this strategy include the use of salubrinal which inhibits the phosphatase GADD34 from reactivating eIF2α, thereby maintaining the PERK pathway-induced translation block (Figure 1). Other possible targets for intervention would include proinflammatory and/or proapoptotic factors such as ASK1, p38MAPK, or GADD153/CHOP. Indeed, GADD153/CHOP-deficient mice are resistant to accelerated atherosclerosis [125, 126]. Recently we have identified glutamine:fructose-6-phosphate amidotransferase (GFAT) and glycogen synthase kinase (GSK)-3 as two enzymes involved in ER stress and potential targets for therapeutic intervention.

6.1. Glutamine:Fructose-6-phosphate Amidotransferase (GFAT). The potential role of glucosamine-induced ER stress in diabetic atherogenesis highlights the importance of glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme in the conversion of glucose to glucosamine, also known as the hexosamine pathway [127, 128]. A central role for GFAT activity in the ER stress pathway is supported by our finding that inhibition of GFAT attenuates glucose-induced ER stress [49] and that overexpression of GFAT is sufficient to promote ER stress in HepG2 cells cultured in normoglycemic conditions [86]. We are currently developing strategies to modulate GFAT activity *in vitro* and in our mouse models. These tools will be used to investigate the potential effects of regulating GFAT activity on the UPR and on activation of proatherogenic processes.

6.2. Glycogen Synthase Kinase (GSK)-3. The mechanisms that link conditions of ER stress to the activation of proatherogenic pathways are not known. GSK-3α and β are two homologous serine/threonine kinases that are involved in a diverse number of intracellular signaling pathways [129]. We have shown using small molecule inhibitors and GSK-3$\alpha^{-/-}$ and GSK-3$\beta^{-/-}$ mouse embryonic fibroblasts that GSK-3-deficiency attenuates ER-stress-induced apoptosis and lipid accumulation [114, 130–132]. *In vivo* we have shown that hyperglycemic mice fed a diet supplemented with valproate, a compound that inhibits GSK-3 activity, have reduced hepatic GSK-3 activity and reduced lesion volume at the aortic sinus [114]. Together, the above findings support our hypothesis that glucosamine-induced ER stress plays a role in accelerated atherogenesis and identifies GSK-3 as a potential target for antiatherogenic therapy. The limitation of targeting GSK-3 arises from the central role that this kinase plays in many diverse metabolic processes and the possibility of detrimental side-effects of small molecular inhibitors [118].

7. Conclusions

Because of the cardiovascular risks of diabetes and the increasing prevalence of type 2 diabetes, it is essential that we further our knowledge of how and why diabetes mellitus and hyperglycemia promote cardiovascular disease.

Currently, and for the near future, the primary strategy for managing cardiovascular disease in the diabetic population will be through the control of hyperglycemia and through the treatment of associated complications such as hypertension and dyslipidemia using established medications such as ACE inhibitors, statins, and fibrates.

The continued identification and investigation of pathways linking hyperglycemia and diabetes mellitus to atherosclerosis is important to the development of new and effective antiatherosclerotic therapies that are tailored to individuals with diabetes. A great deal of research has been focused upon the role of hyperglycemia in the development and progression of atherosclerosis in cell culture and animal model systems. Several mechanisms have been identified that appear to link glucose to proatherogenic processes. The most promising of these, the polyol pathway, PKC activation, the hexosamine pathway, and the AGE-RAGE interaction, show potential and are actively being evaluated as targets for putative antiatherogenic therapies. Thus far, however, all interventions targeting the effects of hyperglycemia, including direct glucose lowering, appear to show greater effect in the treatment of microvascular complications than in the control of macrovascular disease. This is likely due, at least in part, to the complexities of atherosclerosis and current limitations of the animal models available to researchers who study the development and progression of atherosclerosis. Additional studies are obviously required to further our understanding of this important disease.

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Review Article

The Role of Endoplasmic Reticulum Stress in Autoimmune-Mediated Beta-Cell Destruction in Type 1 Diabetes

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Unlike type 2 diabetes which is caused by the loss of insulin sensitivity, type 1 diabetes (T1D) is manifested by the absolute deficiency of insulin secretion due to the loss of β mass by autoimmune response against β-cell self-antigens. Although significant advancement has been made in understanding the pathoetiology for type 1 diabetes, the exact mechanisms underlying autoimmune-mediated β-cell destruction, however, are yet to be fully addressed. Accumulated evidence demonstrates that endoplasmic reticulum (ER) stress plays an essential role in autoimmune-mediated β-cell destruction. There is also evidence supporting that ER stress regulates the functionality of immune cells relevant to autoimmune progression during T1D development. In this paper, we intend to address the role of ER stress in autoimmune-mediated β-cell destruction during the course of type 1 diabetes. The potential implication of ER stress in modulating autoimmune response will be also discussed. We will further dissect the possible pathways implicated in the induction of ER stress and summarize the potential mechanisms underlying ER stress for mediation of β-cell destruction. A better understanding of the role for ER stress in T1D pathoetiology would have great potential aimed at developing effective therapeutic approaches for the prevention/intervention of this devastating disorder.

1. Introduction

Recent epidemiologic studies revealed that the incidence of type 1 diabetes (T1D) in most regions worldwide has been increasing by 2% to 5% [1]. Particularly, in some developing countries such as China, the rapid economic development along with changes in lifestyle and presumably the living environment has rendered this country with an annual increase of 7.4% for T1D prevalence [2]. Given that T1D is typically developed in children and juveniles, its impact on the quality of life is far more significant than that of type 2 diabetes, in which it usually occurs in adults. Although exogenous insulin therapy partly compensates the function of β cells, it cannot regulate blood glucose as accurately as the action of endogenous insulin. As a result, long-term improperly control of blood glucose homeostasis predisposes T1D patients to the development of diverse complications such as diabetic retinopathy [3–5], nephropathy [6, 7], neuropathy [8–10], foot ulcers [11–13], and cardiovascular diseases [14–16]. Although the underlying mechanisms leading to T1D have yet to be fully addressed, extensive studies have consistently demonstrated that endoplasmic reticulum (ER) stress plays a critical role in autoimmune-mediated β-cell destruction during the course of T1D development.

The pancreatic β-cells are equipped with highly developed endoplasmic reticulum (ER) to fulfill the requirement of secreting a large amount of insulin. This physiological feature renders β cells particularly vulnerable to ER stress [17]. Exhaustion of β cells is essential for the onset of T1D, which requires the residual β cells for compensated insulin secretion. While this compensated action is beneficial for control of blood glucose homeostasis, it also increases ER burden

associated with the induction of unfolded protein response (UPR) and ER stress, which further exacerbates β-cell death. Although the implication of ER stress in β-cell death has been extensively emphasized, the underlying mechanisms, however, are yet to be fully elucidated. As such, understanding the role of ER stress in the loss of β mass and dissecting the mechanisms underlying ER stress would be important for developing therapeutic approaches aimed at prevention and intervention of type 1 diabetes. In the present paper, we will first intend to address the overall role of ER stress in autoimmune-mediated β-cell destruction based on published genetic and experimental data. The impact of ER stress on modulation of autoimmune response during the course of T1D development will be next discussed. We will finally focus on the possible pathways implicated in the induction of ER stress and summarize the potential mechanisms underlying ER stress for mediation of β-cell destruction.

2. The Endoplasmic Reticulum (ER)

ER is a membranous network of tubules, vesicles, and cisternae that are interconnected by the cytoskeleton in the cytoplasm of eukaryotic cells. ER is responsible for many general cellular functions, including the facilitation of protein folding and assembly [18–20], manufacture of the membranes [21], biosynthesis of lipid and sterol, storage of intracellular Ca^{2+}, and transport of synthesized proteins in cisternae.

ER can be categorized into rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER). RER is responsible for protein synthesis, while SER is in charge of the synthesis of lipids and steroids, regulation of calcium concentration, attachment of receptors on cell membrane proteins, and detoxification of drugs. As featured by its name, RER bears ribosomes on the outer surfaces of the cisternae and looks bumpy and rough under a microscope. The newly synthesized proteins by RER are sequestered in cisternae and sent to Golgi complex or membrane via small vesicles. In contrast, SER does not have ribosomes on its cisternae and appears to have a smooth surface under the microscope. SER is found commonly in places such as in the liver and muscle. It is important for the liver to detoxify poisonous substances. Sarcoplasmic reticulum (SR) is a special type of SER, which is found in smooth and striated muscle. SR is responsible for the regulation of calcium levels. It sequesters a large store of calcium and releases them when the muscle cell is stimulated.

3. ER Stress

ER stress is the cellular responses to the disturbances of normal function of ER. The most focused and well-studied ER stress is that caused by protein misfolding. The accumulation of unfolded proteins leads to a protective pathway to restore ER function, termed as unfolded protein response (UPR). ER employs a type of special proteins called chaperones as a quality control mechanism. Chaperones attach to the newly synthesized proteins and assist them to fold into their native conformations. In addition, chaperones also help to break down unfolded or incorrectly folded proteins in the ER via a process called ER-associated degradation (ERAD). Protein folding requires a serial of ER-resident protein folding machinery. Exhaustion of those protein folding machineries or insufficient energy supply increases the accumulation of unfolded or misfolded proteins in ER, leading to the activation of UPR. Various physiological and pathological insults such as increased general protein synthesis, failure of posttranslational modifications, hypoxia, nutrient/glucose starvation, and alterations in calcium homeostasis can result in the accumulation of unfolded or misfolded proteins in ER which then causes ER stress [22]. For example, altered expression of antithrombin III [23, 24] or blood coagulation factor VIII [25, 26] results in the exhaustion of protein-folding machinery and thus induces UPR. Some physiological processes such as the differentiation of B lymphocytes into plasma cells along with the development of highly specialized secretory capacity can also cause accumulation of unfolded proteins and induce UPR [27–29]. In response to certain physiological and pathological insults, cells undergo UPR to get rid of the unfolded or misfolded proteins. Therefore, UPR is a protective mechanism by which it monitors and maintains the homeostasis of ER. For instance, UPR increases the folding capacity by upregulating ER chaperones and foldases, and attenuates the biosynthetic burden of secretory pathway through downregulating the expression of secreted proteins [30–32]. In addition, UPR also activates ERAD to eliminate unfolded proteins [33–35] (Figure 1). However, once the stress is beyond the compensatory capacity of UPR, the cells would undergo apoptosis. As such, UPR and ER stress are reported to be implicated in a variety of pathological processes, including diabetes, neurodegenerative diseases, pathogenic infections, atherosclerosis, and ischemia [22, 36].

As aforementioned, there is a monitoring mechanism to ensure the correct protein folding in ER. The unfolded proteins usually have a higher number of hydrophobic surface patches than that of proteins with native conformation [37]. Thus, unfolded proteins are prone to aggregate with each other in a crowed environment and directed to degradative pathway [38]. Molecular chaperones in ER are the major mechanisms to promote protein folding. They preferentially interact with hydrophobic surface patches on unfolded proteins and create a private folding environment by preventing unfolded proteins from interaction and aggregation with other unfolded proteins. In addition, the concentration of Ca^{2+} in ER also impairs protein folding by inhibiting the activity of ER-resident chaperones and foldases [39–42]. ER is the major site for Ca^{2+} storage in mammalian cells. The concentration of Ca^{2+} in ER is thousands times higher than that in the cytosol [43]. Most chaperones and foldases in ER are vigorous Ca^{2+} binding proteins. Their activity, therefore, is affected by the concentration of Ca^{2+} in ER. A variety of posttranslational modifications including N-linked glycosylation, disulfide bond formation, lipidation, hydroxylation, and oligomerization occur in ER. Disruption of those posttranslational modifications can also result in the accumulation of incorrectly folded proteins and thereby induce UPR or ER stress. For example, glucose deprivation impairs

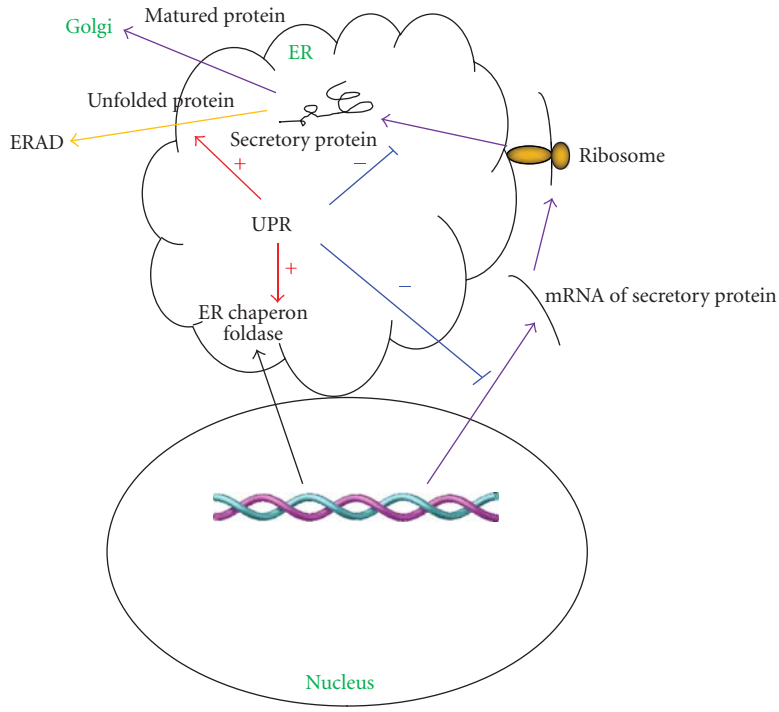

Figure 1: The regulatory role of unfolded protein response (UPR). Various physiological and pathological insults can result in the accumulation of unfolded proteins which then induces UPR and ER stress. In response to stressful insults, UPR regulates secretory pathway via following mechanisms: (1) enhancing (red arrow) the expression of ER chaperones and foldases to increase the folding capacity of ER; (2) attenuating (blue) the biosynthetic burden of secretory pathway through downregulating the expression of secreted proteins (purple arrow); (3) promoting the clearance of unfolded proteins by activating ERAD (orange arrow).

the process for N-linked protein glycosylation and thus leads to ER stress [44].

UPR is mediated by three major pathways, which are initiated by the three transmembrane signaling proteins located on the ER membrane. Those transmembrane proteins function as a bridge to link cytosol and ER with their C-terminal in the cytosol and N-terminal in the ER lumen. The N-terminal is usually engaged by an ER-resident chaperone BiP (Grp78) to avoid aggregation. When unfolded proteins accumulate in ER, chaperons are occupied by unfolded proteins and release the transmembrane signaling proteins: which include the following three axes of signals: the pancreatic endoplasmic reticulum kinase (PERK), the inositol-requiring enzyme 1 (IRE1), and the activating transcription factor 6 (ATF6). The release of these proteins triggers UPR and ER stress (Figure 2). PERK is a Ser/Thr protein kinase uniquely present in ER. Once released from BiP, PERK becomes oligomerized and autophosphorylated. PERK inactivates eukaryotic initiation factor 2α (eIF2α) by phosphorylation of Ser51 to reduce mRNA translation and protein load on ER. Deficiency of PERK results in an abnormally elevated protein synthesis in response to the accumulation of unfolded proteins in ER. IRE1 is another axis of signal involved in UPR. IRE1 increases the production of X box protein-1 (XBP-1), a bZIP-family transcriptional factor, by promoting its mRNA splicing [45]. XBP-1 heterodimerizes with NF-Y and enhances gene transcription by binding to the ER stress enhancer (ERSE) and unfolded protein response element (UPRE) in the promoters of targeted genes. Unlike PERK and IRE1 which oligomerize upon UPR, when released from BiP, ATF6, the third axis of signal, translocates into the Golgi apparatus where its transmembrane domain is cleaved [46]. The cleaved ATF6 is then relocated into the nucleus to regulate the expression of targeted genes. For example, once released from the ER membrane, ATF6 enhances the transcription of XBP-1 mRNA which is further regulated by IRE1 [45].

4. ER Stress in Autoimmune-Mediated β-Cell Destruction

Accumulative evidence supports that ER stress is implicated in autoimmune-mediated β-cell destruction in type 1 diabetes [47, 48]. It was noted that loss of β cells is the direct causing factor for insufficient insulin secretion in T1D patients. As described earlier, pancreatic β cells have a very well-developed ER to fulfill their biological function for secreting insulin and other glycoproteins, and therefore, β cells are highly sensitive to ER stress and the subsequent unfolded protein response (UPR). Severe or long-term ER stress would direct β cells undergoing apoptosis [47]. For example, mice deficient in PERK, a molecule responsible for regulating UPR, are extremely susceptible to diabetes. The *null* mice display a progressive loss of β mass and hyperglycemia with aging [49]. Consistent with the observations in these mice, some infant-onset diabetes in humans have also been

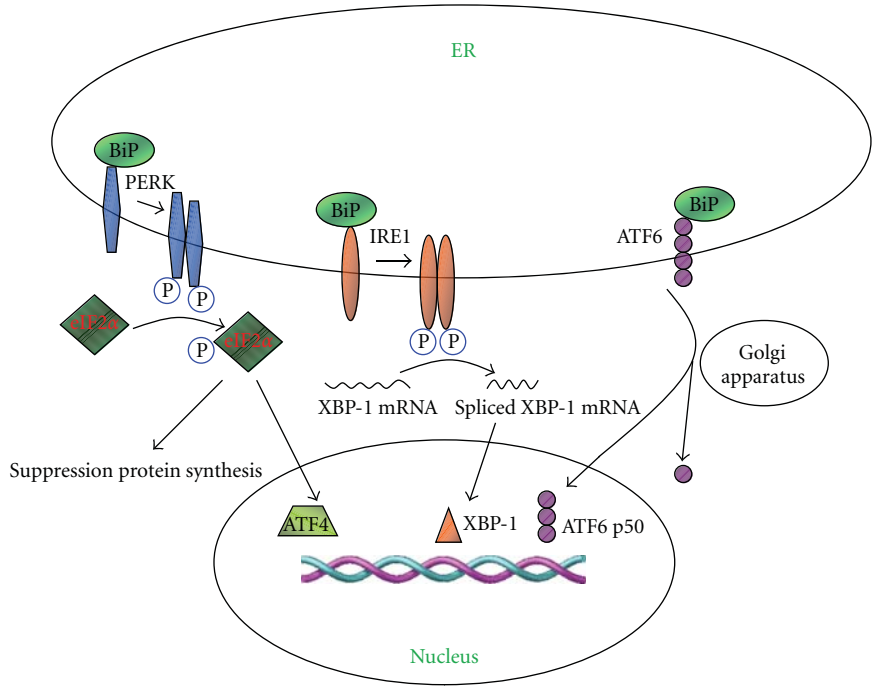

Figure 2: Signaling pathways relevant to UPR. PERK, IRE1, and ATF6 act as ER stress sensors by binding to the ER chaperone BiP, and by which they remain inactive under normal condition. Upon the accumulation of unfolded proteins, BiP preferentially binds to the unfolded proteins, which results in the release of PERK, IRE1, and ATF6. Once released from BiP, PERK becomes activated and dimerized. Activated PERK phosphorylates eIF2α to suppress the overall transcription of mRNAs while selectively enhance the transcription of genes implicated in UPR such as the ATF4 mRNA, and through which ATF4 initiates the transcription of UPR target genes. Similar to PERK, IRE1 is dimerized and activated after detached from BiP. IRE1 induces XBP-1 by promoting the splicing of its mRNA. XBP-1 activates the transcription of its target genes to enhance UPR. The release of ATF6 from BiP results in the translocation of ATF6 to the Golgi apparatus, where ATF6 is cleaved and then translocates into the nucleus, and by which ATF6 initiates the transcription of target genes.

confirmed to be associated with the mutations in PERK. For example, loss of *EIF2AK3* (the gene encodes PERK) develops Wolcott-Rallison syndrome, an autosomal recessive disorder characterized by early infancy insulin-dependent diabetes and multisystemic manifestations including growth retardation, hepatic/renal dysfunction, mental retardation, and cardiovascular abnormalities [50, 51]. Similarly, disruption of UPR by mutating eIF2α, a protein that controls mRNA translation upon ER stress, enhances the sensitivity to ER stress-induced apoptosis and results in defective gluconeogenesis. Mice carrying a homozygous Ser51Ala mutation for eIF2α show defective in pancreatic β cells manifested by the smaller core of insulin-secreting β cells and attenuated insulin secretion [52]. Altogether, defects in PERK/eIF2α signaling render β cells highly vulnerable to ER stress in both humans and mice [53, 54].

In type 1 diabetes, ER stress in the pancreatic β cells is primarily induced by proinflammatory cytokines produced by infiltrated immune cells, which then contributes to β-cell destruction. During the course of autoimmunity, proinflammatory cytokines are secreted by the infiltrated autoreactive immune cells in the milieu of pancreatic islets. For example, nitric oxygen (NO) is an inflammatory mediator resulted from autoimmune response during the course of type 1 diabetes. Studies have shown that excessive NO production induces β-cell apoptosis in a CHOP-dependent manner [55]. Other than ER stress caused by autoimmunity, misfolding of insulin in β cells can also directly induce chronic ER stress as evidenced by the observations in Akita mice. The Akita mouse carries a mutation for the *Ins2* gene which disrupts a disulfide bond between the α and β chain of proinsulin, leading to the mis-folding of the mutated insulin, and by which the mutated insulin induces ER stress in β cells to cause diabetes [56].

It is likely that inflammatory cytokines produced by islet-infiltrated autoreactive immune cells are the major factors causing β-cell death in type 1 diabetes [57]. In the early stage of type 1 diabetes, the autoreactive immune cells such as macrophages and T lymphocytes infiltrate into the pancreatic islets along with the secretion of inflammatory cytokines such as IL-1β, IFN-γ, and TNF-α, which then induce ER stress to mediate β-cell destruction. The damaged or dying β cells also release danger signals such as high-mobility group box 1 and heat shock proteins (HSPs), to alert the immune system for the presence of β-cell injury, which in turn further promotes autoimmune progression [57–60]. Studies have shown that stimulation of β cells with IL-1β and IFN-γ induces the expression of death protein 5 (DP5), and through which these cytokines mediate β-cell apoptosis via ER stress [61]. Knockdown of DP5 provides protection for β cells against inflammatory cytokine-induced ER stress [61]. Insult of β cells with IL-1β and IFN-γ has also been found to

decrease the expression of sarcoendoplasmic reticulum pump Ca^{2+} ATPase (SERCA) 2b, which controls the storage of ER Ca^{2+} [62]. It has been well demonstrated that altered ER Ca^{2+} concentration induces the accumulation of unfolded proteins in ER associated with the induction of UPR and ER stress in β cells [63].

Given that hyperglycemia only occurs when β cells fail to compensate the increased demand for insulin, β cells are usually "exhausted" in T1D patients [54]. Therefore, other than the ER stress induced by autoimmune response, β cells in T1D patients are also under ER stress caused by altered insulin synthesis. In later case, the increased insulin demand requires the remaining functional β cells to increase insulin synthesis to compensate the decrease of β mass. While this process in short term is beneficial for control of blood glucose homeostasis, it also induces ER stress, which in turn exacerbates β-cell dysfunction to promote disease progression and diabetes onset. Collectively, there is convincing evidence that ER stress plays an essential role in β-cell destruction during the course of T1D development.

5. The Impact of ER Stress on Modulation of Autoimmune Response

Unlike its well-defined effect on autoimmune-mediated β-cell destruction in type 1 diabetes, the impact of ER stress in modulating autoimmune response during the course of type 1 diabetes, however, remains poorly elucidated. There is evidence supporting that other than its critical roles played in β-cell destruction, ER stress also modulates the functionality of immune cells with implications in autoimmune response in type 1 diabetes.

It has been well accepted that the presence of β-cell-specific autoantibodies serves as a marker for the initiation and progression of autoimmunity in type 1 diabetes [64]. Studies have shown that IRE1, a key molecule in UPR, modulates the differentiation of antibody-producing B lymphocytes. Deficiency of IRE1 hampers pro-B cells differentiating into pre-B cells [65], and XBP-1, an IRE1 downstream molecule, is required for antibody production by mature B cells [66]. It was found that the engagement of B-cell receptor (BCR) induces ubiquitin-mediated degradation of BCL-6, a repressor for B-lymphocyte-induced maturation protein 1 (BLIMP1) [67], while BLIMP1 negatively regulates the expression of B-cell-lineage-specific activator protein (BSAP) [68], and BSAP is suggested to function as a repressor for XBP-1 [69]. In line with these results, B lymphocytes deficient in BLIMP1 failed to express XBP-1 in response to LPS stimulation [66].

Recent studies highlighted the importance of innate immunity in the pathogenesis of type 1 diabetes [59, 60], while elements of the UPR pathway are found to regulate innate immune response [70]. The expression of CREBH, an ER stress-associated transcription factor, can be induced by inflammatory cytokines such as IL-1β and IL-6, which in turn regulates the transcription of serum amyloid P-component and C-reactive protein, the two critical factors implicated in innate immune responses [71]. Furthermore, the differentiation of dendritic cells (DCs), the most critical innate immune cells, is regulated by UPR signaling element, XBP-1 [72]. High levels of mRNA splicing for XBP-1 are found in DCs, and mice deficient in XBP-1 show altered development of both conventional and plasmacytoid DCs. Loss of XBP-1 renders DCs vulnerable to ER stress-induced apoptosis [72]. Moreover, the capacity for DCs secretion of inflammatory cytokine IL-23 is regulated by CHOP, a UPR mediator. CHOP can directly bind to the *IL-23* gene and regulate its transcription. ER stress combined with Toll-like receptor (TLR) agonists was found to markedly increase the mRNA of IL-23 p19 subunit and the secretion of IL-23, while knockdown of CHOP suppressed the induction of IL-23 by ER stress and TLR signaling [73].

Richardson and coworkers reported that innate immune response induced by *P. aeruginosa* infection causes ER stress in *C. elegans*, and mutations with loss of function for XBP-1 lead to larval lethality [74]. In consistent with this result, the polymorphisms within the *XBP-1* gene were found to be associated with Crohn's disease and ulcerative colitis in humans [75], and the two autoimmune diseases share similar properties as type 1 diabetes. Loss of XBP-1 in intestinal epithelial cells induces Paneth cell dysfunction and overactive epithelium, leading to impaired mucosal defense to *Listeria monocytogenes* and increased sensitivity to colitis [75].

Other than the IRE1/XBP-1 axis, the PERK/eIF2α/ATF4 axis of UPR is also found to be associated with innate response. TLR signaling, the most important innate signaling pathway, is reported to induce selective suppression of the ATF-4/CHOP axis of UPR pathway [76]. TLR signaling decreases eIF2α-induced ATF4 translation. For example, pretreatment of LPS, an agonist for TLR4, suppressed ATF4/CHOP signaling and prevented systemic ER stress-induced apoptosis in macrophages, renal tubule cells, and hepatocytes [76]. In contrast, loss of Toll-IL-1R-containing adaptor inducing IFN-β (TRIF), an important adapter for TLR signaling, abrogated the protective effect of LPS on systemic ER stress-induced renal dysfunction and hepatosteatosis, suggesting that TLR signaling suppresses ATF4/CHOP via a TRIF-dependent pathway [76].

6. Pathways for Cytokines Induction of ER Stress

Upon the insults of pathogens, mutated self-antigens, or tissue damage, the immune system initiates inflammatory response by releasing copious amount of cytokines. UPR and ER stress are interconnected with inflammatory cytokines through multiple mechanisms including reactive oxygen species (ROS), NFκB, and JNK (Figure 3). ROS are highly reactive small molecules with unpaired electrons. They are important mediators of inflammatory response. The accumulation of ROS, referred to as oxidative stress, was confirmed to be associated with ER stress [77]. Oxidizing condition is required for the disulphide bond formation during the process of protein folding [78]. Increased protein folding load may lead to oxidative stress. The PERK axis of UPR signaling is reported to be able to activate antioxidant pathway by promoting ATF4 and nuclear factor-erythroid-derived

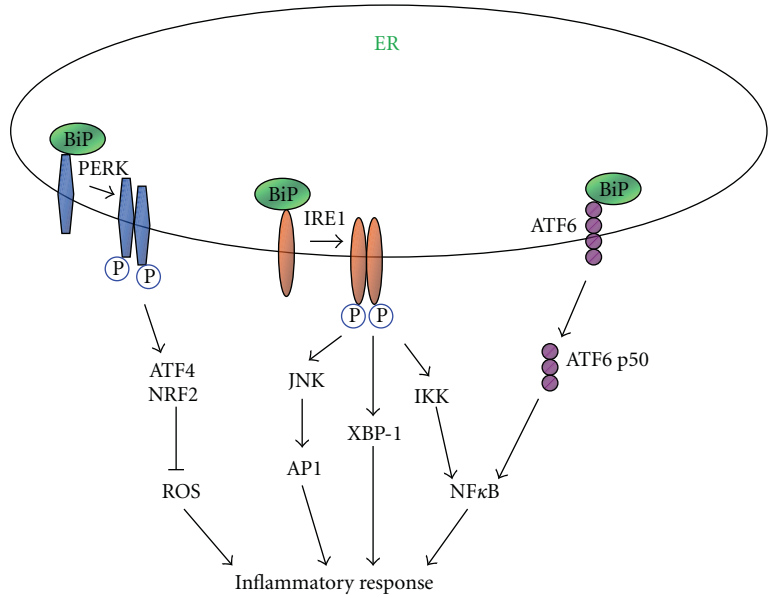

Figure 3: The possible implication of UPR in inflammatory response. UPR is associated with inflammation via a variety of mechanisms involving ROS, JNK, and NFκB. PERK promotes ATF4 and NRF2, which then suppress ROS production by activating antioxidant pathway. Upon activation, IRE1/TRAF2 recruits IKK, leading to the phosphorylation of IκBα and subsequent activation of NFκB. IRE1/TRAF2 can also activate AP1, resulting in the activation of JNK. XBP-1 induced by IRE1 can further induce the expression of various genes implicated inflammation. Furthermore, ATF6, the other axis of UPR signaling, can promote inflammation via activating NFκB.

2-related factor 2 (NRF2) [79, 80]. Therefore, loss of PERK markedly increases ROS accumulation induced by toxic chemicals [79, 81]. The IRE1/TRAF2 axis of UPR can recruit IκB kinase (IKK), leading to the activation of NFκB, a key regulator in inflammation [82]. As a result, NFκB activation and TNF-α production are reduced in cells lacking IRE1 [82]. Furthermore, the IRE1/TRAF2 axis can activate JNK, and by which it induces the expression of inflammatory genes by activating activator protein 1 (AP1) [83]. ATF6, the third axis of UPR signaling, can also activate NFκB pathway, in which suppression of ATF6 reduces NFκB activation caused by BiP degradation [84].

Other than the above described pathways, cytokines may also induce ER stress via inducible nitric oxide (NO) synthase (iNOS) and JNK pathway. JNK pathway is activated by IL-1β. Suppression of JNK by its inhibitor SP600125 protected β cells from IL-1β-induced apoptosis [85]. Cytokines have been evidenced to induce the expression of iNOS, leading to excessive NO production. Stimulation with IL-1β and IFN-γ activates ER stress pathway and induces β-cell apoptosis via NO synthesis [62]. NO has been suggested to be an important mediator of β-cell death in type 1 diabetes. Inflammatory cytokines including IL-1β, IFN-γ, and TNF-α can induce iNOS expression in β cells which then produces copious amount of NO [50]. Excessive NO induces DNA damage and thus results in β-cell apoptosis through p53 pathway or necrosis through poly(ADP-ribose) polymerase (PARP) pathway [54]. Moreover, NO depletes ER Ca^{2+} stores via activating Ca^{2+} channels or inhibiting Ca^{2+} pumps [86–88]. Depletion of Ca^{2+} then leads to ER stress and apoptosis in β cells via the induction of CHOP signaling [55, 89].

7. Mechanisms Underlying ER-Stress-Induced β-Cell Death

ER stress is a key mediator for β-cell death in type 1 diabetes. The primary purpose of ER stress or UPR is to compensate the damage caused by the disturbances of normal ER function. However, continuous ER dysfunction would eventually render cells undergoing apoptosis. The mechanisms by which ER stress induces cell death are not fully elucidated, due to the fact that multiple potential participants involved but little clarity on the dominant death effectors in a particular cellular context. In general, ER stress induction of cell death can be illustrated in three phases: adaptation, alarm, and apoptosis [44].

The phase for adaptation response is initiated to restore the homeostasis of ER and to protect cells from damage induced by the disturbances of ER function. As described earlier, the signaling for UPR involves three axes of responses: IRE1, PERK, and ATF6. These axes interact between each other and form a feedback regulatory mechanism to control the activity of UPR. The accumulation of unfolded proteins in ER results in the engagement of ER-resident chaperon BiP, and as a consequence, IRE1, PERK, and ATF6 are released from BiP. Therefore, overexpression of BiP can prevent cell death induced by oxidative stress, Ca^{2+} disturbances, and hypoxia [90]. PERK is oligomerized and phosphorylated when released from BiP. Activated PERK inactivates eIF2α to reduce mRNA translation and protein load on ER. Therefore, PERK deficiency results in an abnormally elevated protein synthesis in response to the accumulation of unfolded proteins in ER, which renders cells highly sensitive to ER stress

and ER stress-induced apoptosis [91]. Similarly, as a downstream molecule of PERK, eIF2α is required for cell survival upon the insult of ER stress, and a mutation at the phosphorylation site of eIF2α (Ser51Ala) abolishes the translational suppression in response to ER stress [52]. Similar as PERK, IRE1 becomes dimerized and activated once released from BiP. IRE1 induces XBP-1 by promoting the splicing of its mRNA [45]. XBP-1 is a transcriptional factor belonging to the bZIP-family and is responsible for the transcription of many adaptation genes implicated in UPR. Unlike PERK and IRE1, ATF6 translocates into the Golgi apparatus upon the release from BiP. The transmembrane domain of ATF6 is cleaved in the Golgi apparatus and is then relocated into the nucleus, by which it regulates gene expression [46].

During the alarm phase, many signal pathways are activated, and the expression of responsive genes has been induced to alert the system. For example, the cytoplasmic part of IRE1 binds to TNF receptor-associated factor 2 (TRAF2), a key adaptor for TNF-mediated innate immune signaling. TRAF2 would then activate NFκB pathway via activating IKK and activate the signaling for c-Jun N-terminal kinases (JNK) by apoptosis signal-regulating kinase 1 (Ask1). Studies have shown that dominant negative TRAF2 suppresses the activation of JNK by IRE1 in response to ER stress [92]. Importantly, TRAF2 is also a critical component for E3 ubiquitin-protein ligase complex [93], which binds to Ubc13 and promotes the noncanonical ubiquitination of substrates. The Ubc13-dependent ubiquitination of TRAF2 is suggested to be required for the activation of JNK [94]. In addition, IRE1 can further activate JNK signaling through interacting with c-Jun N-terminal inhibitory kinase (JIK) [95].

Although the purpose for the initiation of adaptation response is to restore the homeostasis of ER, apoptosis however could occur, once the accumulation of unfolded proteins exceeds the cellular regulatory capacity. The action for apoptosis is initiated by the activation of several proteases such as caspase-12, caspase-4, caspase-2, and caspase-9. Studies in rodents provided evidence supporting that caspase-12 is involved in ER stress-induced apoptosis. Caspase-12 is activated by IRE1 upon the insult of ER stress. Mice deficient in caspase-12 are resistant to ER stress-induced apoptosis, but remain susceptible to apoptosis induced by other stimuli [96]. There is evidence that caspase-12 can also be activated by interacting with TRAF2, a signaling molecule downstream of IRE1 [95]. In response to ER stress, caspase-7 is translocated from the cytosol to the ER surface, which then activates procaspase-12 as well [97]. The human caspase-4 is the closest paralog of rodent caspase-12, which is normally located on the ER membrane. However, caspase-4 can only be activated by ER stress-inducing reagents not by the other apoptotic reagents, and knockdown of caspase-4 by siRNA reduces ER stress-induced apoptosis in neuroblastoma cells [98]. Similarly, caspase-2 and caspase-9 are found to be activated in the early phase of ER stress and inhibition of their activation either by inhibitors or siRNA reduces ER stress-induced apoptosis [99]. Studies also suggest that some members of inhibitor of apoptosis protein family prevent ER stress-induced cell death via interacting with caspase-2 and caspase-9 [99].

Other than the implication of caspases, Ask1 kinase and CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) are also critical mediators for ER stress-induced cell death. IRE1/TRAF2 complex recruits Ask1 and activates subsequent JNK signaling. Studies have shown that the activation of JNK inhibits antiapoptotic protein BCL-2 [100] and induces proapoptotic protein Bim [101, 102]. Loss of *Ask1* suppresses ER stress-induced JNK activation and provides protection for cells against ER stress-induced death [103]. CHOP is a transcription factor belonging to basic leucine zipper transcription factor (bZIP) family. Many inducers of UPR including ATF4, ATF6, and XBP-1 up-regulate CHOP expression, and phosphorylation of CHOP at ser78 and ser81 by p38 MAPK enhances its transcriptional activity [44, 104]. Upon its activation, CHOP suppresses anti-apoptotic protein BCL-2 which in turn induces cells undergoing apoptosis [105–107].

8. Conclusion and Future Directions

There is convincing evidence that ER stress plays an essential role in autoimmune-mediated β-cell destruction. Feasible evidence also supports that ER stress modulates autoimmune response during T1D development (Table 1). ER stress in β cells can be either triggered by autoimmune responses against β-cell self-antigens and/or by the increase of compensated insulin synthesis. During the course of type 1 diabetes, autoreactive immune cells secrete copious amount of inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ into the islet milieu, which stimulate excessive production of NO in β cells and mediate β-cell destruction by inducing ER stress. Recent studies further suggest that ER stress also modulates the functionality of immune cells with implications in autoimmune progression. The absolute insulin deficiency in T1D patients renders the residual β cells for compensated insulin secretion to meet the demands of insulin for maintaining blood glucose homeostasis. This increase in insulin biosynthesis could overwhelm the folding capacity of ER, leading to UPR and ER stress in β cells, which in turn exacerbates β-cell dysfunction and T1D onset.

It should be kept in mind that the mechanisms underlying autoimmune-mediated β-cell destruction in type 1 diabetes are complex, and ER stress is unlikely the exclusive mechanism implicated in disease process. Despite recent significant advancement in this field, there are still many questions yet to be addressed. Can ER stress be served as a biomarker for β-cell destruction and autoimmune progression in the clinic setting? Are there additional factors for induction of ER stress in β cells during T1D development? Does modulation of ER stress in immune cells attenuate autoimmune progression? Does blockade of ER stress protect β cells from autoimmune-mediated destruction? Future studies aimed at dissecting these questions would provide a broadened insight for T1D pathogenesis and would have great potential for developing novel therapeutic strategies against this devastating disorder.

Table 1: Publications relevant to ER stress in the regulation of immune response and β-cell destruction.

Author	Defective/mutant gene	Species	Major finding	Reference
Harding et al.	PERK$^{-/-}$	Mouse	PERK-deficient mice are extremely susceptible to diabetes. They display a progressive β-cell loss and hyperglycemia with aging.	[49]
Delépine et al.	PERK$^{-/-}$	Human	Deficiency of PERK in human results in Wolcott-Rallison syndrome, which is characterized by early infancy insulin-dependent diabetes and multisystemic dysfunction.	[50]
Scheuner et al.	eIF2α mutant (Ser51Ala)	Mouse	Ser51Ala mutation of eIF2α shows a deficiency in pancreatic β cells manifested by the smaller core of insulin-secreting β cells and attenuated insulin secretion, and the mice die from hypoglycemia at their early infancy.	[52]
Ron et al.	Ins2 mutation	Mouse	Ins2 mutation in Akita mice disrupts disulfide bond between the α and β chain of proinsulin, which leads to the mis-folding of the mutated insulin and further induces ER stress in β cells and diabetes.	[56]
Zhang et al.	IRE1$^{-/-}$	Mouse	Pro-B cells failed to differentiate into pre-B cells when deficient for IRE1.	[65]
Iwakoshi et al.	XBP-1$^{-/-}$	Mouse	Deficiency of XBP-1 results in the impacted development of both conventional and plasmacytoid DCs. Loss of XBP-1 renders DCs vulnerable to ER stress-induced apoptosis.	[72]
Goodall et al.	CHOP knockdown		Knockdown of CHOP suppressed the production of IL-23 induced by ER stress and TLR signaling.	[73]
Richardson et al.	XBP-1 mutation	*C. elegans*	Innate immune response induced by *P. aeruginosa* infection causes ER stress in *C. elegans*, and mutations with loss of function for XBP-1 lead to larval lethality.	[74]
Kaser et al.	XBP-1 polymorphisms	Human	Loss of XBP-1 in intestinal epithelial cells induces Paneth cell dysfunction and overactive epithelium, leading to impaired mucosal defense to Listeria monocytogenes and increased sensitivity to colitis, an inflammatory disease sharing similar properties with T1D. The polymorphisms within the XBP-1 gene are associated with Crohn's disease and ulcerative colitis in humans.	[75]
Nakagawa et al.	Caspase-12$^{-/-}$	Mouse	Caspase-12 is involved in ER stress-induced apoptosis. Mice deficient in caspase-12 are resistant to ER stress-induced apoptosis, but remain susceptible to apoptosis induced by other stimuli.	[96]
Hitomi et al.	Caspase-4 knockdown	Human	Human caspase-4, the closest paralog of rodent caspase-12, is involved in ER stress-induced apoptosis. Knockdown of caspase-4 by siRNA reduces ER stress-induced apoptosis.	[98]
Nishitoh et al.	Ask1$^{-/-}$	Mouse	Loss of Ask1 suppresses ER stress-induced JNK activation and protects cells from ER stress-induced death.	[103]

Abbreviations

AP1: Activator protein 1
Ask1: Apoptosis signal-regulating kinase 1
ATF6: Activating transcription factor 6
BCR: B-cell receptor
BLIMP1: B-lymphocyte-induced maturation protein 1
BSAP: B-cell-lineage-specific activator protein
bZIP: Basic leucine zipper transcription factor
C/EBP: CCAAT/enhancer binding protein
CHOP: C/EBP homologous protein
CPA: Cyclopiazonic acid
CREBH: Cyclic-AMP-responsive-element-binding protein H
DC: Dendritic cell
DP5: Death protein 5
eIF2α: Eukaryotic initiation factor 2α
ER: Endoplasmic reticulum
ER stress: Endoplasmic reticulum stress
ERAD: ER-associated degradation
ERSE: ER stress enhancer
HMGB1: High-mobility group box 1
HSPs: Heat shock proteins
IKK: IκB kinase
iNOS: Inducible nitric oxide synthase
IRE1: Inositol-requiring enzyme 1
IRS-1: Insulin receptor substrate-1
JIK: c-Jun N-terminal inhibitory kinase
JNK: c-Jun N-terminal kinases
NO: Nitric oxygen
NRF2: Nuclear factor-erythroid-derived 2-related factor 2

PARP: Poly(ADP-ribose) polymerase
PERK: Pancreatic endoplasmic reticulum kinase
RER: Rough endoplasmic reticulum
ROS: Reactive oxygen species
SER: Smooth endoplasmic reticulum
SERCA: Sarcoendoplasmic reticulum pump Ca^{2+} ATPase
SR: Sarcoplasmic reticulum
T1D: Type 1 diabetes
TLR: Toll-like receptor
TRAF2: TNF receptor-associated factor 2
TRIF: Toll-IL-1R-containing adaptor inducing IFN-β
UPR: Unfolded protein response
UPRE: Unfolded protein response element
XBP-1: X box protein-1.

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Research Article

Pentoxifylline Attenuates Methionine- and Choline-Deficient-Diet-Induced Steatohepatitis by Suppressing TNF-α Expression and Endoplasmic Reticulum Stress

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Background. Pentoxifylline (PTX) anti-TNF properties are known to exert hepatoprotective effects in various liver injury models. The aim of this study was to investigate whether PTX has beneficial roles in the development of methionine- and choline-deficient-(MCD-) diet-induced NAFLD SD rats *in vivo* and TNF-α-induced Hep3B cells *in vitro*. *Methods*. SD Rats were classified according to diet (chow or MCD diet) and treatment (normal saline or PTX injection) over a period of 4 weeks: group I (chow + saline, $n = 4$), group II (chow + PTX), group III (MCD + saline), and group IV (MCD + PTX). Hep3B cells were treated with 100 ng/ml TNF-α (24 h) in the absence or presence of PTX (1 mM). *Results*. PTX attenuated MCD-diet-induced serum ALT levels and hepatic steatosis. In real-time PCR and western blotting analysis, PTX decreased MCD-diet-induced TNF-alpha mRNA expression and proapoptotic unfolded protein response by ER stress (GRP78, p-eIF2, ATF4, IRE1α, CHOP, and p-JNK activation) *in vivo*. PTX (1 mM) reduced TNF-α-induced activation of GRP78, p-eIF2, ATF4, IRE1α, and CHOP *in vitro*. *Conclusion*. PTX has beneficial roles in the development of MCD-diet-induced steatohepatitis through partial suppression of TNF-α and ER stress.

1. Introduction

Although the proposed theory [1, 2] of pathogenesis of nonalcoholic fatty liver disease (NAFLD) has been challenged, chronic inflammation plays a pivotal role in the development of fatty liver disease. TNF-α and IL-6, which are cytokines associated with inflammation, are found in higher levels in subjects with nonalcoholic steatohepatitis (NASH) than in those without simple NASH [3]. Consequently, a therapeutic strategy targeting TNF-α has been attempted to reduce fat accumulation and improve AST (aspartate aminotransferase) and ALT (alanine aminotransferases) in subjects with fatty liver disease [4–6]. Among the drugs used to suppress or inhibit TNF-α expression, pentoxifylline (PTX), a nonselective phosphodiesterase inhibitor reported to decrease TNF-α gene transcription as well as affect multiple steps in the cytokine pathway by direct or indirect inhibition of TNF-α, is currently used in the clinical field for treatment of cardiovascular disease [4, 7–9]. Several pilot studies have shown the beneficial effects of pentoxifylline on NAFLD and NASH [4, 5, 10]. The endoplasmic reticulum and oxidative stress of the initiation and progression of hepatic steatosis and inflammation have been implicated under metabolic stress conditions [11]. Recently, Zhang et al. [9] demonstrated ER stress-induced hepatic steatosis.

Based on these reports, we hypothesized that fat storage, triggered by cytokine-mediated inflammation, could be decreased through the alleviation of ER stress. Therefore, we investigated whether pentoxifylline has a beneficial role in methionine- and choline-deficient-(MCD-) diet-induced

fatty liver disease in a rat animal model and examined molecular pathways related to ER stress.

2. Method

2.1. Cell Culture. Human Hep3B cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Welgene, Daegu, Republic of Korea) with 4.5 g/L glucose and 2 mM glutamine supplemented with 10% FBS, 1.5 g/L sodium bicarbonate, 100 IU/mL penicillin, and 100 ug/mL streptomycin. The medium was changed twice a week, and the cells were maintained in a 37°C incubator with 5% CO_2. The cells were subcultured when confluent (every 5∼7 days) using trypsin (2.5 g/L) and EDTA (1 g/L).

For the western blot analysis, the cells were plated at 3 $\times$ 10^5/well in 6-well plates and then treated with 100 ng/mL TNF-α (R&D system, Minneapolis, MN) in the absence or presence of 1 mM PTX (Sigma).

2.2. Animals and Experimental Protocol. Sprague Dawley (SD) rats (male, 220–280 g body weight) purchased from Orient Bio Inc. (Sungnam, Republic of Korea) were randomly divided into four groups (six rats per group) as follows. Group I: chow diet plus saline injection (once/day, i.p.); group II: chow diet plus PTX injection (50 mg/kg, once/day, i.p; PTX); group III: MCD diet plus saline injection (once/day, i.p.); group IV: MCD diet plus PTX injection (50 mg/kg, once/day, i.p.) for four weeks. Pentoxifylline (PTX) was purchased from Handock Pharmaceuticals (Seoul, Republic of Korea), and MCD diet was purchased from Dyets Inc. (Bethlehem, Pennsylvania). The rats were maintained at 60 ± 5% relative humidity and 22 ± 2°C, with a 12-hour light/dark cycle. Blood was obtained by cardiac puncture, and the livers were removed and weighed. The livers were fixed in 10% formalin or snap frozen in liquid nitrogen and then stored at −70°C for histologic analysis. All experimental procedures were performed under sterile conditions and approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine.

2.3. Determination of Serum and Hepatic Biochemistry Levels. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-CHO), and triglyceride (TG) were quantified in serum using a commercial kit (Asanpharm Inc., Seoul, Republic of Korea). Frozen liver tissue was homogenized in 0.9% NaCl solution, and the homogenate was diluted to solution of 1 : 2 chloroform:methanol. The homogenate was mixed vigorously with vortex mixer and centrifuged at 1,000 rpm for 20 min. The upper phase was aspirated, and then the chloroform phase was used for the analysis of a variety of metabolite.

2.4. Histological Analysis. Fresh tissues were frozen immediately after each animal was sacrificed, and the tissue was placed in prelabeled base molds filled with embedding medium used for frozen tissue to ensure optimal cutting temperature (OCT). Routine frozen sections (7 μm) were stained with oil-red O (Sigma, St Louis, MO). The paraffin-embedded sections were stained with hematoxylin & eosin (H-E) and Masson's trichrome. To evaluate hepatic steatosis, morphometric analysis was performed on two randomly selected fields (at 200 $\times$ magnification) of each animal section using an Olympus IX71 microscope with an Olympus DP70 camera (Olympus Optical Company, Tokyo, Japan).

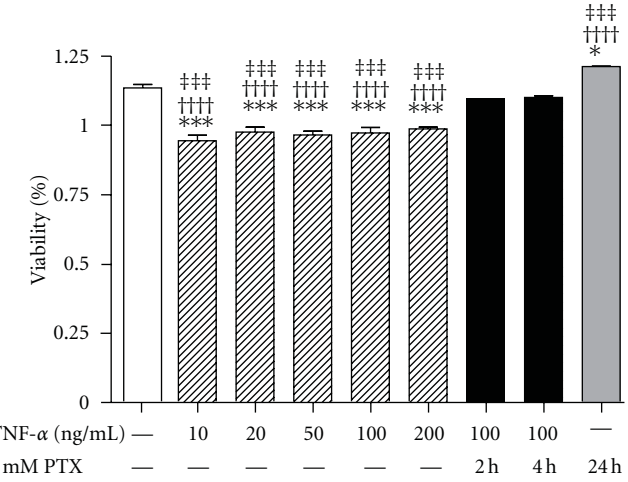

Figure 1: Viability of Hep3B cells after treatment with TNF-α and pentoxifylline. Hep3B cells exposed to 10, 20, 50, 100, and 200 ng/mL TNF-α for 24 hours showed a significant decrease in cell viability, assessed by MTT. Pretreatment with 1 mM pentoxifylline for 2 and 4 h prevented TNF-α-induced Hep3B toxicity. The bars represent percent cytotoxicity versus untreated controls. The experiments were performed four times under identical conditions. Results are shown as mean ± SEM. $^{***}P < 0.001$ *versus* untreated control cells; $^{\dagger\dagger\dagger}P < 0.001$ *versus* 1 mM pentoxifylline for 2 h; $^{\ddagger\ddagger\ddagger}P < 0.001$ *versus* 1 mM pentoxifylline for 4 h.

2.5. Real-Time PCR. Total RNA from liver was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The hepatic mRNA levels of TNF-α were quantified by real-time PCR using the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster, CA) with TaqMan fluorogenic probes and primers for TNF-α: forward, 5′-AAT GGC CTC CCT CAT CAG TT-3′; reverse, 5′-CCA CTT GGT GGT TTG CTA CGA-3′. PCR reactions and analyses were obtained using Sequence Detector Software (Applied Biosystems, Foster, CA).

2.6. Western Blot. Homogenized liver tissues or Hep3B were lysed in lysis buffer (Intron Biotechnology, Sungnam, Republic of Korea) containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 μM phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL aprotinin, and 5 μg/mL leupeptin. The protein extracts were quantified using Bradford assay (Bio-Rad, Hercules, CA). The protein extracts were loaded into 10% SDS-PAGE, followed by transfer to nitrocellulose membrane (Bio-Rad). After blocking with 5% skim milk in 1XPBS, the membranes were incubated with each specific primary antibody, including GRP78 (Santa Cruz Biotechnology, Santa Cruz,

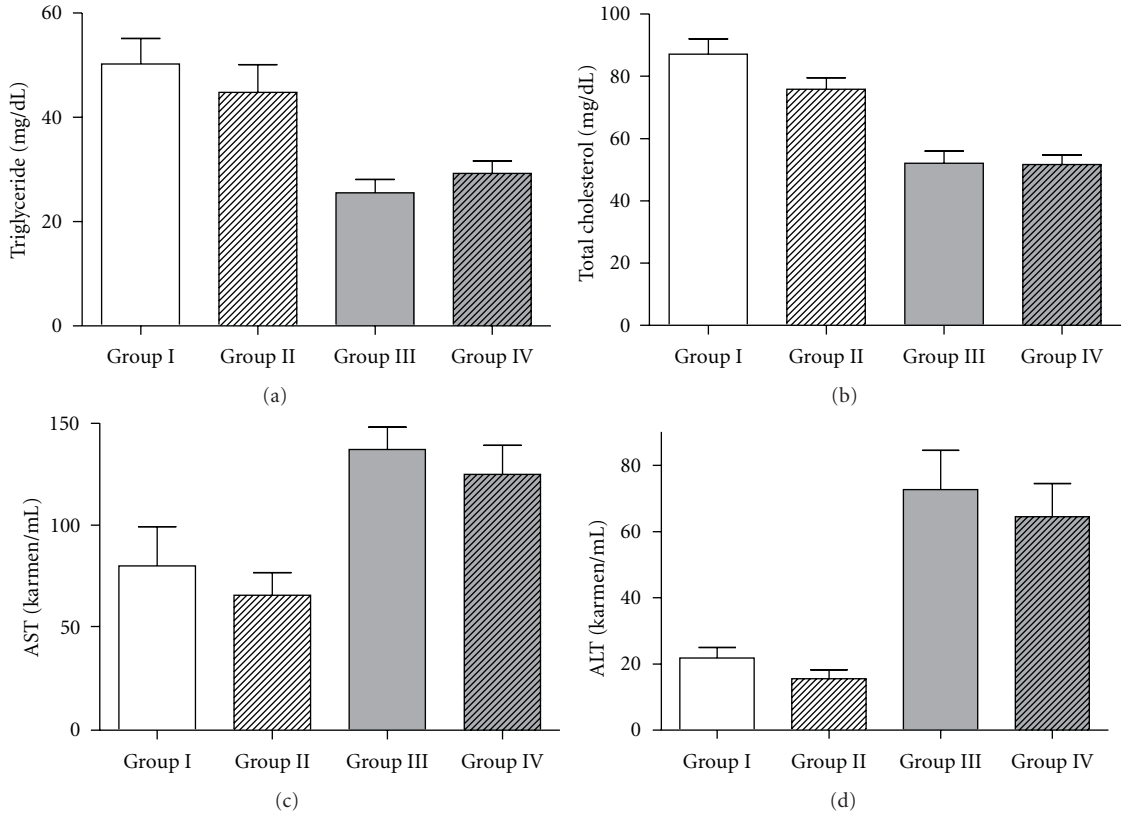

Figure 2: Serum chemistry values after 4 weeks of administration with an MCD diet and pentoxifylline. There was a significant decrease in serum triglyceride and total cholesterol in rats in the MCD plus saline (group III) and MCD plus PTX (group IV) groups relative to rats in control group (group I). A significant increase of ALT levels in groups III and IV was observed. Results are shown as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* untreated control group.

CA), total and phospho-eIF2α (Cell Signaling Technology, Danvers, MA), ATF4 (Santa Cruz), ATF6 (ABNOVA, Taipei city, Taiwan), IRE1 (Santa Cruz), phospho-JNK (Cell signaling), CHOP, and β-actin (Santa Cruz), at 4°C overnight. The membranes were washed with TBS-T and incubated with peroxidase conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz) for one hour at room temperature. The membrane was washed and incubated with detection solution (GE Healthcare, Buckinghamshire, NA, UK) for one minute and was then exposed to film. The signal intensity for each specific band on the western blots was quantified using National Institutes of Image J density analysis software (version 1.20).

2.7. Statistical Analysis. Statistical analysis was performed using PRISM (GraphPad Software Inc., San Diego, CA). Results are expressed as mean ± SD. Statistical significance was calculated using one-way analysis of variance (ANOVA) with a post hoc Bonferroni multiple comparison test to assess the differences between groups. Statistical significance was defined as the conventional P value of < 0.05.

3. Results

3.1. Effect of TNF-α and Pentoxifylline on Viability Hep3B Cells. Hep3B cells exposed to 10, 20, 50, 100, and 200 ng/mL TNF-α for 24 hours showed significantly decreased viability as assessed by MTT (1.00 ± 0.02 *versus* 0.83 ± 0.04, 0.86 ± 0.03, 0.85 ± 0.02, 0.86 ± 0.04, 0.87 ± 0.01, $P < 0.001$ for all). Compared to untreated controls, Hep3B cells treated with 1 mM PTX for 24 h showed statistically increased viability (1.00 ± 0.02 *versus* 1.07 ± 0.01, $P < 0.05$). Pretreatment with 1 mM pentoxifylline for 2 (0.97 ± 0.03) and 4 h (0.97 ± 0.01) significantly reduced TNF-α- induced Hep3B cell viability ($P < 0.001$ *versus* 10, 20, 50, 100, and 200 ng/mL TNF-α). Based on these results, we chose the concentrations of 100 ng/mL TNF-α and 1 mM PTX for this experiment (Figure 1).

3.2. Metabolic Effects of MCD Diet and Pentoxifylline on SD Rats. The amount of weight loss was different between rats given MCD plus saline (group III) and MCD plus PTX (group IV) for four weeks: -53.6 ± 9.2 g (19.6%) and -63.4 ± 10.2 g (23.4%) from their initial body weights. However, the difference was not significant. Such degree of weight loss is similar to previously reported data where rats were placed on MCD diets [1–3]. In contrast, rats in the control group gained a minimal amount of weight (4.6%) during the study period. Liver weight was not different among the four groups. The proportion of liver weight to body weight was similar between groups III and IV (Table 1).

The blood concentrations of TG, T-CHO, AST, and ALT were analyzed using serum. Rats in groups III and IV showed

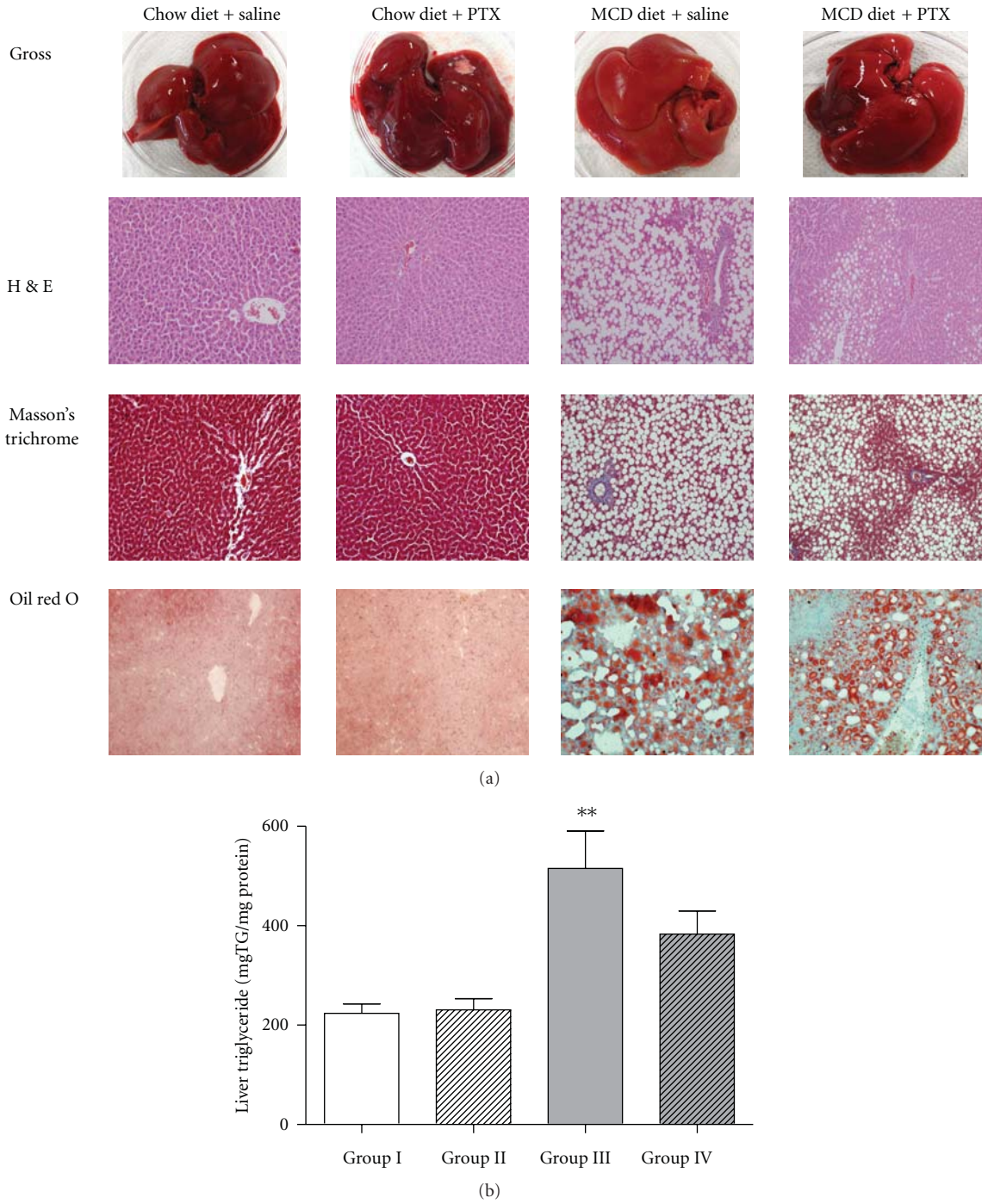

Figure 3: Hepatic histology and triglyceride content after 4 weeks of administration with an MCD diet and pentoxifylline. The liver sections were stained with H&E, Masson's trichrome, and oil-red O staining (×200). The liver of MCD-diet-fed rats showed yellowish markings. Rats administrated pentoxifylline showed the reduced yellow hepatic color and fat accumulation (a). Rats in groups III and IV have increased hepatic triglyceride content relative to rats in the control group. There was no statistical difference between groups III and IV (b). Results are shown as mean ± SEM. $^{**}P < 0.01$ *versus* untreated control group.

statistically decreased serum TG and T-CHO levels relative to rats in the control group. ALT levels were significantly increased in MCD-fed rats (groups III & IV) compared to the control groups, but there were no differences between groups III and IV (Figure 2).

3.3. Hepatic Effects of MCD Diet and Pentoxifylline on SD Rats. Macroscopic and histological images of liver pathology were obtained. Liver sections were stained with H&E, Masson's trichrome, and oil-red O staining. In rats fed chow diet (groups I and II), there was no detectable fatty change

Table 1: Metabolic effects of MCD diet and pentoxifylline on SD rats.

Group	Chow + saline	Chow + PTX	MCD + saline	MCD + PTX
Initial BW (g)	269.2 ± 39.3	265.1 ± 37.6	268.4 ± 34.9	270.7 ± 39.0
Final BW (g)	402.1 ± 32.0	385.6 ± 33.5	214.8 ± 30.4	207.3 ± 32.1
Weight change (g)	132.9 ± 14.2	120.5 ± 10.5	−53.6 ± 9.2	−63.4 ± 10.2
Liver weight (g)	12.4 ± 1.5	11.7 ± 1.0	10.1 ± 2.8	9.8 ± 2.2
LW/BW (%)	3.1 ± 0.0	3.0 ± 0.0	4.7 ± 0.0	4.7 ± 0.0

LW: liver weight, BW: body weight.

in gross appearance and microscopic image as assessed by H&E and oil-red O staining. In contrast, the liver of MCD-diet-fed rats showed yellowish markings, typical of steatosis. Consistent with statistically increased serum ALT levels, extensive macrovesicular steatosis and minimal inflammation around perisinusoidal area were present in group III. In rats that were administrated pentoxifylline, the intensity of yellow color as well as fat accumulation was reduced. In addition to liver discoloration and fat accumulation, minimal perivenular fibrosis typically seen in NASH was found in MCD-fed rats (Figure 3(a)).

Rats in groups III and IV had increased hepatic triglyceride content relative to rats in the control group (515.1 ± 200.5, 375.7 ± 35.92 *versus* 223.6 ± 50.02 mg TG/mg protein), and significant increase was found in group III ($P < 0.01$). However, there were no statistical differences between groups III and IV (Figure 3(b)).

3.4. Effect of MCD-Diet-Induced Hepatic TNF-α Expression. The hepatic TNF-α mRNAs from the four groups were measured using real-time PCR. Hepatic TNF-alpha gene expression was significantly increased in the MCD diet groups (groups III and IV) relative to the control group (1.14 ± 0.15 *versus* 8.12 ± 0.45, 3.45 ± 0.24, $P < 0.001$ for both). Administration of PTX (50 mg/kg, once/day, i.p) in MCD diet rats (group IV) significantly decreased TNF-α mRNA expression ($P < 0.001$) as compared to MCD-diet-fed rats in group III (Figure 4).

3.5. Effect of Pentoxifylline on TNF-α-Induced ER Stress in Hepatocytes and Hep3B Cells. Western blotting assay was performed to elucidate the hypothesis that downregulation of direct or indirect TNF-α-induced ER stress markers by pentoxifylline would attenuate hepatosteatosis.

ER stress markers were measured in liver protein levels *in vivo*. Compared to chow-diet-fed rats (group I), rats fed MCD diet (group III) showed increased levels of GRP78, phosphor-eIF2α, ATF4, ATF6, IRE1, p-JNK, and CHOP, with significant increases found in ATF4, ATF6, and IRE1 ($P < 0.05$ for ATF6, $P < 0.001$ for ATF4 and IRE1). Compared to MCD-diet-fed rats in group III, the expression of GRP78, phosphor-eIF2α, ATF4, ATF6, IRE1, p-JNK, and CHOP was attenuated in PTX-administered, MCD-fed rat (group IV), significantly in GRP78, p-eIF2α, ATF4, and ATF6 ($P < 0.05$ for GRP78 and p-eIF2α, $P < 0.01$ for ATF4 and ATF6, resp.) (Figure 5(a)).

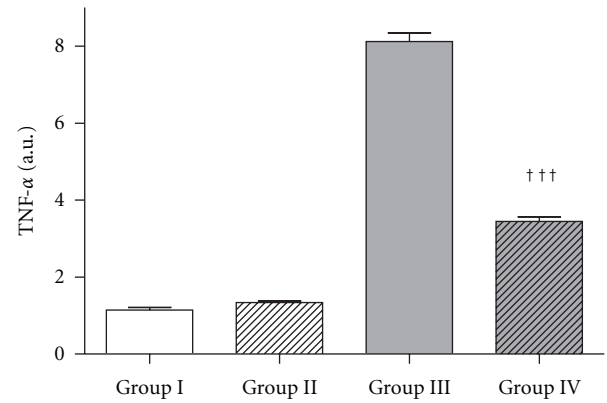

Figure 4: Hepatic TNF-α gene expression after 4 weeks of treatment with an MCD diet and pentoxifylline. There was a significant increase in liver TNF-α mRNA levels in rats fed MCD (groups III and IV). Administration of PTX in MCD diet rats significantly decreased the TNF-α mRNA expression. Results are shown as mean ± SEM. $^{***}P < 0.001$ *versus* untreated control group. $^{\dagger\dagger\dagger}P < 0.001$: MCD plus saline *versus* MCD plus PTX group.

Hep3B cells exposed to 100 ng/mL TNF-α for 24 h activated GRP78, phosphor-eIF2α, ATF4, IRE1, and CHOP *in vitro*. Significant increases were found in phosphor-eIF2α, ATF4, IRE1, p-JNK, and CHOP ($P < 0.05$ for p-eIF2α, ATF4 and $P < 0.001$ for IRE1, p-JNK, and CHOP). Pretreatment with 1 mM PTX for 18 and 24 h reduced TNF-α-induced ER stress in Hep3B cells, significantly in p-eIF2α, ATF4, IRE1, p-JNK, and CHOP (Figure 5(b)).

4. Discussion

Similar to unsatisfactory explanation for pathophysiology of nonalcoholic fatty liver disease (NAFLD) encompassing steatosis plus necroinflammation, controversy remains regarding the efficacy of therapeutic strategy targeting oxidative stress and TNF-α [4, 5] in the treatment of NAFLD. Although heterogenous results of pentoxifylline have been reported from randomized controlled trials of NAFLD treatments [4], consistent evidence in pentoxifylline-treated patients regarding improved liver aminotransferase levels, its safety of treatment for the treatment of alcohol-related liver disease, and significant improvement on mortality suggest pentoxifylline as a candidate for NAFLD treatment [6]. Few

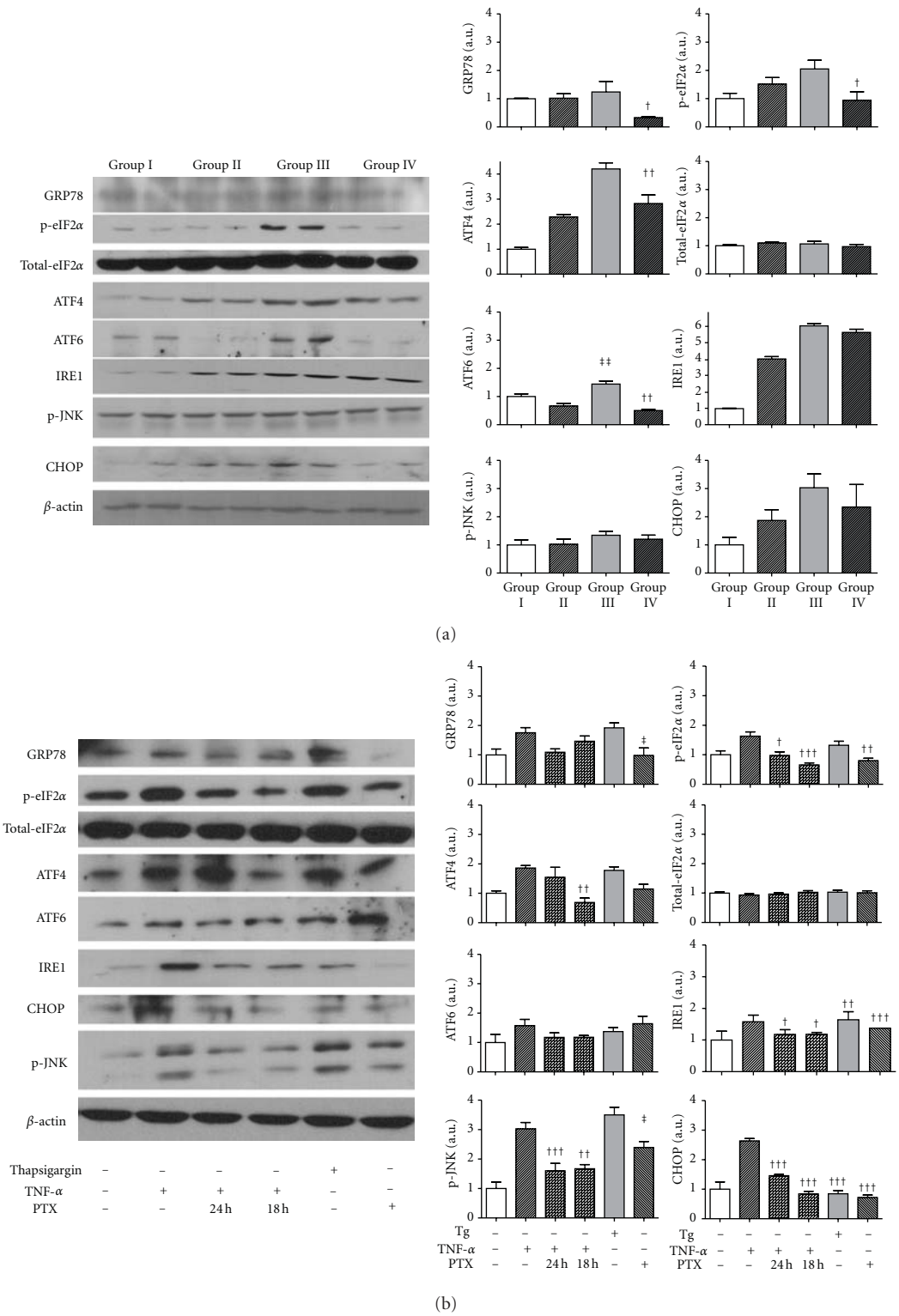

Figure 5: Effect of pentoxifylline on TNF-α-induced ER stress in Hep3B cells and hepatocytes. Rats fed MCD diet (group III) showed activated levels of GRP78, phosphor-eIF2α, ATF4, ATF6, IRE1, p-JNK, and CHOP with significance in ATF4, ATF6, and IRE1. Compared to group III, administration of PTX in MCD diet rats (group IV) attenuated the expression of GRP78, phosphor-eIF2α, ATF4, ATF6, p-JNK, and CHOP (a). The experiments were performed five times under identical conditions. Results are shown as mean ± SEM. $^{***}P < 0.001$ *versus* untreated control group. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.001$: MCD plus saline *versus* MCD plus PTX group. Hep3B cells exposed to 100 ng/mL TNF-α for 24 h activated GRP78, p-eIF2α, ATF4, ATF6, IRE1, p-JNK, and CHOP. Pretreatment of 1 mM PTX for 18 and 24 h reduced TNF-α-induced ER stress in Hep3B cells (b). The experiments were performed four times under identical conditions.

studies have investigated the effects of pentoxifylline on inflammation-induced NAFLD animal model. In view of these facts, our attentions were focused on the antihepatotoxic effect of pentoxifylline (PTX), a nonselective TNF-α inhibitor, on improvement of inflammation and fat droplets accumulation in methionine- and choline-deficient-(MCD-) diet-induced steatohepatitis. We hypothesized that PTX may inhibit TNF-α-induced endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) pathway, allowing for alleviation of steatohepatitis *in vitro* and *in vivo*. Concerning animal models used to investigate the pathogenesis or treatment efficacy of steatohepatitis characterizing human condition, we adopted MCD-diet-induced rat steatohepatitis model. MCD diet depletes hepatic antioxidants, such as GSH and S-adenosylmethionine (S-AMe), and induces production of TNF-α and other proinflammatory cytokines [7]. According to one study, after 26 days of MCD feeding, serum ALT levels increased consistently, and steatohepatitis ultimately developed [1]. In this study, the rats were fed either chow or MCD diet for 4 weeks.

This study has two main findings. First, in accordance with previous reports, the administration of MCD diet for four weeks induced steatosis, inflammation and ballooning degeneration of hepatocytes, but not pericellular fibrosis. Endoplasmic reticulum (ER) stress was found to cause the degradation of misfolded or unfolded proteins in the ER through three pathways, including double-stranded RNA-activated protein kinase—like ER-resident kinase (PERK), inositol-requiring protein-1 (IRE), and activating transcription factor-6 (ATF6) pathwaies [8]. Similar to previous reports on ER stress in development of NAFLD [8, 9], MCD diet increased hepatic TNF-α expression and activated ER stress factors such as Bip/GRP78, p-eIF2, ATF4, ATF6, IRE1, and CHOP. Treatment with 100 ng/mL TNF-α *in vivo* also caused ER stress. The discrepancies of statistical significance between hepatocytes acquired from MCD diet rats and Hep3B cells on the expression of ER stress markers in the group of TNF-α only and the groups of both TNF-α and PTX may be attributed to nonuniform pattern of downstream of ER stress markers, especially ATF4, CHOP, and GADD34 in human and animal subjects with NAFLD and NASH [10].

In contrast with the human metabolic profiles of hepatic consequences of metabolic syndrome [12], MCD-fed animals demonstrate weight loss associated with atrophy of adipose tissue [2]. In this experiment, chow diet resulted in an increase in rat weight by approximately 132.9 g, but MCD diet resulted in a decrease in weight of approximately 53.6 g after four weeks of diet. This weight loss in the MCD diet group led to 50.8% and 58.3% decrease in serum triglyceride and total cholesterol level compared to the chow diet group, with statistical significance. Compared to the controls, MCD-induced steatohepatitis also led to 50.8% and 233.0% increase in AST and ALT levels, with statistical significance found only in ALT level.

Second, intraperitoneal treatment with pentoxifylline in rats fed MCD diet decreased fat accumulation. However, the effects of PTX on either body and liver weight or serum lipid profile and aminotransferases were not profound. The minimal effects of PTX on metabolic profiles, while contradictory to the expected results, may be attributed to the overwhelming effect of MCD diet due to prolonged 4-week feeding model adopted in this study. Conventional duration of the MCD diet ranges from 2 to 3 weeks [1–3]. However, compared to MCD-fed rats, rats fed PTX showed significantly decreased hepatocellular expression of TNF-α mRNA. *In vitro*, pretreatment with 1 mM PTX in the presence of 100 ng/mL TNF-α attenuated ER stress. We have suggested that pentoxifylline has anti-inflammatory effects against cytokine-induced steatohepatitis. Therefore, as we and others have found, pentoxifylline might exert its main effects through alleviation on ER stress.

To summarize our data, we observed that pentoxifylline (PTX) alleviated ER stress in TNF-α-induced cytotoxic human hepatocarcinoma Hep3B cells *in vitro* and had protective effect on the development of steatohepatitis in a methionine- and choline-deficient (MCD) diet animal model *in vivo*. Further clinical studies are needed to evaluate the use of PTX for nonalcoholic fatty liver disease.

Conflict of Interests

The authors declare that there is no duality of interests associated with this paper.

Authors' Contribution

M. K. Chae and S. G. Park contributed equally to this work.

Acknowledgments

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Review Article

ER Stress and Apoptosis: A New Mechanism for Retinal Cell Death

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The endoplasmic reticulum (ER) is the primary subcellular organelle where proteins are synthesized and folded. When the homeostasis of the ER is disturbed, unfolded or misfolded proteins accumulate in the ER lumen, resulting in ER stress. In response to ER stress, cells activate a set of tightly controlled regulatory programs, known as the unfolded protein response (UPR), to restore the normal function of the ER. However, if ER stress is sustained and the adaptive UPR fails to eliminate unfolded/misfolded proteins, apoptosis will occur to remove the stressed cells. In recent years, a large body of studies has shown that ER stress-induced apoptosis is implicated in numerous human diseases, such as diabetes and neurogenerative diseases. Moreover, emerging evidence supports a role of ER stress in retinal apoptosis and cell death in blinding disorders such as age-related macular degeneration and diabetic retinopathy. In the present review, we summarize recent progress on ER stress and apoptosis in retinal diseases, focusing on various proapoptotic and antiapoptotic pathways that are activated by the UPR, and discuss how these pathways contribute to ER stress-induced apoptosis in retinal cells.

1. Introduction

Retinal cell death has been widely held as a central event that leads to retinal neurodegeneration, vascular dysfunction, and eventually irreversible blindness in ocular diseases, such as glaucoma, retinal degeneration, diabetic retinopathy, and uveitis. Loss of retinal ganglion cells is considered to be a direct cause of vision loss in experimental glaucoma [1], correlating with elevated intraocular pressure (IOP) [2]. Injury of retinal pigment epithelial (RPE) cells and photoreceptors leads to photoreceptor dysfunction and retinal degeneration [3], as seen in both inherited and acquired degenerative retinal diseases such as Stargardt's Disease [4], retinitis pigmentosa [5], and age-related macular degeneration [6]. In diabetic retinopathy, high glucose and other diabetic insults, such as oxidants, advanced glycation end products (AGEs), and inflammatory cytokines, result in neural and vascular cell death [7, 8]. In streptozotocin-induced diabetic rats, both retinal neurons and vascular cells become apoptotic soon after the onset of diabetes [9]. Inflammation-driven neural and vascular cell death is also a hallmark characteristic of uveitis, a chronic eye disease that cause vision loss [10–13]. Together, these findings support a pivotal role of cell death in the pathogenesis of retinal diseases. Apoptosis, that is, programmed cell death, is the most common form of cell death in various cell types, including retinal cells. Apoptosis is tightly controlled by a variety of signaling pathways that either promote or inhibit the apoptotic cascades. Among the most extensively studied proapoptotic factors in retinal cells are oxidative stress, mitochondrial dysfunction, inflammation, ischemia, hyperglycemia, and excitotoxicity [14–18]. Intriguingly, recent evidence suggests that disturbed protein homeostasis and endoplasmic reticulum (ER) stress also contribute to apoptosis of retinal cells [19]. Moreover, ER stress activates a large number of genes involved in the control of cell fate, including antiapoptotic and proapoptotic molecules such as Bax and Bcl-2 [20, 21]. Therefore, elucidating the role and mechanisms of ER stress

in retinal cell apoptosis may provide important insight into the pathogenesis of retinal diseases and help in developing new drugs to protect retinal cells and to prevent vision loss. In the present review, we discuss the potential implication of ER stress in retinal cell apoptosis, with a primary focus on the signaling transduction pathways that link ER stress with apoptosis in general as well as specific for retinal cells.

2. ER Stress and the Unfolded Protein Response (UPR)

The endoplasmic reticulum (ER) is the primary intracellular organelle responsible for protein folding, maturation, and trafficking [22, 23]. The ER consists of a network of folded membranes in which secretory and most membrane proteins are synthesized, posttranslationally modified, and folded into their correct three-dimensional conformations. Only properly folded (mature) proteins can be transported to the Golgi apparatus for further processing. In addition, the ER also serves as a dynamic pool of calcium, governing the intracellular calcium homeostasis [24]. Other major functions of the ER include lipid and steroid hormone synthesis, carbohydrate metabolism, and drug detoxification. Importantly, compelling evidence indicates that the ER is one of the major machinery that senses subtle environmental changes and cellular stresses, coordinates signaling pathways, and modulates cell function and cell survival. Various physiological and pathological circumstances, such as excessive mutant proteins, viral infection, energy or nutrient deprivation, as well as alteration in the redox status, can compromise the ER capacity in protein folding, resulting in the accumulation of unfolded or misfolded proteins in the ER lumen, or ER stress. In turn, misfolded proteins aggregate to form insoluble intracellular or extracellular deposit, which is toxic to the cell. It has been demonstrated that a number of age-related diseases, such as Alzheimer's diseases; inflammatory disorders, such as diabetes; and neurodegenerative diseases, such as Parkinson's disease, are associated with the build-up of misfolded or unfolded protein aggregates [25–28]. To eliminate the toxic protein components, cells activate an adaptive mechanism that consists of a number of intracellular signaling pathways, collectively known as unfolded protein response (UPR). The UPR relieves ER stress and restores the protein homeostasis through three complementary strategies: (1) halt the generation of more unfolded proteins by suppression of protein translation; (2) induce ER-related molecular chaperones to promote refolding of the unfolded proteins, and (3) activate the ER-associated protein degradation (ERAD) system to remove the unfolded proteins.

There are three branches of UPR that are initiated by distinct ER stress transducers located on the ER membrane: PKR-like endoplasmic reticulum kinase (PERK) [29], inositol-requiring enzyme 1 (IRE1) [30, 31], and activating transcription factor 6 (ATF6) [22]. In nonstressed cells, all three ER stress transducers are kept in an inactive state through binding to the ER chaperon glucose-regulated protein 78 (Bip), which is also known as immunoglobulin binding protein (Bip) [32, 33]. Upon ER stress, excessive unfolded proteins accumulate in the ER lumen, resulting in the dissociation of GPR78 from the ER stress transducers [34], which triggers activation of the UPR branches. In eukaryotic cells, UPR is an adaptive cellular response to the disturbance of normal ER functions, which attenuate the aggregation of unfolded or misfolded proteins and promote cell survival [35]. However, during prolonged or overwhelming ER stress, UPR fails to restore the normal function of the ER, and apoptotic cascade will be activated [36, 37] (Figure 1). The exact mechanism underlying the switch of the UPR from a prosurvival mechanism to a proapoptotic response is not clear.

2.1. The IRE1/XBP1 Pathway. IRE1 was firstly identified as an ER transmembrane protein kinase that is essential for signaling transduction from the ER to the nucleus [38] and was subsequently found to be involved in the initiation of the UPR [39]. There are two different IRE1 proteins in mammalian cells, both of which participate in the ER stress response or UPR. IRE1α is ubiquitously expressed while IRE1β is tissue-specific [30, 40]. During ER stress, IRE1 dissociates with Bip/Bip and becomes activated. Activated IRE1 acquires the function as endogenous ribonuclease (RNase) and splices a 26-nucleotide intron from the mRNA of XBP1. The splicing results in a shift in the translational frame of the XBP1 gene, leading to the translation of a new protein, named spliced XBP1 [31, 41]. The newly generated spliced XBP1 is an active transcription factor, which in turn induces diverse downstream genes, such as ER chaperones [42] and proteins involved in ER-associated protein degradation (ERAD) [43]. These proteins work together to restore the ER homeostasis and promote cell survival. Indeed, cells deficient of XBP1 are susceptible to oxidative stress- and inflammation-induced cell death [44, 45], suggesting that XBP1-mediated adaptive UPR is an important mechanism that protects the cell from apoptosis during ER stress. In addition, the IRE1/XBP1 pathway is also essential for embryonic development. Genetic deletion of IRE1 or XBP1 is lethal to mouse embryo due to fetal liver hyperplasia [46, 47]. In addition, a recent study shows that loss of IRE1 results in severe dysfunction of the placenta and also contributes to the embryonic lethality of IRE1 KO mice [48].

2.2. The PERK/eIF2α/ATF4 Pathway. PERK is a serine/threonine protein kinase located on the ER membrane. Like IRE1, PERK is activated by ER stress via dimerization and autophosphorylation upon the dissociation with Bip. Activated PERK phosphorylates its downstream target protein, eIF2α, resulting in the inhibition of global protein translation [49]. However, some genes with upstream open reading frames (uORFs) in its 5′ untranslated region could escape from the eIF2α-initiated translational attenuation. A representative example is activating transcription factor 4 (ATF4)—human ATF4 gene contains multiple uORFs in its 5′UTR whereas the murine mRNA has two uORFs [50]. These uORFs prevent the translation of ATF4 under

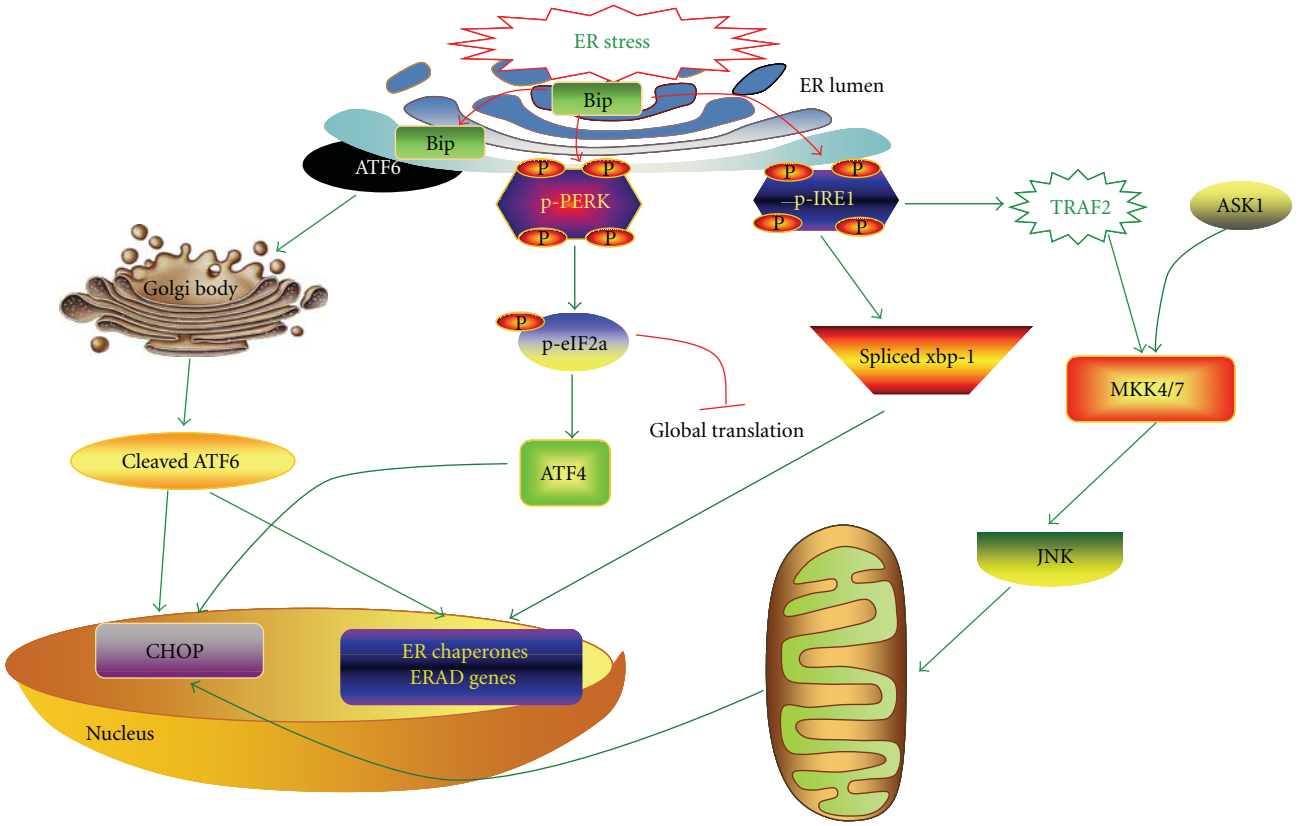

Figure 1: Signaling pathways of the UPR. Accumulation of unfolded proteins in ER lumen results in the ER stress. In response to ER stress, Bip dissociates from ER stress transducers and binds to unfolded and misfolded proteins, resulting in the activation of ER stress transducers- IRE1, PERK and ATF6. Upon activation, IRE1 splices the mRNA of XBP1, and produces an active transcription factor named spliced XBP1 (XBP1-S), which upregulates ER chaperones and proteins implicated in the ER-associated protein degradation (ERAD). In addition, IRE1 recruits TRAF2 and ASK1, resulting in JNK activation. The activation of PERK increases phosphorylation of eIF2α, leading to a global attenuation of protein synthesis and a concomitant increase in ATF4 translation. In turn, ATF4 induces CHOP, a proapoptotic transcription factor. After the dissociation of Bip, ATF6 translocates to Golgi apparatus, where it is activated by proteolysis. Activated ATF6 transcriptionally induces ERAD genes and upregulates CHOP expression.

normal conditions but enhance its expression when eIF2α is phosphorylated [51, 52]. ATF4 belongs to the superfamily of DNA-binding proteins that includes the activator protein-1 (AP-1) family, cAMP-response element binding proteins (CREBs), and CREB-like proteins. As a transcription factor, ATF4 binds to the CRE site in the promoter region of target genes, inducing a battery of stress response genes involved in oxidative stress, amino acid synthesis, and transportation. In addition, ATF4 is a major inducer of C/EBP homologous protein (CHOP), which has been considered as a central mediator of ER stress-induced apoptosis. The role of CHOP in coordinating the apoptotic pathways will be discussed in detail in the following chapters.

2.3. The ATF6 Pathway. Besides XBP-1 and ATF4, ATF6 has been identified as another basic leucine zipper- (bZIP-) containing transcription factor induced by ER stress. ATF6 is a type II ER transmembrane protein. Like IRE1 and PERK, ATF6 binds to Bip and remains in an inactive state in unstressed cells. In response to ER stress, the Bip/ATF6 complex is dissociated, resulting in the translocation of ATF6 from ER membrane to Golgi apparatus. In Golgi apparatus, ATF6 is cleaved by two proteases, serine protease site-1 protease (S1P) and the metalloprotease site-2 protease (S2P), to produce the active form of the transcription factor [53–55]. The active ATF6 then moves to the nucleus and activates the ER stress response element- (ERSE-) related genes through binding their promoters [35]. ATF6 also regulates other URP genes, such as XBP-1 and CHOP [56].

3. ER Stress-Associated Apoptosis in Retina Cells

Previous studies suggest that the cell fate is dependent on the balance between the extent/severity of ER stress and the capacity of the ER to restore ER homeostasis through the UPR [57–59]. Temporal and mild ER stress can be overcome by the adaptive UPR, cell function maintained, and cells survive. However, if the stress condition is prolonged and the UPR fails to restore the ER homeostasis, the apoptotic signaling pathways will be initiated to remove the unhealthy cells. Recently, several independent studies have provided ample

evidence that ER stress is a potential cause of retinal vascular and neuronal cell death in diseases such as glaucoma, diabetic retinopathy and age-related macular degeneration [19, 60–62]. ER stress has been observed in both cultured retinal cells (vascular endothelial cells, pericytes, ganglion cells, Muller cells, as well as RPE cells) and in the retina from animal models of various diseases. Not surprisingly, the role of ER stress has been extensively studied in the pathogenesis of retinitis pigmentosa (RP) with mutations of various retinal genes. In 2004, Rebello and associates reported that expression of a mutant (R14W) of carbonic anhydrase IV, a glycosylphosphatidylinositol-anchored protein that is highly expressed in the choriocapillaris of the human eye, induced upregulation of Bip, PERK, and CHOP, markers of ER stress and the unfolded protein response, accompanied by apoptosis [63]. Similarly, enhanced ER stress was reported in RP induced by the rhodopsin mutation P23H in Xenopus laevis [64] and in rats [65, 66]. Further, stimulation of the UPR in the retina or cultured retinal cells by preexposure to mild ER stress protected photoreceptor neurons from oxidative damage and cell death [67]. Moreover, overexpression of Bip, an ER chaperone that facilitates protein folding and reduces ER stress, attenuated retinal expression of CHOP and the activation of apoptotic cascade and restored retinal photoreceptor function in P23H rats [61]. In addition to the genetic models of RP, ER stress was found remarkably enhanced in retinal photoreceptors, coincident with photoreceptor cell apoptosis, in a rodent model of light damage-induced retinal degeneration [62]. These findings collectively support a causal role of ER stress in photoreceptor cell death and retinal degeneration.

Another well-studied area for ER stress-related retinal cell death is glaucoma. Increased ER stress markers were observed in retinal ganglion cells in animal models of ischemia-reperfusion and chronic glaucoma [68]. Cultured retinal ganglion cells (RGC-5, a transformed rat ganglion cell line) treated with tunicamycin, a common ER stress inducer, undergo apoptosis, accompanied by increased production of ER stress-related proteins [19, 69]. *In vivo*, intravitreal injection of tunicamycin resulted in loss of retinal ganglion cells and reduced thickness of the inner retina. Moreover, raising IOP or intravitreal injection of N-methyl-D-aspartate (NMDA), an excitotoxin that binds to the NMDA receptor and induces neuron cell death, also increased the expression of ER stress markers in retinal ganglion cells, amacrine cells, and microglial cells [19]. Pharmaceutical induction of Bip significantly attenuated tunicamycin- or NMDA-induced apoptosis in retinal ganglion cells, suggesting a pivotal role of ER stress in retinal neuron cell death [69].

Loss of retinal vascular cells and apoptosis of retinal neurons have been recognized as critical events and pathological features of diabetic retinopathy [70–72]. Although currently it remains to be investigated how ER stress signaling pathways contribute to retinal cell death induced by diabetes, recent studies by our group and others demonstrated that ER stress was induced in early stage of diabetic retinopathy and was implicated in retinal inflammation and vascular damage [73–76]. In 2009, we reported increased ER stress markers in the retina of diabetic Akita mice, in parallel with elevated expression of inflammatory genes [73]. In cultured retinal endothelial cells, ER stress was induced by hypoxia, a potent stimulator of inflammation and angiogenesis, and prevented by chemical chaperones. Moreover, we showed that induction of ER stress in the retina was sufficient to trigger an upregulation of inflammatory genes. Conversely, inhibiting ER stress protected the retina and retinal endothelial cells from inflammatory damage. In addition, activation of the adaptive UPR by preconditioning with ER stress also successfully prevented inflammatory damage to retinal endothelial cells and vascular leakage induced by diabetic stimulus [74]. These results suggest that ER stress is implicated in retinal cell damage caused by diabetes.

4. Signaling Pathways of ER Stress-Associated Apoptosis

It is currently unclear how ER stress induces apoptosis in various retinal cells. Generally, there are two major pathways for the initiation of apoptosis: extrinsic and intrinsic pathways [77]. The extrinsic pathway is mediated by the cell membrane death receptors. Activation of the death receptor recruits adaptor molecules and activates caspase-8 or caspase-10, which cleaves the downstream substrates, that is, other caspases including caspase-3, resulting in apoptosis [78]. The intrinsic pathway is closely related to factors anchored on the mitochondria. The insertion of these proapoptotic proteins changes the mitochondrial membrane permeability, resulting in the release of cytochrome *c* from mitochondria into the cytosol. Then cytochrome *c* binds to Apaf-1 and activates caspase-9 and then caspase-3, leading to the execution of cell death. In addition, accumulating evidence suggests that calcium release from the ER can also initiate the cell death signals, either by directly activating death receptors or by altering the sensitivity of mitochondria. Finally, these apoptotic pathways converge on caspase-3, resulting in the cleavage of other proteases and leading to apoptosis. In addition to caspase-dependent pathways, caspase-independent pathway is also implicated in retina apoptosis [79]. In the following sections, we discuss the potential pathways that may play a role in ER stress-associated apoptosis in various retinal diseases, such as age-related macular degeneration, glaucoma, and diabetic retinopathy (Figure 2).

4.1. CHOP: A Key Mediator of ER Stress-Induced Apoptosis. CHOP, also named as growth-arrest and DNA-damage-inducible gene 153 (GADD153), is a major stress-inducible proapoptotic gene in ER stress-induced apoptosis [80]. All three branches of the UPR regulate the activation of CHOP; however, ATF4 is considered as the major inducer of CHOP expression. CHOP is expressed at a very low level under physiological conditions but its expression level significantly increases in the presence of severe or persistent ER stress. Notably, the induction of CHOP well correlates with the onset of ER stress-associated apoptosis [81, 82], silencing CHOP expression protects cells against apoptosis induced by prolonged ER stress [83]. As a transcription factor, CHOP

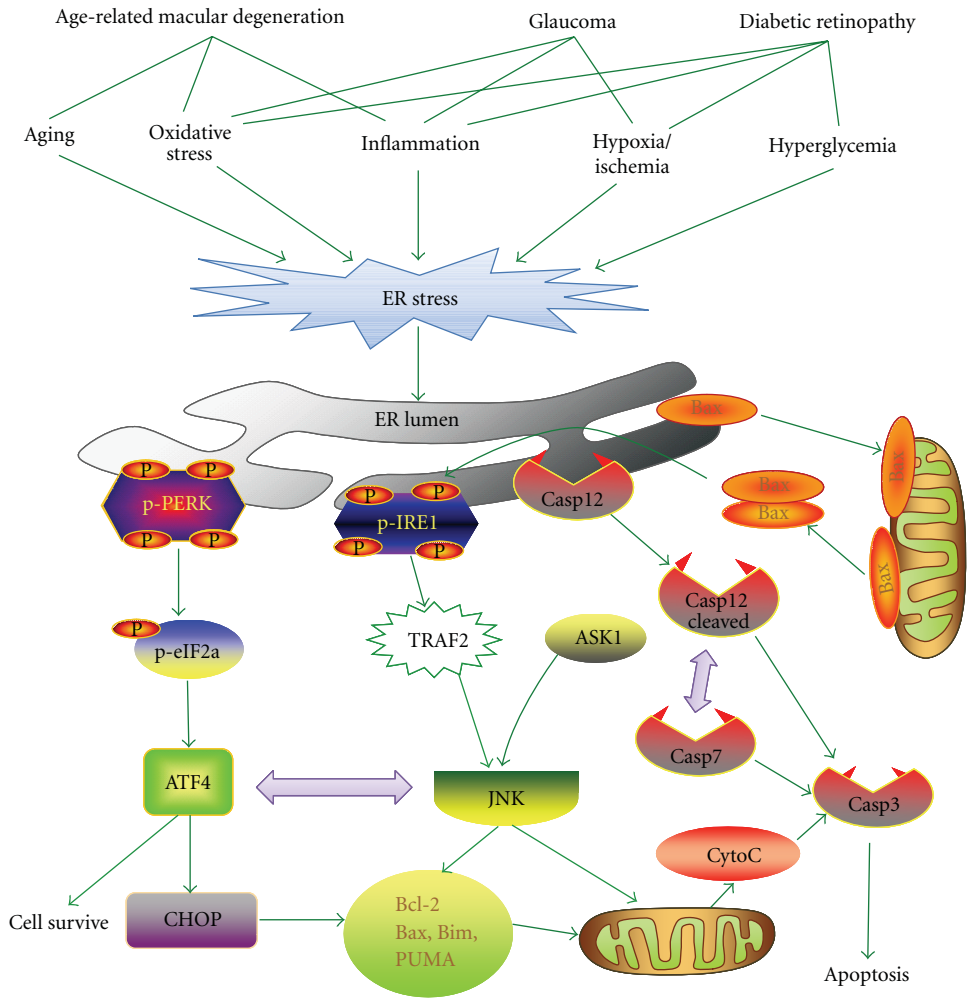

Figure 2: ER stress-associated apoptotic pathways in retinal diseases. A variety of pathogenic factors in chronic retinal degenerative diseases (e.g., age-related macular degeneration, glaucomatous retinopathy and diabetic retinopathy), including aging, oxidative stress, hypoxia, inflammatory factors, and hyperglycemia and others, can disturb ER function and compromise the adaptive UPR, resulting in persistent ER stress in retinal cells. This leads to sustained activation of the ATF4/CHOP pathway and the IRE1/TRAF2/ASK/JNK pathway. Both JNK and CHOP attenuate the function of the pro-survival factor Bcl-2, but enhances the activity of proapoptotic Bcl-2 proteins such as Bim, Bax, and PUMA, resulting in mitochondrial dysfunction and cytochrome *c* release. In addition, caspase-12 is activated during ER stress, which sequentially activates caspase-7 and/or caspase-3, leading to mitochondria-independent apoptosis.

has been shown to regulate numerous pro- and antiapoptotic genes, including Bcl-2, GADD34, and TRB3 [84]. CHOP directly binds the promoter of TRB3 gene and upregulates its expression [84], which in turn inhibits AKT activation, resulting in apoptosis and cell death [85]. Intriguingly, TRB3 also regulates CHOP expression through negative feedback. Overexpressing TRB3 inhibits the transcriptional induction of CHOP while silencing TRB3 results in upregulation of CHOP under both normal and stressed conditions [86]. Treatment with PBA, a chemical chaperone that attenuates ER stress, restores AKT phosphorylation, reduces CHOP and TRB3 expression, and prevents apoptosis. These findings indicate that CHOP is a key mediator of ER stress-induced apoptosis and is tightly regulated by multiple factors, including UPR components such as ATF4 and its downstream genes such as TRB3.

4.2. Mitochondria and the Bcl-2 Family. Recent evidence suggests that mitochondrial dysfunction plays a role in ER stress-induced apoptosis [20, 87]. ER stress, via the UPR, also regulates a number of apoptosis-associated proteins that localize on the mitochondrial membrane, notably the members of the Bcl-2 family. These proteins are widely held as the central coordinators of mitochondria-mediated apoptotic pathways. The Bcl-2 family consists of antiapoptotic members, such as Bcl-2 and Bcl-xL, and proapoptotic proteins, such as Bax, Bak, and Bik [88]. The balance between the anti- and proapoptotic proteins is important for maintaining normal mitochondrial function as well as cell survival. Cells overexpressing Bcl-2 or deficient of Bax and Bak are resistant to ER stress-induced apoptosis [89]. Conversely, overexpressing Bax promotes cytochrome *c* release and activates apoptotic enzymes, leading to cell

death [90]. BH3-only proteins, such as Bim and Bax, are proapoptotic members of the Bcl-2 protein family, playing an essential role in the initiation of programmed cell death and stress-induced apoptosis [91]. Recent studies show that both the antiapoptotic gene Bcl-2 and the proapoptotic proteins, for example, Bim and Bax, are regulated by CHOP during ER stress [87, 92]. CHOP downregulates Bcl-2 expression but upregulates Bim and promotes the translocation of Bax into the mitochondria [93]. Another BH3-only protein, p53-upregulated modulator of apoptosis (PUMA), is induced by p53 during ER stress, and PUMA-deficient cells are resistant to ER stress-elicited apoptosis. These results imply an important role of p53 and PUMA in ER stress-associated cell death [94]. In addition to mediating ER stress-driven apoptosis, the Bcl-2 family also regulates ER stress through physical interaction with ER stress sensors and UPR components. For example, both Bax and Bak have been reported to form a protein complex with IRE1α, which is essential for IRE1α activation [95]. Double knockout mice that lack Bax and Bak exhibited decreased expression of XBP1, a substrate of IRE1, and developed extensive tissue damage in the liver in response to ER stress induced by tunicamycin [95]. Thus, the mediators and pathways implicated in ER stress-related apoptosis are very complex. Nevertheless, the interdependent regulation of Bcl-2 proteins and the UPR appears to be a key event in the process of fine tuning of pro- and antiapoptotic system during ER stress.

4.3. Caspase-12: An ER-Resident Caspase. Caspase-12 is a member of the inflammatory group of the caspase family, localized to the ER. Moreover, it has been shown that caspase-12 is specifically activated by ER stress, including disruption of ER calcium homeostasis and accumulation of excess proteins in ER, but not by membrane- or mitochondrial-targeted apoptotic signals [96]. Mice deficient of caspase-12 are resistant to ER stress-induced apoptosis, suggesting that caspase-12 plays a critical role in this process [96]. However, the human caspase-12 gene has a single nucleotide polymorphism, which results in the production of either a truncated caspase-12 protein or a full-length protein with no enzymatic activity [97]. In human, caspase-4, a member of caspase-1 subfamily that includes caspase-12, was found localized to the ER membrane and activated specifically by ER stress-inducers [98]. Cleavage of caspase-4 was not affected by overexpression of Bcl-2, which prevents signal transduction on the mitochondria, suggesting that caspase-4 is primarily activated in ER stress-induced apoptosis [98]. Furthermore, a reduction of caspase-4 expression by small interfering RNA decreased ER stress-induced apoptosis in some cell lines, but not other ER stress-independent apoptosis [98]. Although the role of caspase-12 (caspase-4 in human) has been well established, it remains unclear how caspase-12 is activated during ER stress. Recent studies suggest that caspase-12 activation requires the IRE1 signal [99]. Upon activation by ER stress, the cytosolic domain of IRE1 recruits TNF receptor-associated factor 2 (TRAF2), which interacts with caspase-12 and induces the cleavage and activation of the enzyme [100]. In turn, activated caspase-12 cleaves procaspase-9 into active caspase-9, which further cleaves and activates caspase-3, resulting in apoptosis [101]. In addition, caspase-12 can also be activated by its downstream executioner caspase-7, indicating a possible amplification loop in the apoptotic cascades though caspase-12 [102]. Notably, in caspase-12-mediated apoptotic process, cytochrome *c* is not released from mitochondria, which suggests that cytochrome *c* is not involved in the caspase-12-dependent apoptosis [101].

4.4. The JNK Pathway in ER Stress-Mediated Apoptosis. The c-Jun N-terminal kinases/stress-activated protein kinase (JNK/SAPK) pathway is one of three members of the mitogen-activated protein kinase (MAPK) superfamily which also includes the ERK and the p38 MAPKinases [103]. JNK is originally identified for specifically phosphorylating the transcription factor c-jun in its N-terminal transactivation domain [103]. There are three different isoforms of JNK (JNK1, 2, and 3). Among these isoforms, JNK1 and JNK2 are ubiquitously expressed while the expression of JNK3 is tissue specific [104]. It has been reported that JNK is activated by various stress factors and contributes to apoptosis and cell death [105, 106]. Recent evidence suggests that JNK activation is also involved in ER stress-initiated apoptotic cascades [107]. For example, activation of IRE1 by ER stress recruits and activates tumor necrosis factor receptor-associated factor 2 (TRAF2), which further activates JNK [99], resulting in caspase-12 activation and apoptosis [100]. IL-1β, a proinflammatory cytokine, stimulates JNK activation and enhances ER stress in pancreatic epithelial cells [108]. Pretreatment with JNK inhibitor abrogates IL-1β-induced ER stress, indicated by phosphorylation of eIF2α, and increased expression of CHOP, GADD34, ATF4, and spliced XBP-1 while inhibition of ER stress does not affect JNK activation by IL-1β [108]. This suggests that JNK activation is required for IL-1β-induced ER stress. In addition, recent studies demonstrate that inhibiting JNK resulted in reduced ATF4 expression during osteoblast differentiation. JNK inhibition also alleviated Bcl-2 antagonist-induced ER stress in a lymphoma cell line [109, 110]. These findings collectively indicate a pivotal role of JNK in induction of ER stress and in mediating ER stress-induced apoptosis, which is yet to be studied in retinal cell apoptosis and retinal diseases.

4.5. Fas-FasL-Induced Apoptosis. The Fas death receptor belongs to the TNF receptor superfamily and is known as important inducer of apoptosis. Fas, through binding to its ligand FasL, recruits and activates the zymogen (precursor) form of cysteine protease caspases, particularly procaspase-8 and -10, which in turn activate caspase-3 and the downstream apoptotic cascades [111, 112]. Previous studies reported that Fas-FasL system is activated in diabetic retinopathy and is implicated in retinal vascular cell death in diabetic animals [113]. Treatment of retinal endothelial cells with neutrophils isolated from patients with diabetic retinopathy induced adhesion of neutrophils to endothelium and caused endothelial apoptosis [114]. Blockade of the Fas-FasL interaction prevented retinal endothelial apoptosis [114]. In an *in vivo* study, inhibiting FasL potently reduced

retinal vascular endothelial cell injury, apoptosis, and blood-retinal barrier breakdown in diabetic animals [115]. Increased immunoreactivity of Fas/FasL and Fas-associated death domain (FADD) was observed in retinal glial cells and ganglion cells in rats with experimental glaucoma [116]. These findings suggest an important role of Fas-FasL system in retinal cell death. While it is widely held that binding and interaction of Fas and FasL are important for activation of the Fas signaling, recent studies suggest that Fas can also be regulated independently of FasL. Timmins and associates reported that Fas expression was induced by ER stress through a pathway involving calcium/calmodulin-dependent protein kinase IIgamma (CaMKIIgamma) and JNK [117]. In addition, activation of CaMKII by ER stress also activated STAT1, a proapoptotic signal transducer, and induced mitochondrial-dependent apoptosis, including release of mitochondrial cytochrome *c* and loss of mitochondrial membrane potential [117]. It was proposed that prolonged CHOP expression leads to the release of ER calcium stores, which increases cytosolic calcium concentration, resulting in CaMKII activation and apoptotsis. The role of CaMKII in retinal cell apoptosis remains to be elucidated.

5. Perspectives

Emerging evidence suggests that ER stress plays a pivotal role in retinal apoptosis and cell death. Studies in other fields have identified a number of signaling pathways that are implicated in ER stress-mediated apoptotic process. These include CHOP induction, caspase-12 activation, mitochondria dysfunction, JNK activation, Fas-FasL system and the STAT1 pathway. Blocking each of these pathways reduces or prevents ER stress-induced apoptosis to a certain extent; however, induction of an individual proapoptotic pathway may not be sufficient to induce apoptosis [117]. This suggests that prolonged ER stress may activate multiple subthreshold proapoptotic pathways, and these pathways interact and regulate each other to execute apoptosis. In addition, prolonged ER stress may also suppress the compensatory cell survival pathways induced by the UPR, such as the IFN-β and Akt-p38α pathways [117]. Recently, we demonstrated that enhancing the endogenous adaptive UPR system by preconditioning retinal cells with mild ER stress was able to reduce vascular inflammation and retinal vascular leakage [74]. Moreover, inducing molecular chaperones, for example, heat shock protein 90 (Hsp90), by antibiotics [118] prevents protein aggregation and that protects photoreceptors against retinal degeneration in a murine model of autosomal dominant retinitis pigmentosa (ADRP) [119]. In addition, overexpressing Bip/Bip in retinal photoreceptors alleviated ER stress, reduced CHOP expression, and mitigated photoreceptor apoptosis in P23H rhodopsin transgenic rats [61]. These findings support an essential role of the adaptive URP system and the URP-activated survival pathways in protecting retinal cells against apoptosis and cell death. Therefore, identifying the key proapoptotic and antiapoptotic pathways implicated in ER stress-associated apoptosis and addressing how these pathways are involved in different pathological conditions of retinal cells may offer the opportunity for developing new drugs to treat retinal diseases.

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Review Article

Endoplasmic Reticulum Stress-Related Factors Protect against Diabetic Retinopathy

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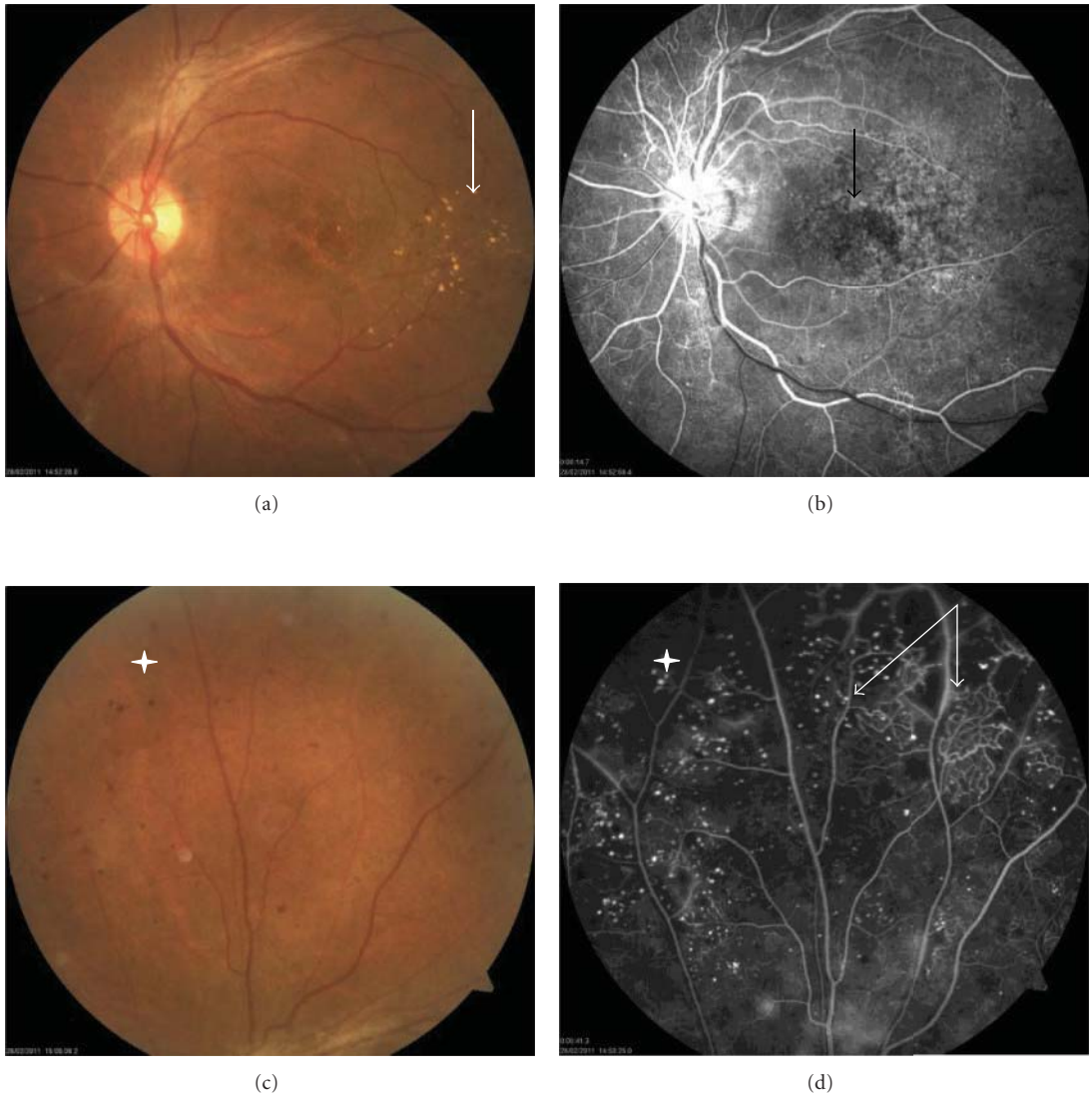
The endoplasmic reticulum (ER) is a principal mediator of signal transduction in the cell, and disruption of its normal function (a mechanism known as ER stress) has been associated with the pathogenesis of several diseases. ER stress has been demonstrated to contribute to onset and progression of diabetic retinopathy (DR) by induction of multiple inflammatory signaling pathways. Recent studies have begun to describe the gene expression profile of ER stress-related genes in DR; moreover, genes that play a protective role against DR have been identified. $P58^{IPK}$ was determined to be able to reduce retinal vascular leakage under high glucose conditions, thus protecting retinal cells. It has also been found by our lab that ER-associated protein degradation factors exhibit significantly different expression patterns in rat retinas under sustained high glucose conditions. Future research based upon these collective genomic findings will contribute to our overall understanding of DR pathogenesis as well as identify potential therapeutic targets.

1. Introduction

Diabetic retinopathy (DR) is one of the most common complications of diabetes mellitus. The surge in diabetes across the globe has led to DR becoming one of the main causes of blindness. Early clinical manifestations of DR include apoptosis of retinal pericytes and increase in permeability of ocular blood vessels. As a consequence, the protective blood-retinal barrier is broken down, which further results in microaneurysm, hard exudates, retinal edema, and minor bleeding. As the disease progresses, the retinal capillary structure becomes so perturbed that all normally perfusable areas are eventually lost (Figure 1). Collectively, these initial clinical changes are diagnosed as nonproliferative (NP) DR, and the disease is considered to have advanced to a proliferative stage (PDR) once revascularization of the affected region has commenced. At this point, successive clinical observations include retinal neovascular proliferation, vitreous hemorrhage, retinal detachment, and loss of vision. Ultimately, the patient succumbs to blindness. Interestingly, retinal edema progression to involve the macula, the central area of the retina, is considered the principal underlying cause of impaired vision.

Many large-population, multicenter clinical studies have been performed to identify the most significant risk factors of DR onset and progression. It appears that the features of diabetes duration, severity of hyperglycaemic episodes, and elevated blood pressure are directly related to DR [1–4]. Many DR pathoincidence theories have been proposed on the basis of these findings, but none have yet to result in an effective "gold-standard" prophylactic or therapeutic strategy. At present, the preferred clinical treatment process is careful maintenance of blood glucose levels and of blood pressure. Events of neovascularization and capillary nonperfusion are commonly treated by partial retinal photocoagulation and pan-retinal photocoagulation, and macular edema is treated by laser and vitrectomy. At the latest stages of DR, retinal reattachment surgery is available.

Despite a vast amount of investigative effort, the pathoincidence of DR is not completely understood. Research findings have implicated roles for the polyol pathway (aldose reductase-mediated conversion of glucose to sorbitol) [5, 6], protein kinase C (a known mediator of glucose transport) [7, 8], advanced glycation end products (AGEs, forming from accumulated glucose), and oxidative stress



(a) (b)

(c) (d)

Figure 1: Fundus photographs (a, c) and retinal fluorescence angiography (b, d) of a patient with proliferative diabetic retinopathy. (a, b) and (c, d) are images of the same part of the retina. The Fundus photographs revealed widely scattered spots on the retina, which represent microaneurysms (white star in c). In addition, we observed that the macular foveal reflex had disappeared. Yellow-white exudates were apparent on the temporal area of the macula (white arrow in a). The angiograms were obtained during the arterial phase (b) and the late arteriovenous phase (d), after injection of dye into an antecubital vein. Retinal neovascularization was observed adjacent to areas of vascular nonperfusion (white arrow in d). The multiple, tiny fluorescent dots (white star in d) are microaneurysms. The blood-retinal barrier breakdown manifests as neovascular lesions, which fluoresce brightly and appear blurred as the dye leaks from the vascular lumina (black arrow in b).

(a by-product of glucose metabolism) [9, 10]. Moreover, specific immune/inflammatory factors and angioincidence factors [11, 12] have been implicated in the incidence and development of DR [13–18].

The incidence of DR has also gained the interest of clinical geneticists interested in determining whether heredity may significantly contribute to DR risk. Indeed, many DR-susceptibility genes have been identified by their efforts [19–21]. Therefore, DR is believed to have a genetic component, and further research into this mechanism will advance our overall understanding of DR pathogenesis and help to identify targets for potential genome-based therapy.

2. ER Stress and DR

Recently, studies into the underlying molecular mechanisms of DR have suggested that endoplasmic reticulum (ER) stress may play important roles in triggering and maintaining the disease state. The ER organelle mediates processing of newly translated proteins, from synthesis and modification to transport. ER stress is the process of the ER adjusting its function, accelerating or decelerating internal machinery to effectively meet the precise needs of the cell under dynamic conditions. For example, the cellular unfolded protein response (UPR) acts to reduce overall protein

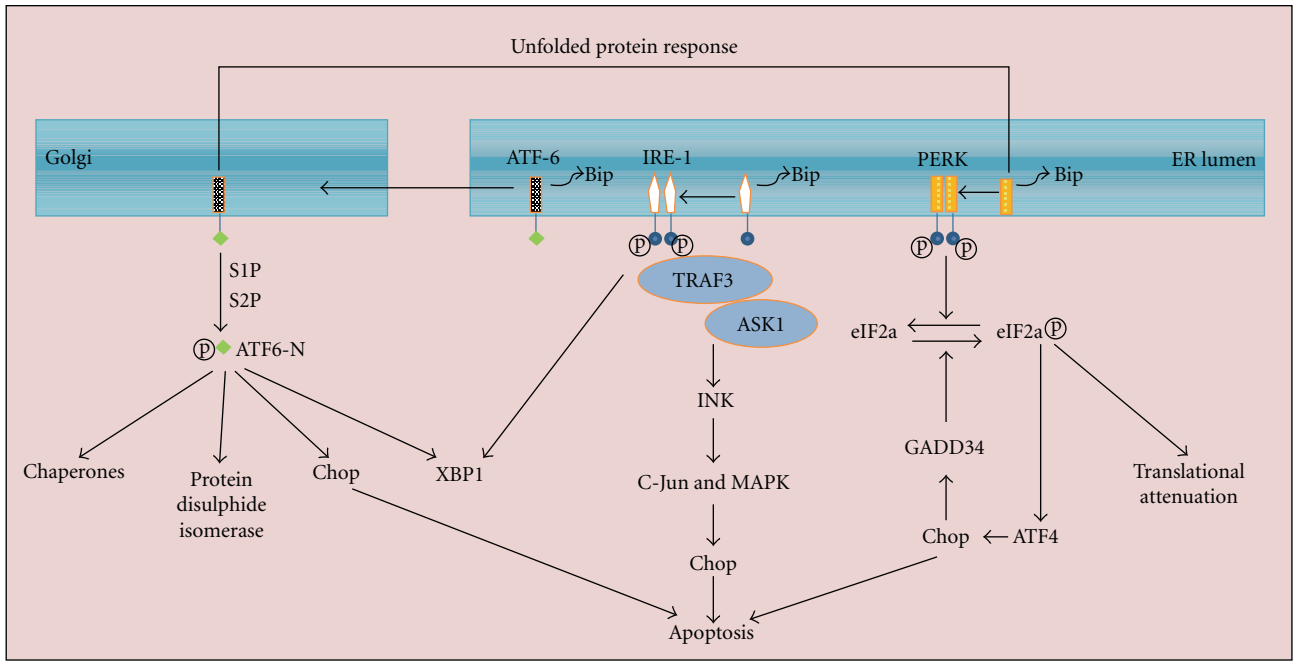


Figure 2: In response to ER stress, Bip separates from the three transmembrane mediators, causing the unfolded protein response to be activated. Unbound PERK then phosphorylates eIF2α, leading both to inhibition of new protein translation and to induction of the ATF4 transcription factor. ATF4, in turn, activates CHOP gene expression, which then promotes apoptosis and induces the growth arrest and DNA damage-inducible gene 34 (GADD34). The GADD34 phosphatase dephosphorylates eIF2α, thereby completing a negative feedback loop. Meanwhile, the unbound IRE1 initiates splicing of the XBP-1 mRNA. Recruitment of the TNF receptor-associated factor3 (TRAF3) and apoptosis signal-regulating kinase 1 (ASK1) to IRE1 leads to activation of c-Jun amino-terminal kinase (JNK), which in turn activates c-Jun and mitogen-activated protein kinase (MAPK) and ultimately promotes CHOP activity. Unbound ATF6 is cleaved within the Golgi apparatus by the site-1 protease (S1P) and the site-2 protease (S2P) to produce an active transcription factor fragment known as ATF6-N, which in turn activates XBP1 and CHOP; in addition, transcription of ER chaperones and protein disulphide isomerase is increased.

synthesis speed, which in turn decreases protein components entering into the ER. Subsequently, expression of the ER molecular chaperones is upregulated, and the protein folding function is accelerated in an attempt for the cell to recover homeostasis. In the event that the UPR becomes too robust or prolonged [22–25], three ER stress factors are induced to facilitate quelling of the process. The pancreatic kinase-(PKR-) like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring enzyme 1 (IRE1) act by binding to the key ER protein immunoglobulin heavy chain protein/glucose-regulated protein, which has a molecular weight of 78 kDa (Bip/glucose-regulated protein 78 (GRP78)) [26]. However, Bip will dissociate from PERK, ATF6 and IRE1 under conditions of accumulated unfolded or misfolded proteins inside the ER. After such dislocation, the released PERK, ATF6, and IRE1 become activated, and relevant signal transmission is initiated (Figure 2).

A variety of other factors (over 200 known to date) participate in the process of ER stress. These factors have been divided into 11 subclasses, according to their functions: unfolded protein binding, ER protein folding quality control, regulation of cholesterol metabolism, regulation of translation, endoplasmic reticulum-associated degradation (ERAD), ubiquitination, transcription factors, protein folding, protein disulfide isomerization, heat shock proteins (HSPs), and apoptosis [27].

In 2004, Roybal et al. found that activating transcription factor 4 (ATF4), which has been identified as an important factor of ER stress, is capable of directly increasing expression of the vascular endothelial growth factor (VEGF) gene [28]. Since VEGF itself plays an important role in DR, it is possible that ER stress acts through this factor to influence the pathogenesis of DR. In fact, Ikesugi et al. demonstrated that glucose deprivation conditions induced ER stress in retinal pericytes [29], supporting the notion that ER stress participates in incidence and development of DR. Oshitari et al. also found that ER stress was involved in ocular vascular abnormalities in human DR patients [30, 31]. Further experimental investigation in animal models of diabetes and oxygen-induced retinopathy (OIR), carried out by Zhang et al., demonstrated that ER stress was activated in the affected retinas and indicated that ER Stress is a potential mediator of retinal inflammation in DR [32]. The authors also demonstrated that ER stress preconditioning could protect against retinal endothelial inflammation through activation of X-box-binding protein (XBP)1-mediated UPR and inhibition of NF-κB activation [33].

ER stress is well known to elicit induction effects on inflammatory factors, and inflammation is believed to play a critical role in DR; thus, many researchers are currently exploring the effects of ER stress on DR via the actions of

inflammatory factors [34]. Our laboratory also focuses on this mechanism of DR.

3. Identifying DR-Susceptibility and DR-Protective Genes

The bulk of research on DR hereditary factors performed to date has focused on patients with preexisting illness (i.e., diabetes), complicating the discovery of true DR-susceptibility genes. Yet, it has been clearly observed that the occurrence and severity of DR in diabetic patients varies among individuals. Some patients develop DR relatively soon after their diabetes diagnosis, while others do not develop DR for decades. In the same sense, some cases of DR are mild, while others are severe. Neither the aggressiveness nor severity of DR have been related to control of blood sugar levels or extent of loss of control, further indicating an underlying role for genetic susceptibility. Therefore, it appears that some diabetic patients have an inherent resistance to developing the DR complication or are better equipped to limit its pathogenesis.

It has been estimated that up to 20% of patients who have suffered from diabetes for 20 years remain free of the DR complication [35]. Since there are no medicines available which can effectively control DR and mitigate its progression, it can be concluded that expression of a particular gene or set of genes in these DR-resistant patients can help to protect against diabetes/glucose-related injury to retinal vessels. Even if these genes do not protect patients from DR over their entire lifetime, they may substantially delay onset or lessen severity of DR. Therefore, identification of these genes and gaining a detailed understanding of their expression patterns will likely lead to development of new therapeutic targets for genetic-based DR treatment.

On the basis upon previously published study designs for detecting genes associated with a disease state [36, 37], we chose a cohort of Type 2 diabetes patients ($n = 59$) with long-standing diagnosis (20 years or more). These patients were divided into two groups: not complicated by DR (normal) and complicated by DR. Gene expression analysis was performed by microarray (GeneChip human genome U133 plus 2.0; Affymetrix, Santa Clara, Calif, USA), and statistically significant differences in expression profiles were determined by comparative analysis among the two groups. Careful analysis of the enrolled patients' demographics led to exclusion of 22 patients from the normal group and 37 patients from PDR group. From the remaining patients, 20 were selected from each group, and venous blood samples were obtained for total RNA extraction. The RNAs of six patients from each group were preferentially selected to conduct gene chip detection. We found that diabetic patients without DR complication presented with 173 overexpressed genes ($P < 0.05$), compared to the PDR group [38]. These differential expression results were confirmed by quantitative PCR.

Thereafter, the functions of these 173 genes were analyzed, and it was found that 46 were related to protein degradation and structure modification. In addition, several factors known to be involved in the ER stress process were also found. Thus, we concluded that these genes that were suppressed in PDR patients may represent genes that provide a protective effect against DR.

4. The Role of $P58^{IPK}$ in DR

One of the differentially expressed genes in our study of non-DR diabetic patients versus PRD-afflicted diabetic patients that piqued our interest was $P58^{IPK}$. This gene encodes a 58 kDa inhibitor of the interferon-induced double-stranded RNA-activated protein kinase (also known as DNAJC3) and is a member of the Hsp40 family. First characterized for its activities as an inhibitor of the key translation-mediator eukaryotic initiation factor 2α (eIF2α) [39], $P58^{IPK}$ has since been determined to play an essential role in preventing ER stress [40, 41]. The mechanism by which $P58^{IPK}$ affects ER stress was determined to involve inhibition of PERK activation [42, 43], suggesting that $P58^{IPK}$ acts as a key mediator of cotranslocational ER protein degradation; moreover, this process is likely to contribute to ER homeostasis in stressed cells. Mutant mouse strains with $P58^{IPK}$ gene deletion displayed glucosuria, hyperglycemia, and hypoinsulinemia [44], suggesting that $P58^{IPK}$ plays important roles in maintaining normal glucose levels. $P58^{IPK}$ must enter the endoplasmic reticulum through a translocon in order to perform its protein synthesis and folding functions. Interestingly, $P58^{IPK}$ can also prevent a polypeptide chain from entering the ER through the translocon, thereby reducing protein loading of the ER and protecting cells from the stress state [45, 46]. When polypeptide chains are excluded from the ER, they are subject to degradation by the ubiquitin system. Accordingly, it has been reported that ER stress can be experimentally induced in rat pancreatic β cells by eliminating $P58^{IPK}$ as a result, the islet cells of these rats experience significant apoptosis and develop diabetes [47].

We were the first to investigate the effect of $P58^{IPK}$ on DR by performing extracorporeal experiments. Human retinal capillary endothelial cells (HRCECs) were cultured *in vitro* and transfected with a $P58^{IPK}$ overexpressing vector or $P58^{IPK}$ RNA interference (RNAi) to suppress expression. As expected, $P58^{IPK}$ expression was significantly increased in cells transfected with adeno-associated virus vector-(rAAV2-) $P58^{IPK}$ (0.63 ± 0.02), as compared to those transfected with pGIPZ-$P58^{IPK}$ RNAi (0.23 ± 0.01). $P58^{IPK}$ expression was not different between the control transfected cells (rAAV2-GFP and pGIPZ-GFP). ER stress was induced in the transfected cells by treating with tunicamycin and changes in the expression of $P58^{IPK}$ were determined, along with that of VEGF, core/emopamil-binding protein (C/EBP) homologous protein (CHOP), ATF4, and GRP78. Apoptosis levels were also determined for the $P58^{IPK}$ overexpressing cells and suppressed cells. ER stress had no effect on gene expression in cells overexpressing $P58^{IPK}$, as evidenced by no difference in expression levels of ATF-4, GRP78, CHOP, and VEGF as compared to those in unstressed control cells. However, the inhibitory effect of $P58^{IPK}$ on the expression

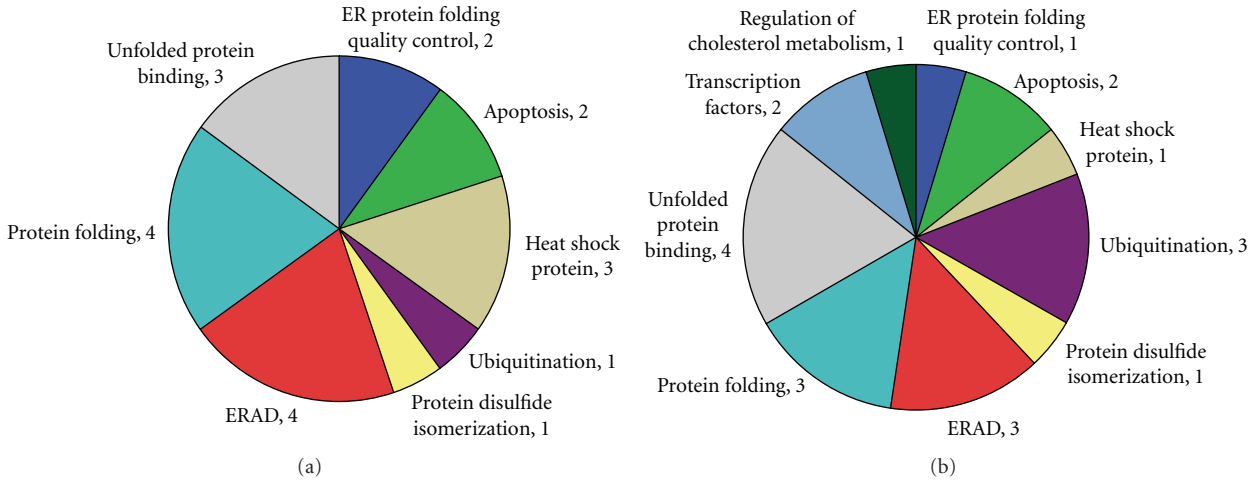


Figure 3: Associated functions of ER stress-related factors differentially expressed in diabetic retina in the first (a) and third (b) month after development of diabetes (numbers of proteins identified for each function are indicated). Expression levels were determined by quantitative real-time RNA polymerase chain reaction microarrays and compared to those of normal nondiabetic rats; differential expression was designated if a gene was detected at 2-fold lower levels.

of ER stress-related factors was suppressed in cells transfected with pGIPZ- P58^{IPK} RNAi. Apoptosis was also found to be significantly increased in cells transfected with pGIPZ-P58^{IPK} RNAi and not in those transfected with rAAV2-P58^{IPK} [48].

We also investigated the effects of P58^{IPK} overexpression on the retinas of rats with sustained high glucose. A rat diabetic model was established by intraperitoneal injection of streptozotocin. Overexpression of P58^{IPK} was achieved by intravitreal injection of purified recombinant rAAV2-P58^{IPK} or transfection into cultured rat retinal capillary endothelial cells. Retinal vascular permeability was determined by assessing the Evans Blue retinal leakage. To downregulate the P58^{IPK} level in cultured rat retinal capillary endothelial cells, pGIPZP58^{IPK} RNAi was introduced in these cells. Real-time reverse transcription- (RT-) PCR and Western blot analyses were performed to evaluate the mRNA and protein levels, respectively, of CHOP, VEGF, and tumor necrosis factor-α (TNF-α). Results showed that retinal blood vessel leakage was significantly decreased in diabetic rats overexpressing P58^{IPK}, as compared with the control diabetic rats. Both mRNA and protein levels of CHOP, TNF-α, and VEGF were remarkably reduced in the retinas of diabetic rats overexpressing P58^{IPK}. *In vitro* study further demonstrated that overexpression of P58^{IPK} led to the downregulation of CHOP, TNF-α, and VEGF gene expression under high glucose conditions, whereas RNAi suppression of P58^{IPK} enhanced the expression of CHOP, TNF-α, and VEGF [49].

Collectively, these studies indicated that P58^{IPK} functions include protecting the integrity of retinal vessels and resisting development and progression of DR. Its key role as a stabilizing endoplasmic reticulum factor led us to presume that P58^{IPK} contributes to DR by reducing incidence of ER stress through maintaining stability of the ER; these studies are underway. Nonetheless, P58^{IPK} appears to be a particularly promising genetic target to develop therapy to protect against DR.

5. Which ER Stress-Related Factors Protect against DR?

Research by our group and others are continuing to investigate the contribution of the full panel of ER stress-related factors to DR. We established a high glucose rat model in order to observe the expression changes of these ER stress-related factors. At the same time, Ikesugi and colleagues and Roybal and colleagues [28, 29] reported that in the early stages of high glucose in rats the expressions of VEGF and CHOP were upregulated, but the expressions of GRP78 and ATF4 remained stable. Again, the animal experiment results were consistent with those from the extracorporeal system [50]. Therefore, the studies to determine exactly which ER stress factors play critical roles in resisting DR and the mechanisms by which they act are of clinical interest.

It is ultimately necessary to gain a comprehensive understanding of the effects of ER stress-related factors on DR. To this end, we selected 89 factors from the entire panel of known ER stress-related factors representing each of the 11 subclasses of function in ER stress, [27] and on the basis of relevant studies of ER stress from the literature [28, 32, 51–69]. Custom-made real-time PCR chips based on the rat sequences for these 89 genes were designed (SABiosciences, Gaithersburg, Md, USA) and employed for accurate detection of temporal expression changes of these factors in the retina of high glucose rats. The results indicated that 13 genes, including the P58^{IPK} gene, were significantly down-regulated in the high glucose rat model at 1 month old. In three-month-old high glucose rats, 12 genes were downregulated (Figure 3). In addition, we found that three key signaling pathways of ER stress (PERK, IRE1 and ATF6) were not activated in the early stage of high glucose in these rats. This phenomenon is consistent with our earlier research findings [50]. According to the results of our gene chip studies, the ERAD pathway-related

factors were of particular interest. The effects of $P58^{IPK}$ and ERAD in ER stress were discussed in this paper. Firstly, both have effects on maintaining functional balance of the endoplasmic reticulum and in preventing ER stress. $P58^{IPK}$ mediates the transport of new unfolded proteins entering into the endoplasmic reticulum [45], while ERAD degrades the unfolded proteins that have accumulated in the endoplasmic reticulum. Removal (processing) of unfolded proteins from the endoplasmic reticulum is crucial to prevent ER stress, and is a more direct control mechanism than the transport modulation by $P58^{IPK}$. Therefore, we have theorized that overexpression of ERAD-related factors in the retina might be able to boost the ERAD signaling pathways to a more robust level and prevent ER stress under high glucose conditions and halt or slow down the progress of DR.

6. Perspectives for Future Studies

Much like the information gleaned from studies to identify disease-susceptibility genes, data on disease-protective genes provide the foundational knowledge by which our understanding of human health is advanced and effective healthcare strategies are developed. If the pathological mechanism of DR is the proverbial "black box", then defining the gene expression profile of DR will represent a window through which we may observe and assess the situations inside of it. Using this idea as a guide to our own studies, we have gained significant insights into which ER stress-related factors participate in the onset and pathogenic process of DR and identified a promising target for DR treatment.

However, like most disease processes, DR involves many signaling pathways and physiological factors. Cross-talk and functional interactions among these factors certainly form a complex and dynamic network structure. Each individual gene that is characterized as having a protective role against DR effectively represents a single node in that entire network. Therefore, the collective analysis of the DR gene expression profile must be continued and augmented with studies to understand the influence of clinical (comorbidities) and nonphysiologic aspects (environmental factors).

Acknowledgments

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