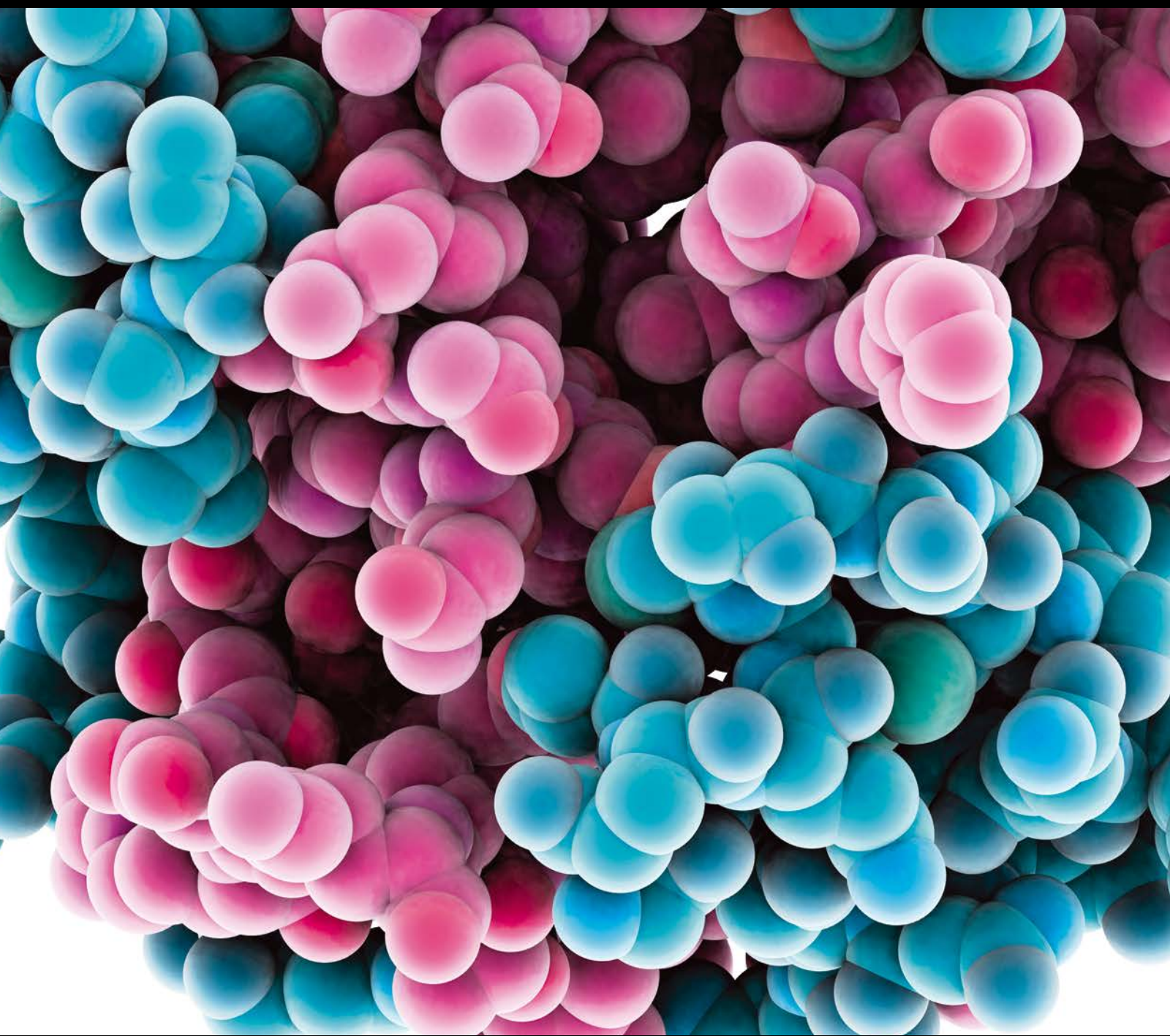
Journal of Diabetes Research

Role of Tissue and Systemic Hypoxia in Obesity and Type 2 Diabetes

Guest Editors: Lei Xi, Chin-Moi Chow, and Xingxing Kong



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Editorial

Role of Tissue and Systemic Hypoxia in Obesity and Type 2 Diabetes

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Human lifestyle in most modern and developing societies has dramatically changed over past decades. Physical inactivity along with unrestricted access to calorie dense foods has established an "obesogenic" environment and contributed to a serious epidemic of obesity and type 2 diabetes (T2D), associated with increased morbidity and mortality. In 2005 a population-based study conducted by Reichmuth et al. of University of Wisconsin with a cross-sectional and longitudinal analysis identified that among 1387 participants the odds ratio for T2D with an apnea-hypopnea index (AHI) > 15 versus an AHI < 5 was 2.30 (1.28–4.11; $p < 0.01$) after adjustment for age, sex, and body habitus [1]. Therefore it has been assumed that intermittent hypoxic periods associated with obstructive sleep apnea (OSA) may play a pathogenic role in inducing insulin resistance and T2D. At organ/tissue levels, in 2007–2009 Ye and colleagues first proposed a central role played by adipose tissue hypoxia resulting from adipocyte expansion in promoting chronic inflammation, adiponectin reduction, adipocyte dysfunction, and death in obese individuals [2, 3]. This group of researchers later identified the mediator roles played by hypoxia inducible factor 1α (HIF-1α) [4] and other hypoxia-triggered signaling mechanisms that may promote free fatty acid release and inhibit glucose uptake in adipocytes by inhibition of the insulin-signaling pathway and induction of cell death [5].

Furthermore, at the whole-body level, systemic nocturnal intermittent hypoxia was shown to be associated with increased risk of developing T2D in middle-aged men [6]. Most recently, another elegant study examined 601 participants who were originally enrolled into the Wisconsin Sleep Cohort around 18 years ago and found that hypoxia may be a stimulus for cardiac hypertrophy in individuals with OSA [7]. This study showed that the decade-long occurrence of OSA was associated independently with decreasing left ventricular systolic function and with reduced right ventricular function. Echocardiographic measures of adverse cardiac remodeling were strongly associated with OSA but were confounded by obesity. Nevertheless, it remains elusive how systemic versus local tissue hypoxia affects the key pathophysiological processes of obesity and T2D, such as metabolic imbalance, inflammation, dysglycemia, and insulin resistance. Conversely, the changes resulting from T2D may also alter the adaptation ability or resistance of an organ against tissue injuries caused by hypoxia or ischemia and in turn affect the progression and outcome of many chronic diseases. For example, the studies from our and other groups revealed that obese and/or T2D animals were refractory to ischemic postconditioning, a promising cardioprotective modality against myocardial infarction [8–10].

Under this context, our primary goal for guest-editing this special issue is to provide a platform for insightful discussions and exchanges of divergent ideas concerning systemic and local hypoxia as trigger or treatment (in some cases) of adipose tissue dysfunction and other organ injuries in obesity and T2D, to invite and showcase the cutting edge original research articles and reviews focusing on the impact

of continuous or intermittent systemic hypoxia (e.g., altitude training and OSA), as well as localized tissue hypoxia (in adipose and other types of tissues), on the pathogenesis, progression, and possible novel treatments of obesity and T2D. New revelation of brown adipose tissue activity in adult humans has stimulated vigorous investigations on how it can serve as a prevention and treatment target for obesity and insulin resistance; a particular emphasis is to analyze the role of brown adipose tissue in whole-body energy homeostasis and substrate metabolism under normal and hypoxic conditions.

Out of a dozen of submitted manuscripts in response to our call-for-papers, seven papers authored by 35 biomedical researchers or physician scientists from China, Hong Kong, Slovenia, United Kingdom, and United States have been selected through a rigorous peer-review process and finally included in this special issue. The following are some highlights of these accepted works.

Most relevant to the main theme, the review article by R. W. A. Mackenzie and P. Watt has provided insights at the molecular and whole-body levels on the mechanisms surrounding glucose disposal and insulin resistance with systemic exposure to chronic hypoxia. These authors thoughtfully discussed the complex and paradoxically opposing effects of hypoxia on the development of insulin resistance. On the one hand, hypoxia may induce insulin resistance either via the direct action on insulin receptor substrate and protein kinase B/Akt or indirectly through adipose tissue expansion and systemic inflammation. Yet hypoxia may also promote glucose transport via insulin-dependent mechanisms largely reliant on AMP-activated protein kinase (AMPK) and hypoxic exposure could improve glucose control in T2D. The authors' provocative conclusion is that hypoxia may decrease insulin signaling but may not induce whole-body insulin resistance.

Another focus of this special issue has been on adipose tissue, a biologically active organ that is vital for lipid storage, energy homeostasis, and whole-body insulin sensitivity [11]. Three articles have addressed this important topic. P. Zhou et al. explored the effects of inhibitor of DNA binding 2 gene (Id2) deficiency in the regulation of core body temperature over the circadian cycle and the impact of Id2 deficiency on genes involved in insulin signaling and adipogenesis in brown adipose tissue. Their findings lend support for Id2 as an important coordinator of energy homeostasis including insulin signaling, adipogenic programing, and thermoregulation. In addition, at the adipocyte level, J. Sun et al. demonstrated that free fatty acids activated the renin-angiotensin system in mouse 3T3-L1 adipocytes through a nuclear factor-kappa B-dependent signaling pathway, which shed light on the molecular basis for a vicious cycle between obesity and adipose tissue inflammation. Importantly, the authors identified the signaling pathways of activation of local adipose RAS in preadipocyte. They found that free fatty acid (palmitic acid, PA) could upregulate the expression of angiotensinogen and angiotensin type 1 receptor and also stimulate the secretion of angiotensin II in 3T3-L1 adipocytes. Moreover, the activation of renin-angiotensin system in 3T3-L1 adipocytes was inhibited when Toll-like receptor 4 (TLR4) signaling pathway was blocked by TLR4 inhibitor or NF-κB inhibitor, suggesting involvement of the PA/TLR4/NF-κB signaling pathway in regulating the local renin-angiotensin system in adipose tissue. More relevant to the hypoxia-related problems, H. H. Chowdhury et al. provided evidence that hypoxia alters expression of dipeptidyl peptidase 4 (DPP4) and induces developmental remodeling of human preadipocytes. The authors indicated that hypoxia strongly inhibits DPP4 protease activity and insulin-mediated increase in DPP4 suggesting that DPP4 represents an important marker for early detection of insulin resistance.

Two articles in this special issue targeted myocardial ischemia-reperfusion injury, an important pathological condition complicated by obesity and/or T2D. L. Pang et al. thoroughly analyzed the less understood prostaglandin E receptor subtype 4 signaling in the heart, which could profoundly impact ischemia-reperfusion injury and cardiac hypertrophic response in T2D. Metformin, a first-line medication for the treatment of T2D, was reported to be protective against cardiac ischemia-perfusion injury [12]. In this special issue, M. Hu et al. provided further evidence for metformin-induced cytoprotection against hypoxia-reoxygenation injury in H9C2 rat cardiomyocytes under normal and hyperglycemic conditions. The authors demonstrated that the protection may be via an intracellular signaling mechanism involving AMPK and JNK (Jun NH(2)-terminal kinase). Given this commonly prescribed medication, an understanding of its underlying mechanisms is vital both for clinicians and for patients.

Finally, a review by D. Grinnan et al. presented a framework with epidemiological, clinical, biochemical, and molecular evidence to support a role for diabetes mellitus in the pathogenesis of pulmonary arterial hypertension. The review, in this issue, is both timely and significant in trumpeting the need for future robust research to confirm an association between diabetes mellitus and pulmonary hypertension, to demonstrate diabetes as a disease modifier of pulmonary arterial hypertension, and to investigate the outcome of glycemic control on pulmonary hypertension. From an editorial perspective, this review underscores the need for future investigations in endothelial dysfunction in pulmonary arterial hypertension in patients with diabetes who have comorbid conditions of chronic obstructive pulmonary disease and OSA, since hypoxia experienced in these patient groups has an added vasoconstrictive stress on the pulmonary vasculature [13]. Notably, the interaction between intermittent hypoxia and obesity/T2D was not covered in the current issue. This topic deserves greater attention. Future studies are warranted to investigate this often controversial area, that is, a pathogenic role played by OSA in inducing insulin resistance and T2D in humans [1, 14] versus the exposure to moderate intermittent hypoxia that may serve as a therapeutic means for reducing obesity and T2D [15].

We would like to thank all contributors to this special issue for their participation. We believe that the works presented in this special issue may instigate innovative research and development of new therapeutic drugs for the future treatment of diabetes and diabetes complications. There remain many questions to be answered regarding the role of

hypoxia in the pathogenesis and treatment target of obesity and T2D.

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Review Article

The Role of Hyperglycemia and Insulin Resistance in the Development and Progression of Pulmonary Arterial Hypertension

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Pulmonary hypertension is a progressive disorder which often leads to right ventricular failure and death. While the existing classification system for pulmonary hypertension does not account for the impact of diabetes mellitus, evidence is emerging that suggests that diabetes is associated with pulmonary hypertension and that diabetes modifies the course of pulmonary hypertension. There is also growing radiographic, hemodynamic, biochemical, and pathologic data supporting an association between diabetes and pulmonary hypertension. More robust epidemiologic studies are needed to confirm an association between diabetes and pulmonary hypertension and to show that diabetes is a disease modifier in pulmonary hypertension. In addition, evaluating the effects of glucose control in animals with pulmonary hypertension and diabetes (as well as in humans) is warranted.

1. Introduction

Pulmonary hypertension (PH) refers to an abnormally elevated blood pressure in the pulmonary circulation that can lead to right ventricular (RV) failure and death [1]. Interestingly, PH has been partitioned and separated from other vascular disorders, including systemic hypertension. Therefore, while the role of diabetes mellitus in the pathogenesis of systemic microvascular and macrovascular disease has been appreciated for decades, there has been little evaluation of the potential role that diabetes could have in the pathogenesis of PH. The existing classification of PH does not account for the potential influence of diabetes or other components of the metabolic syndrome, and current treatment is focused on the use of pulmonary vasodilators. Only recently have we begun to understand that not only diabetes may predispose to PH, but also it may fundamentally alter the prognosis in those with PH. Below, we will review the current diagnosis and management of PH, the clinical evidence supporting a role of diabetes in the pathophysiology of PH, the biochemical evidence suggesting a role of local hyperglycemia and insulin resistance in the development of PH, and directions for future research.

2. Current Classification and Treatment of Pulmonary Hypertension

Since 1996, there has been a classification system developed by the World Health Organization (WHO) and comprising 5 groups [2]. This system has been subject to minor changes over time, but it has remained relatively stable (Table 1).

Group I is pulmonary arterial hypertension (PAH) and is characterized by progressive obliteration of the pulmonary arterioles due to endothelial and smooth muscle proliferation [3]. Several underlying conditions (connective tissue disease, HIV infection, congenital heart disease, portal hypertension, genetic polymorphisms, and anorexigen or amphetamine use) are known to predispose to PAH. PAH patients have a varying course and prognosis depending on how their right ventricle tolerates the increased afterload [4]. Group II is pulmonary venous hypertension (PVH) occurring when a

Table 1: Current clinical classification of pulmonary hypertension.

Group	Associated conditions
(1) Pulmonary arterial hypertension	Idiopathic, heritable, and connective tissue diseases, congenital heart diseases, drug and toxins, portal hypertension, and schistosomiasis
(1′) Diseases affecting pulmonary capillaries or pulmonary venules	Pulmonary venoocclusive disease or pulmonary capillary hemangiomatosis
(2) Pulmonary venous hypertension	Left ventricular systolic or diastolic dysfunction or left sided valvular heart disease
(3) PH due to lung disease or hypoxemia	Sleep disordered breathing, chronic altitude exposure, chronic obstructive lung disease, and interstitial lung disease
(4) Chronic thromboembolic pulmonary hypertension	
(5) PH due to multifactorial mechanisms	Sarcoidosis, hematologic disorders, chronic renal failure, and glycogen storage disease

left heart condition (systolic, diastolic, or valvular dysfunction) leads to an increase in pulmonary arterial pressure. This is the most common type of PH [5]. Over time, the pulmonary vascular bed can be chronically and permanently changed. Group III is pulmonary hypertension related to chronic hypoxia. This can be from untreated obstructive sleep apnea or from a variety of severe lung diseases (emphysema, pulmonary fibrosis, and bronchiectasis). Group IV is chronic thromboembolic pulmonary hypertension (CTEPH), a condition resulting from unresolved pulmonary thromboembolic disease. CTEPH may require medical or surgical management [6]. Group V is miscellaneous, implying that the underlying physiology and treatment is poorly understood. Group V includes those patients with PH associated with end stage renal disease, sarcoidosis, sickle cell disease, and others. Patients are classified into these "groups" to optimize the chance that pulmonary vasodilators will be effectively used. Patients with PAH are generally treated with pulmonary vasodilators, while patients with groups II or III disease are not known to respond to pulmonary vasodilators. However, there are many group II/III patients who have "pulmonary hypertension out of proportion" to their underlying condition, and it is not established how to treat these patients [1].

Pulmonary vasodilator therapy is expensive and complicated [7]. At the present, there are 13 pulmonary vasodilator therapies approved for use in the United States. However, each therapy falls into one of 3 biochemical pathways. The first approach activates cyclic GMP through the nitric oxide pathway. This group included the phosphodiesterase type 5 inhibitors and the soluble guanylate cyclase agonists. The second approach includes endothelin receptor antagonists, a group of medications that block a potent vasoconstrictor (endothelin) from activating its receptor. The third approach targets the prostacyclin pathway, as prostacyclin causes intense vasodilatation in the pulmonary vascular bed when bound to its receptor. In clinical practice, it is common for patients to be on combination therapy [1, 8], where vasodilators targeting different pathways are combined. Pulmonary vasodilator therapy, when combined with management of right ventricular failure and optimization of oxygen delivery, has resulted in improved patient outcomes. However, PH remains a deadly disease [9].

3. Clinical Impact of Hyperglycemia and Glucose Intolerance on the Development and Progression of Pulmonary Hypertension

The above framework for the classification of PH and management of PAH does not mention a potential role for hyperglycemia or glucose intolerance, as there has been insufficient evidence to link the conditions (hyperglycemia and glucose intolerance) with the disease (PH). However, there is evidence that diabetes is an independent predictor (OR 1.53, $p < 0.001$) for the development of PH, even after other components of the metabolic syndrome are controlled for. In addition, an abnormally high percentage of patients are found to have glucose intolerance at the time they are diagnosed with PAH. Four pulmonary hypertension centers have systematically assessed those patients newly diagnosed with pulmonary hypertension for diabetes mellitus [10–12]. When the experiences from these centers are combined, a total of 415 PH patients were evaluated, of whom 107 (26%) had diabetes. This correlates with data from the UK and Ireland pulmonary hypertension registry, which found that 23% of PH patients over the age of 50 had diabetes [13]. While these studies provide only a snapshot of the PH community, the incidence of diabetes in the PH population appears to be higher than the incidence of diabetes in the general population over the age of 45 (19%) [14] and suggests a connection between diabetes and PH.

In those already diagnosed with PH, diabetes appears to have a significant impact on their disease course. It has been well established that current patients with pulmonary hypertension are older (average age 53.1 in the REVEAL cohort) [15] and have more comorbidities compared with cohorts from the 1980s and 1990s (average age 36 in the NIH cohort) [16]. Not surprisingly, the older PH population is much more likely ($p < 0.001$) to have diabetes compared with the younger PH population [13]. There are now several single-center or two-center studies that have found worse survival in patients with PH and diabetes, compared with those PH patients without diabetes. One showed that, at the time a patient is diagnosed with PH, hemoglobin A1C less than 5.7 was an independent predictor of survival ($p < 0.002$)

[17]. A separate analysis found that patients with PH and diabetes had worse survival (hazard ratio 1.7, $p = 0.04$) compared with other PH patients [18]. A third study found that 10-year survival was worse in those with diabetes and PH compared with those without diabetes ($p = 0.04$) [12]. While these studies only show an association and cannot show that diabetes leads to PH and accelerates the disease, they do raise the question "why would the presence of diabetes be harmful to those with PH?" To answer this question, we will look at existing research that suggests potential involvement of the microvascular circulation of the pulmonary arterioles and the right ventricle as related to diabetes.

4. Right Ventricular Failure and Diabetes Mellitus

Many clinical studies have shown that the prognosis of patients with PH is dependent on the right ventricle's ability to tolerate the increased afterload imposed by pulmonary hypertension. In patients with PH, the RV is resultantly hypertrophied and enlarged. The hypertrophied RV is subject to ischemia, and this ischemia is associated with RV dysfunction and prognosis [19]. Ischemia may be related to increased afterload, to increased myocardial density without a compensatory increase in right ventricular angiogenesis, and to RV microvascular injury impairing oxygen delivery. In addition, it has been well documented that patients with systemic sclerosis have increased right ventricular fibrosis, and the resultant impairment in their RV function is linked to a poor prognosis. Thus, both RV ischemia and RV fibrosis impact the prognosis of patients with PH.

Several imaging studies have suggested that diabetes affects the RV. Cardiac magnetic resonance (CMR) and 3-dimensional echocardiography have emerged as tools that provide important information about the right ventricle. Imaging studies utilizing these techniques have found that right ventricular end-diastolic volume is reduced in patients with diabetes (after controlling for other potential risk factors) [20, 21] and that right ventricular stroke volume is impaired in patients with diabetes and PH (without a change in pulmonary vascular resistance) [12]. These imaging studies correlate with existing hemodynamic data. Patients with PH and diabetes (PH-DM), when compared to patients with PH alone, have repeatedly been found to have higher right atrial pressure despite having a trend toward lower pulmonary arterial pressures [10] and pulmonary vascular resistance [11]. In the AMBITION study, the PH-DM cohort has a worse 6-minute walk distance (a surrogate for RV function in patients with PAH) compared to a matched PH cohort, despite a trend toward lower pulmonary arterial pressures [22]. These results have led to acceptance that patients with PH-DM and other features of the metabolic syndrome behave differently than patients with PH alone, and recent clinical studies have amended their exclusion criteria so that these patients are excluded from participation [23]. While further research is needed, the above radiographic and hemodynamic data suggests that the right ventricle is adversely impacted by diabetes in patients with PH and that RV dysfunction leads to fundamental differences between cohorts of patients with pulmonary arterial hypertension.

The above recognition that patients with PH-DM have physiologic differences in their RV function (compared to those with PH alone) suggests that diabetes may impair the right ventricle in patients with PH-DM by predisposing to fibrosis, ischemia, or both. There are several established biochemical pathways that predispose the RV to fibrotic changes in patients with diabetes (Figure 1).

Platelet derived growth factor (PDGF) is upregulated by local hyperglycemia, in turn increasing transforming growth factor-B (TGF-B), which is profibrotic [24]. TGF-B plays a major role in the fibrosis caused by diabetes in other organs, such as the kidney and the left ventricle [25, 26]. Regional hyperglycemia also causes another profibrotic pathway, induced by endothelin-1, to be activated [27]. Increased levels of endothelin-1 have been linked to patients with PH for many years through its vasoconstrictive effect on the pulmonary arterioles, but the profibrotic role of endothelin-1 in the RV remains poorly understood. In addition, insulin resistance leads to upregulation of the diabetic marker microRNA miR-29 family, which causes the cardiac fibroblast to increase collagen production and myocardial fibrosis [28]. Therefore, it is no surprise that, when myocardial biopsies from the right ventricle of patients with diabetes have been compared with controls without diabetes, there is an increase in RV fibrosis in those with diabetes [29]. While the role of fibrosis in causing left ventricular cardiomyopathy in patients with diabetes has been well established for many years [30], the role of fibrosis in right ventricular failure of patients with PH-DM is just emerging. The above findings suggest that, as more is learned about the role of hyperglycemia and insulin resistance specific to the RV in patients with diabetes, we will find that fibrosis plays a major role in RV dysfunction and clinical outcomes.

RV ischemia is also likely to contribute to the decreased RV function in patients with PH-DM. Capillary rarefaction, defined as a decrease in the density of myocardial arterioles within the right ventricular myocardium, is established in PAH [31]. As the maladaptive RV myocardium has decreased not only capillary density to deliver oxygen to the myocardium but also myocardial hypertrophy and fibrosis, RV ischemia is a common problem in patients with PAH [19]. The symptom of exertional, substernal chest pressure in patients with PAH has also been linked to elevated troponin T levels, a marker of RV ischemia [32]. In patients with PAH who develop RV ischemia (as evidenced by elevated troponin T), survival is worse, thus highlighting the importance of ischemia in this disease [33]. While no existing studies have evaluated the role of diabetes in creating RV ischemia in patients with PH-DM, the role of diabetes in promoting ischemia in other vascular beds (including the left ventricle) [34] is well established.

5. Diabetes Mellitus and the Pulmonary Microvasculature

Pulmonary arterial hypertension is a disease of the pulmonary arterioles, where there is proliferation of endothelial

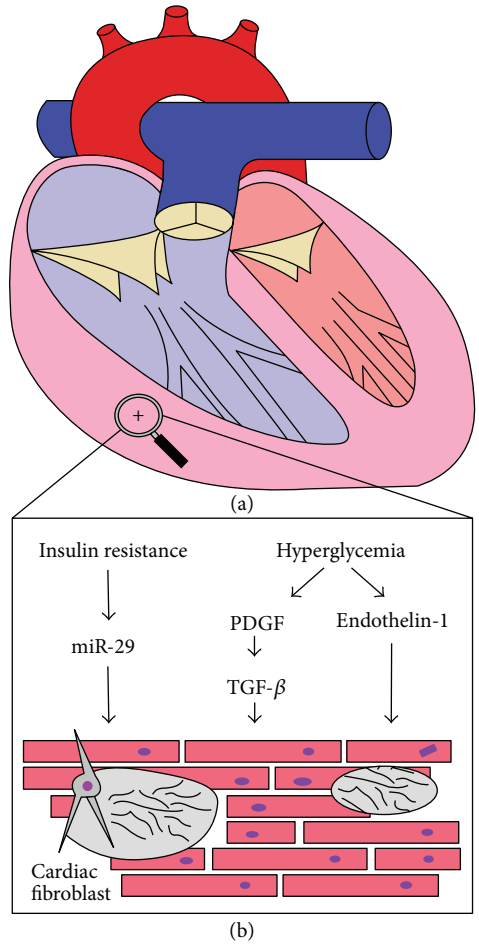

Figure 1: (a) Depiction of a heart with right ventricular hypertrophy and fibrosis. (b) Summary of the known pathways connecting diabetes to RV fibrosis and hypertrophy.

cells and smooth muscle cells, eventually leading to vascular narrowing or even obliteration. There are many established mediators that contribute to the vasoconstriction and pulmonary arteriolar proliferation that characterize PAH. As mentioned above, nitric oxide and prostacyclin are potent vasodilators of the pulmonary circulation. They also inhibit endothelial proliferation within the pulmonary arterioles. Endothelin is a potent vasoconstrictor and mitogen in the pulmonary circulation and it is overexpressed in patients with PAH [35]. In addition to these chemical mediators, mutations in the bone morphogenetic protein receptor 2 (BMPR2) gene have been associated with both familial and idiopathic PAH [36, 37]. These mutations lead to decreased BMPR2 activity within the smooth muscle cells of patients with PAH, and this in turn leads to the overexpression of transforming growth factor-B (TGF-B) and increased smooth muscle and myofibroblast proliferation. Insulin like growth factor (ILGF-1), a mitogen for pulmonary arteriolar smooth muscle proliferation, is locally upregulated in the smooth muscle cells of PAH patients [38]. Last, peroxisome proliferator-activated receptor gamma (PPARγ), a transcription factor which is antiproliferative and proapoptotic, is decreased in pulmonary hypertension and contributes to endothelial proliferation [39].

Local hyperglycemia and insulin resistance influence all of the above pathways that have been implicated in the development of PAH. Hyperglycemia inhibits endothelial nitric oxide synthase (eNOS), thereby decreasing production of nitric oxide within the endothelial cell [40]. In addition, hyperglycemia generates reactive oxygen species [41], which decrease nitric oxide bioavailability independent of eNOS regulation [42]. Hyperglycemia also activates protein kinase C (PKC) within the endothelial cells, which further decreases nitric oxide production while increasing endothelin levels, TGF-B levels, and inflammatory mediators (NADPH and NF-kB) [43]. In addition, activation of PKC inhibits the vasodilatory effect of prostacyclin (Figure 2) [44].

Insulin resistance was developed in Apo E deficient mice fed a high fat diet, and these mice developed PH that was ameliorated with rosiglitazone induced activation of PPARγ [45]. This study suggests that insulin resistance reduces PPAR-γ levels, which predisposed the animals to PH. Another intriguing study found that mice deficient in BMPR2 developed insulin resistance [46]. Moreover, if these mice

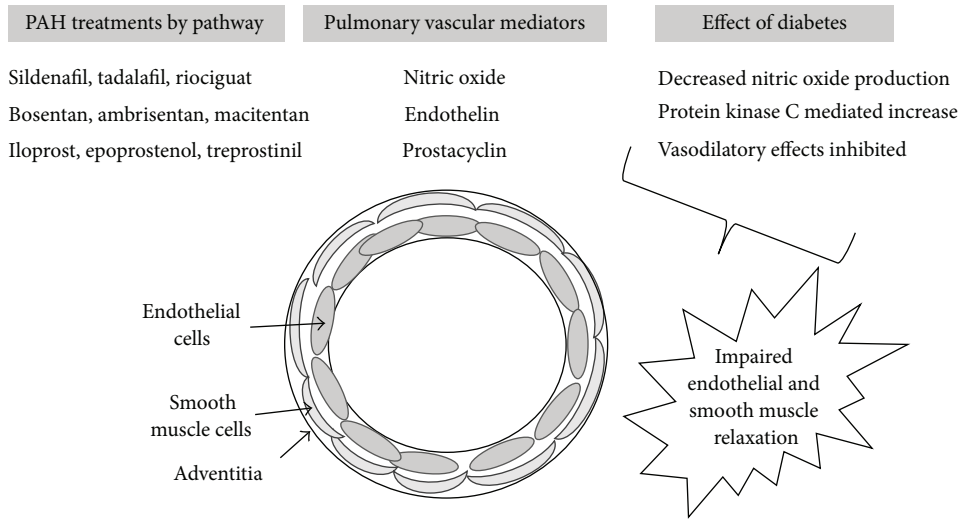

Figure 2: Role of diabetes mellitus in promoting vasoconstriction within the pulmonary arteriole and the potential impact on the efficacy of medications commonly used to treat pulmonary hypertension.

were fed a high fat diet, the penetrance of PAH was increased. All of the above data suggests that insulin resistance and/or hyperglycemia may influence the known molecular pathways involved in the development of PAH, thus providing a physiologic rationale for the increased incidence of diabetes in the PH population that was discussed earlier.

It is also important to note that diabetes has a significant effect on other vascular beds in the body at the level of the capillary bed. Similarly, there is evidence that regional hyperglycemia and resultant oxidative stress increases pulmonary capillary permeability [47]. Therefore, it is possible that damage to the pulmonary vascular system at the level of the capillary bed, in addition to the above-mentioned pulmonary arteriolar involvement, may influence how patients with PH-DM respond to targeted therapy of the pulmonary arterioles.

6. Conclusion

The pulmonary vascular bed is one of the only vascular beds in the body where the effects of diabetes have not been well studied. The current classification system (see Table 1) for pulmonary hypertension does not incorporate diabetes mellitus. However, evidence is emerging that suggests not only that diabetes is associated with PH, but that diabetes also modifies the course of PH in patients who have PH-DM. This is suggested by small epidemiologic studies, by hemodynamic studies, by imaging and pathology studies of the RV, and by a growing number of molecular and biochemical studies showing that the determinants of PAH (endothelial cell and smooth muscle cell proliferation) and RV failure (ischemia and fibrosis) are influenced by hyperglycemia and insulin resistance. The existing evidence supports the role for further research in this field. More robust epidemiologic studies are needed to confirm an association between diabetes and PH and to show that diabetes is a disease modifier in PAH. In addition, evaluating the effects of glucose control in animals with PH-DM and in humans with PH-DM is warranted. After all, why would the pulmonary vasculature be selectively spared from the effects of diabetes?

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Metformin Protects H9C2 Cardiomyocytes from High-Glucose and Hypoxia/Reoxygenation Injury via Inhibition of Reactive Oxygen Species Generation and Inflammatory Responses: Role of AMPK and JNK

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Metformin is a first-line drug for the management of type 2 diabetes. Recent studies suggested cardioprotective effects of metformin against ischemia/reperfusion injury. However, it remains elusive whether metformin provides direct protection against hypoxia/reoxygenation (H/R) injury in cardiomyocytes under normal or hyperglycemic conditions. This study in H9C2 rat cardiomyoblasts was designed to determine cell viability under H/R and high-glucose (HG, 33 mM) conditions and the effects of cotreatment with various concentrations of metformin (0, 1, 5, and 10 mM). We further elucidated molecular mechanisms underlying metformin-induced cytoprotection, especially the possible involvement of AMP-activated protein kinase (AMPK) and Jun NH(2)-terminal kinase (JNK). Results indicated that 5 mM metformin improved cell viability, mitochondrial integrity, and respiratory chain activity under HG and/or H/R ($P < 0.05$). The beneficial effects were associated with reduced levels of reactive oxygen species generation and proinflammatory cytokines (TNF-α, IL-1α, and IL-6) ($P < 0.05$). Metformin enhanced phosphorylation level of AMPK and suppressed HG + H/R induced JNK activation. Inhibitor of AMPK (compound C) or activator of JNK (anisomycin) abolished the cytoprotective effects of metformin. In conclusion, our study demonstrated for the first time that metformin possessed direct cytoprotective effects against HG and H/R injury in cardiac cells via signaling mechanisms involving activation of AMPK and concomitant inhibition of JNK.

1. Introduction

Diabetes mellitus is associated with a number of long-term complications, including nephropathy, retinopathy, stroke, and cardiovascular diseases, which lead to decreased quality of life and reduced life expectancy [1]. Patients with type 2 diabetes mellitus (T2DM) have a higher risk for coronary heart disease [2] and are more susceptible to myocardial ischemia/reperfusion (I/R) injury as compared with non-diabetic individuals [3, 4]. To date, no agent is in routine clinical use to protect the myocardium against I/R injury, although several pharmacological agents have been studied with respect to their ability to attenuate I/R injury [5]. Metformin (1,1-dimethylbiguanide), a biguanide derivate, is the most widely prescribed drug in the treatment of T2DM [6]. Clinical trials demonstrated that metformin reduced diabetes-related death and all-cause mortality [7, 8] and previous exploratory studies suggested that metformin had direct vascular beneficial effects, for example, in a murine model of myocardial I/R, but the underlying mechanisms of this beneficial effect are not completely understood [9, 10].

The pathogenesis of hypoxia/reoxygenation (H/R) injury (a major component of I/R injury) in diabetic hearts is associated with cardiomyocyte apoptosis [11] and overproduction of reactive oxygen species (ROS) [12]. It is widely accepted that metformin leads to activation of AMP-activated protein kinase (AMPK) with increased levels of phosphorylated AMPK [13, 14], which has complex properties on cardiomyocyte functionality and ROS production [12]. In this context, we hypothesized that metformin played a direct protective role against I/R injury in diabetic hearts and we

tested this hypothesis in an in vitro study using H9C2 rat cardiomyoblasts exposed to H/R injury under a simulated hyperglycemic (HG) condition with or without coincubation with various concentrations of metformin. We further investigated the potential cellular and molecular mechanisms underlying metformin-induced cytoprotection against HG and/or H/R injury, particularly those related to the AMPK and JNK related kinase signaling pathways. Cellular ROS generation and proinflammatory cytokines were also investigated.

2. Methods

2.1. Cell Culture and Treatment Protocol. H9C2 rat cardiomyoblast cell line was purchased from the American Type Culture Collection (ATCC) and cultured in mixed growth medium (Dulbecco's modified Eagle's medium (DMEM) (Hyclone)) supplemented with 10% heat-inactivated FBS (Hyclone). Cells were kept in an incubator in an atmosphere of 5% CO_2 and 95% air at 37°C and passaged at 1 : 3 ratio when they reached 80% confluence. For the H/R experimental groups, cells were firstly maintained at 37°C under hypoxic atmosphere of 95% N_2 and 5% CO_2 for 3 hours, and then cells were given fresh medium with serum and maintained in normoxic conditions (i.e., reoxygenation) for the next 3 hours. The oxygen content (~1% O_2) inside the incubator was continuously monitored to maintain a stable level of hypoxia. In the HG (33 mM)- and metformin (0, 1, 5, and 10 mM)-treated groups, the cells were pretreated with glucose for 48 hours with or without an inhibitor of AMPK (compound C (Tocris)) or an activator of JNK (anisomycin (ANISO, Sigma-Aldrich)) followed by preincubation for 30 min before addition of metformin, which was added 60 min prior to H/R until the end of the experiment protocol. JNK inhibitor (SP600125, Sigma-Aldrich) was added 30 min ahead of H/R. The cells in the control group were treated with the same procedure under normal culture conditions.

2.2. Cell Viability. After exposure to the abovementioned HG and/or H/R treatments with or without various concentrations of metformin (0, 1, 5, and 10 mM) and other kinase activators/inhibitors, cell proliferation in the H9C2 cells was assessed using Cell Counting Kit-8 (CCK-8, Sigma-Aldrich) according to the manufacturer's instruction. Briefly, cells were seeded on 96-well plates at a density of 2×10^3 per well in 100 μL of complete medium. After being treated with the corresponding drug(s) and H/R as described above, the cells were further incubated with 10 μL of the CCK-8 reagent for 0.5 to 4 hours. The absorbance at 450 nm wavelength was measured with a microplate reader (BIO-TEX ELx800).

2.3. Measurement of Mitochondrial Membrane Potential. As a mitochondria specific membrane potential-sensitive fluorescent probe, JC-1 (Keygen) accumulates in the mitochondrial matrix when mitochondrial membrane potential is high, forming J-aggregates which emit bright yellow-red fluorescence. In contrast, JC-1 presents itself as a green fluorescent monomer with low levels of aggregation when mitochondrial membrane potential is low. During this assay, cells were seeded on 6-well plates and, following the assigned drug and/or H/R treatments, the cells were loaded with JC-1 for 20 min at 37°C and then harvested to detect fluorescence with a flow cytometer (BD Biosciences).

2.4. Measurement of Activities of Mitochondrial Complex I and Complex III. Mitochondrial complex activities were measured by a commercial assay kit for mitochondrial complex activity (Beyotime Institute of Biotechnology, China) following the manufacturer's instruction. Briefly, NADH-cytochrome c reductase activity (complexes I–III) was measured at 550 nm in a reaction medium containing (mM) 100 phosphate buffer (pH 7.4), 0.2 NADH, 0.1 cytochrome c, and 0.5 KCN at 30°C. Enzyme activity was expressed in nmol cytochrome c reduced per minute per mg of protein. The activity was measured by the absorbance at 340 nm wavelength with a microplate reader (BIO-TEX ELx800) for 3 min.

2.5. Flow Cytometric Evaluation of Intracellular ROS. The ROS generation was monitored by flow cytometry using peroxide-sensitive fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA) as previously described [15]. The cells were loaded with 10 mM DCFH-DA in serum-free medium at 37°C for 30 mins, then washed twice with PBS, and then monitored with a flow cytometer (BD Biosciences) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. ROS was determined by comparing the changes in fluorescence intensity to those of the control wells. The vertical coordinate represents the amount of cells, and the horizontal ordinate represents the mean fluorescence. The more the curve shifts to the right, the stronger the mean fluorescence is.

2.6. Real-Time PCR (Polymerase Chain Reaction) and mRNA Levels of Cytokines. Total RNA from the cultured cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), followed by the synthesis of first-strand cDNA (Thermo). Real-time quantitative PCRs were performed using the SYBR-green I Core Kit (Thermo). PCR products were detected in the ABI PRISM 7700 sequence detection system (Applied Biosystems), and the results were analyzed using the $2^{-\Delta\Delta CT}$ method. The level of expression of mRNA was normalized to mRNA of GAPDH. Sequences of the primers are shown below:

TNF-α:
F5′-GGTCTGAGTACATCAACCTGGA-3′,
R5′-GGTCTGAGTACATCAACCTGGA-3′.
IL-1α:
F5′-AAGACAAGCCTGTGTTGCTGAAGG-3′,
R5′-TCCCAGAAGAAAATGAGGTCGGTC-3′.
IL-6:
F5′-TCAAGGGAAAAGAACCAGACA-3′,
R5′-TCAAGGGAAAAGAACCAGACA-3′.
GAPDH:
F5′-CTCTCTGCTCCTCCCTGTTC-3′,
R′5′-GCCAAATCCGTTCACACCG-3′.

2.7. Western Blotting. Western blotting was performed as previously described [15]. Briefly, equal amount of protein extraction was loaded into an electrophoresis apparatus and, after the completion of electrophoresis, the blot was transferred onto a PVDF membrane followed by timed incubation with the selected primary and secondary antibodies. The scanned images of Western blot bands were analyzed using the Quantity One software (Bio-Rad). The levels of protein expression were quantified by densitometry and normalized to β-actin expression. Primary antibodies including phosphorylated and total AMPK (P-AMPK/T-AMPK) and phosphorylated and total ACC (P-ACC/T-ACC) were purchased from Abcam, and phosphorylated and total JNK (P-JNK/T-JNK) antibodies were from Cell Signaling Technology.

2.8. Statistical Analyses. Each of the cellular and molecular biology assays was replicated with 3 independent experiments and the flow cytometry studies were repeated 6 times. All data were presented as mean ± standard deviation (SD). Comparisons between groups were performed by one-way ANOVA with Student-Newman-Keuls post hoc analyses. The level for significant statistical differences was set at $P < 0.05$.

3. Results

3.1. Metformin Protected against HG and H/R Induced Cardiac Cell Injury. To observe the effect of metformin on cell viability, H9C2 cells were exposed to HG and H/R treatments along with various concentrations of metformin. As evidenced by CCK-8 assay, the cell viability of HG or H/R groups was significantly lower than those of control group and the cells exposed to HG + H/R had greater loss in cell viability as compared with HG and H/R alone groups. Introducing low concentrations (1 or 5 mM) of metformin into the cell culture medium significantly increased the H9C2 cells viability while a higher concentration of metformin (10 mM) aggravated the loss in cell viability induced by HG + H/R treatment (Figure 1), indicating dose-dependence for metformin-induced protective effects, and only a moderate concentration of metformin is cytoprotective against HG + H/R injury.

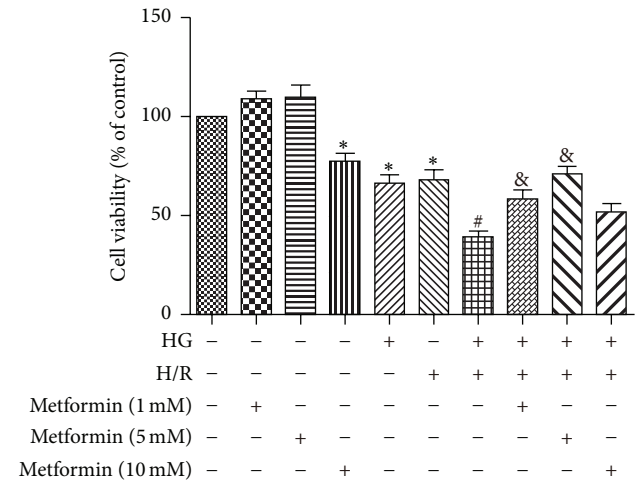


Figure 1: Dose-dependent effects of metformin on HG + H/R induced reduction in cell viability. H9C2 cells were treated with different concentrations of metformin (0, 1, 5, and 10 mM). A "+" symbol indicates presence and a "−" symbol indicates absence of the relevant treatment condition, such as HG (33 mM), H/R, and various concentrations of metformin. Cell viability of each group was estimated using the CCK-8 assay. Data are shown as means ± SD of 3 independent experiments. $^{*}P < 0.05$ versus Control; $^{\#}P < 0.05$ versus HG or H/R; $^{\&}P < 0.05$ versus metformin + HG + H/R.

3.2. Dependence of AMPK Activation in Protection of Metformin against H/R Injury. It was reported that metformin protected cardiomyocytes from injury through activation of AMPK pathway [13, 14]. To examine whether AMPK pathway inactivation was involved in HG and/or H/R induced cardiac cell injury, we tested the phosphorylation state of AMPK and its downstream target ACC. Our results showed that the levels of P-AMPK were markedly lower and its inhibitory downstream target P-ACC were higher in HG or H/R groups than in control group (Figures 2(a) and 2(b)) and the cells in HG + H/R group had the lowest level of P-AMPK (Figure 2(a)) and the resultant highest level of P-ACC (Figure 2(b)). These results suggested that AMPK pathway was inhibited during H/R injury and this effect was exacerbated under HG + H/R injury. Incubation with all three concentrations of metformin (1, 5, and 10 mM) significantly increased P-AMPK expression and the highest level of P-AMPK was found in the cytoprotective dose (5 mM) of metformin (Figure 2(c)). Furthermore, the metformin-enhanced cell viability under HG + H/R conditions was abolished by compound C, an inhibitor of AMPK (Figure 2(d)), confirming that metformin protected against H/R + HG injury by activating AMPK pathway.

3.3. Metformin Attenuates HG + H/R Induced Reduction in Mitochondrial Transmembrane Potential and Increased ROS Formation. Mitochondria act as a nexus for reperfusion injury pathways [16] and diffusion of mitochondrial transmembrane potential ($\Delta\psi m$) indicates mitochondrial dysfunction [17]. In our present study, a mitochondrial membrane potential kit was used to explore the effect of metformin on mitochondrial function in cells exposed to HG and H/R. Metformin ameliorated depolarized $\Delta\psi m$ induced by HG + H/R treatment, which was abolished by compound C evidenced from the data of JC-1 (Figure 3(a)).

I/R injury impairs the mitochondrial respiratory chain, especially complexes I and III, and produces a large amount of ROS [18]. To investigate the effect of metformin on ROS levels in the cells exposed to HG and H/R, DCF-DA assay was performed. The levels of ROS in the HG + H/R groups treated with low dose metformin (5 mM) were significantly lower than that of the HG + H/R group (Figures 3(b)-3(c)). On the other hand, metformin increased the activity of mitochondrial electron transport chain complexes I and III that were reduced by HG + H/R (Figure 3(c)). Consistent with JC-1, the ROS reduction and mitochondrial electron transport chain complex activity increase by metformin treatment were reversed by compound C (Figures 3(b)–3(d)).

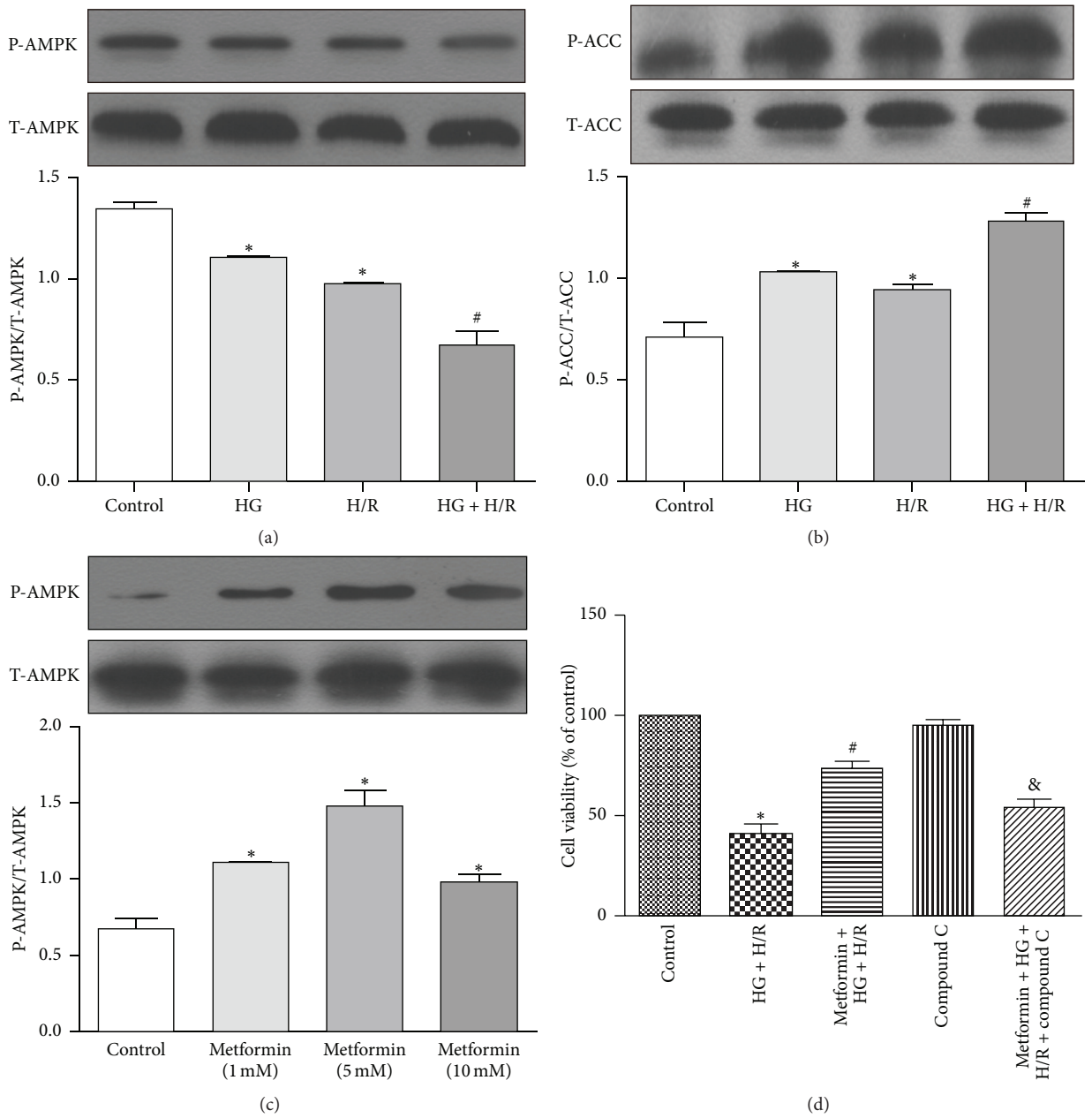


Figure 2: AMPK pathway was inhibited during H/R injury and exacerbated by HG and the cytoprotective effects of metformin were dependent on AMPK activation. H9C2 cells were treated with HG (33 mM), H/R, and HG + H/R. The expression of P-AMPK, T-AMPK (a) and P-ACC, T-ACC (b) was measured by Western blots. (c) Expression of P-AMPK and T-AMPK in the H9C2 cells treated with different concentrations of metformin (0, 1, 5, and 10 mM) was measured by Western blots. (d) Cells were treated with HG + H/R, metformin (5 mM) + HG + H/R, compound C (1 μM), or metformin + HG + H/R + compound C. Cell viability of each group was established using the CCK-8 assay. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus HG or H/R; $^{\&}P < 0.05$ versus metformin + HG + H/R. Data are shown as means ± SD of 3 independent experiments.

3.4. Metformin Inhibits Inflammatory Response to HG and H/R Injury. Exposure to HG + H/R injury induced an inflammatory response, characterized by increasing mRNA levels of IL-1α, IL-6, and TNF-α compared with that of control groups (Figure 4) and administration of metformin (5 mM) reduced these changes. In addition, inhibition of AMPK by compound C reversed this trend, implying that the protective effect of metformin against HG + H/R induced inflammation was mediated by activation of AMPK signaling pathway.

3.5. Metformin Protects against HG + H/R Injury by Inhibiting Phosphorylation of JNK, Which Is a Downstream Target of AMPK. Expression of P-JNK and T-JNK in each of the treatment conditions was measured by Western blots (Figure 5(a)). The results showed that increased protein level of P-JNK during HG + H/R treatment was also decreased by metformin (5 mM) and inhibition of AMPK by compound C reversed these changes in P-JNK expression. In addition, the protective effect of metformin was abolished by cotreatment

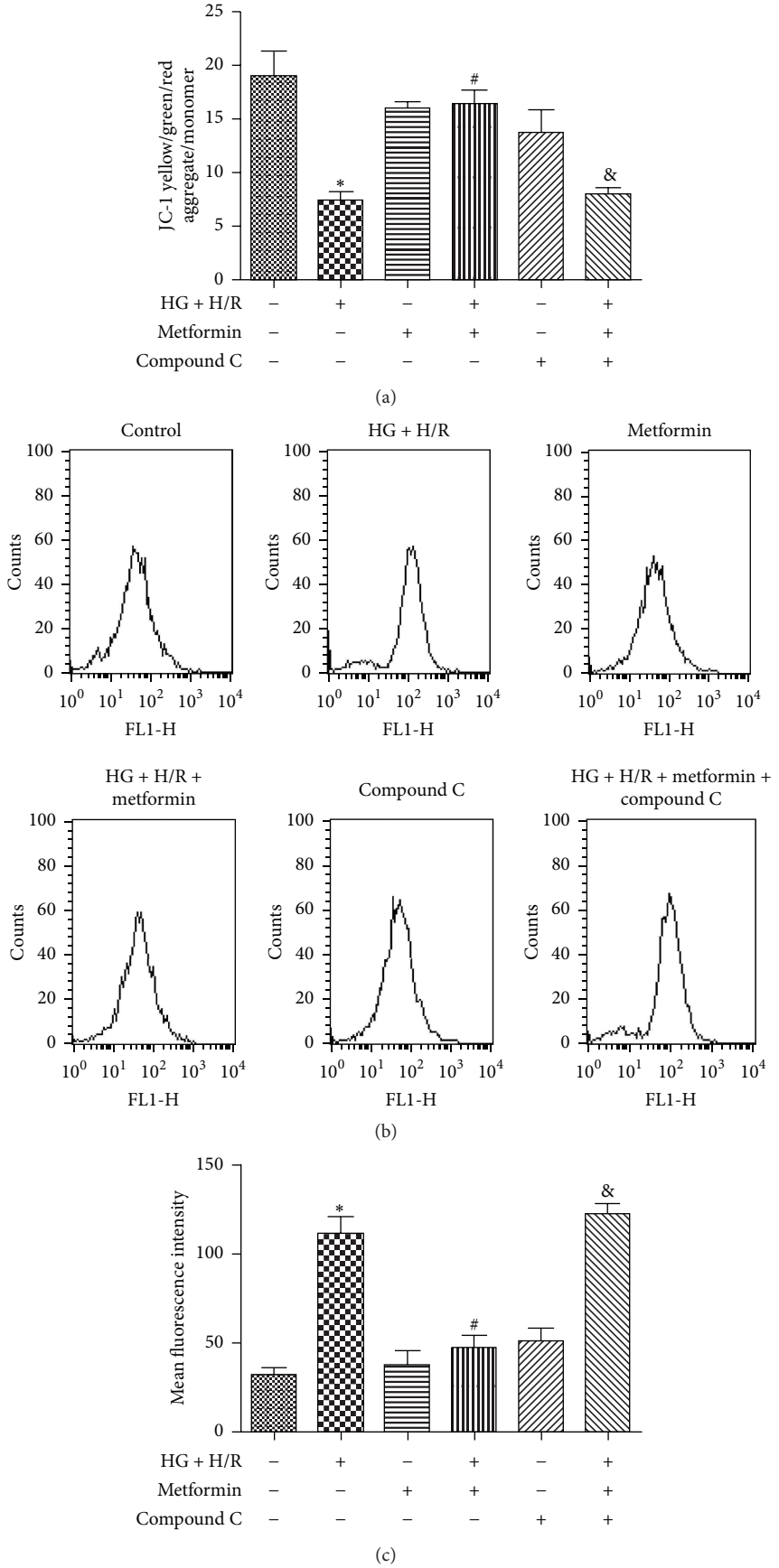
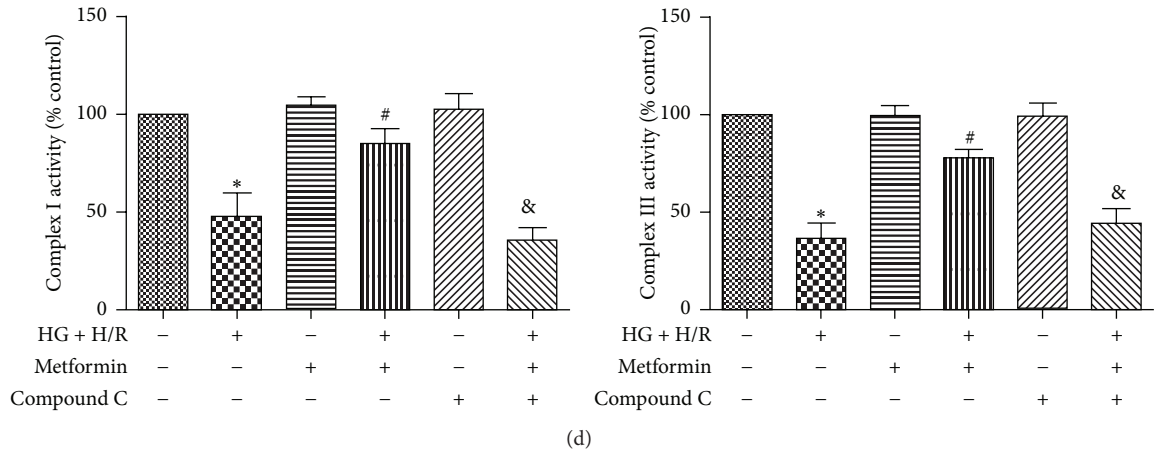
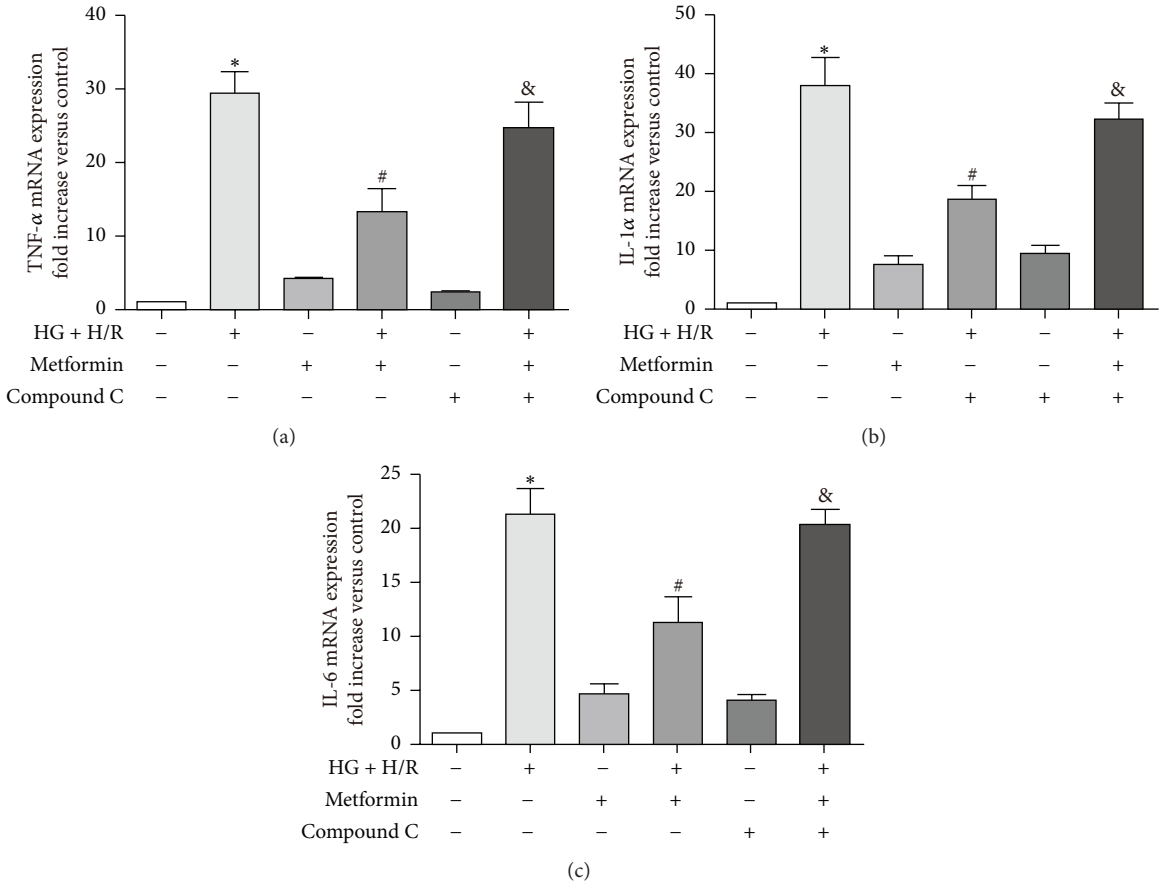


Figure 3: Continued.



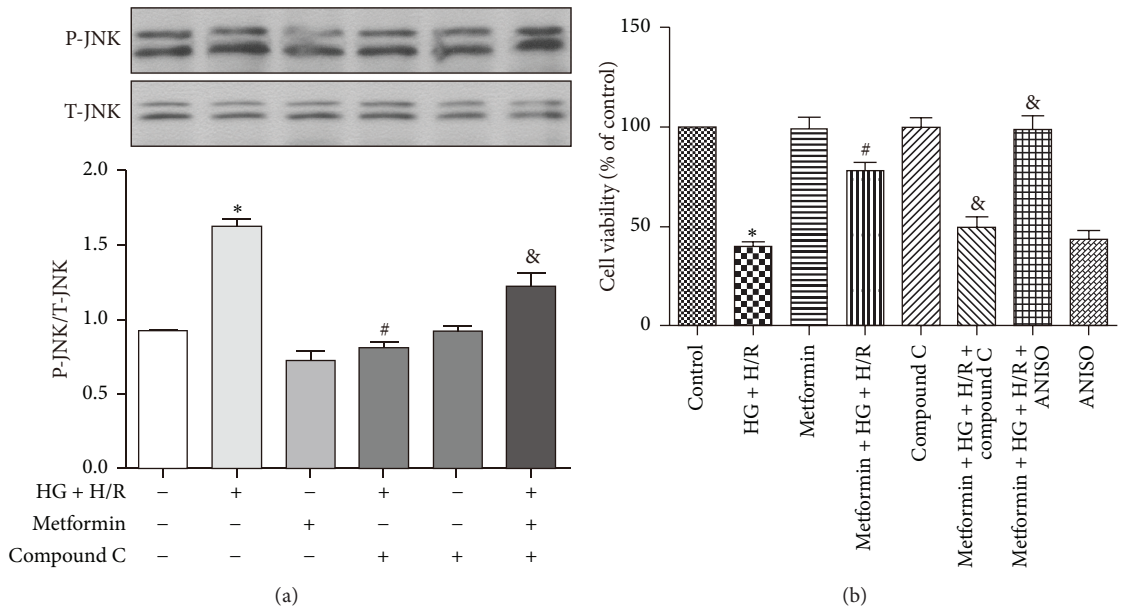
(d)

Figure 3: Metformin attenuated HG + H/R induced decrease in cell viability by preservation of mitochondrial membrane integrity and mitigating oxidative stress. H9C2 cells were treated with HG + H/R, metformin (5 mM), metformin + HG + H/R, compound C (1 μM), and metformin + HG + H/R + compound C. (a) Mitochondrial membrane potential evidenced by JC-1 staining. (b) ROS generation was measured by the DCF fluorescence intensity. (c) Bars represent quantified ROS generation. (d) Mitochondrial electron transport chain complex I and complex III activities were measured with commercial kits. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus HG + H/R; $^{\&}P < 0.05$ versus metformin + HG + H/R. Data in the bar graphs are calculated as means ± SD of 3 independent experiments.



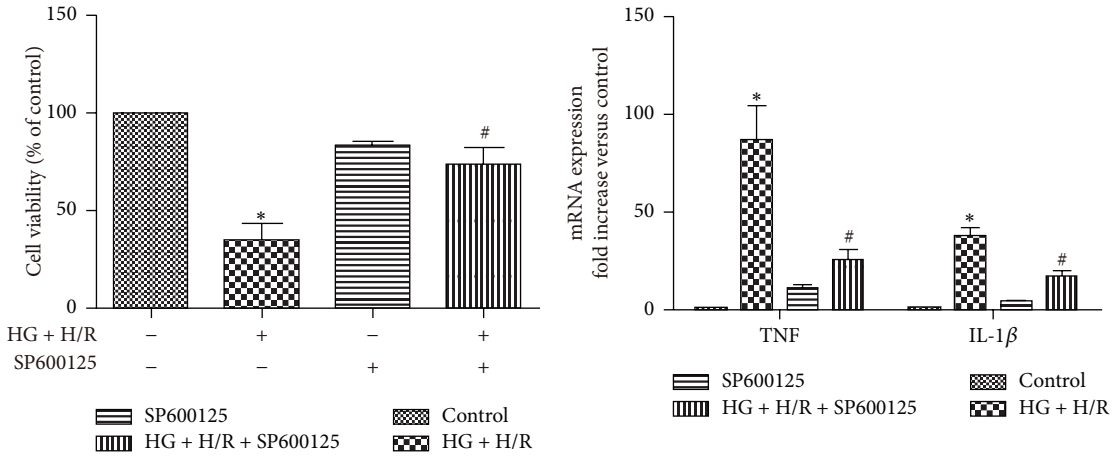
(a) (b) (c)

Figure 4: Metformin attenuated HG and H/R injury by inhibiting proinflammatory cytokine expression in cardiac cells. H9C2 cells were treated as described in Figure 3. (a)–(c) Expressions of TNF-α, IL-1α, and IL-6 mRNA level were measured by real-time PCR. GAPDH was used as the housekeeping gene. Data are shown as means ± SD of 3 independent experiments. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus HG + H/R; $^{\&}P < 0.05$ versus metformin + HG + H/R.



(a) (b)

Figure 5: Metformin protects against HG + H/R induced cardiac cell injury by inhibiting JNK signaling pathway, which acts downstream of AMPK. H9C2 cells were treated with HG + H/R, metformin (5 mM), metformin + HG + H/R, compound C (1 μM), metformin + HG + H/R + compound C, ANISO (10 μM), and metformin + HG + H/R + ANISO. (a) Expression of P-JNK and T-JNK in each of the treatment conditions was measured by Western blots. (b) Effect of a putative JNK activator ANISO on cell viability was determined using the CCK-8 kit. Data are shown as means ± SD of 3 independent experiments. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus HG + H/R; $^{\&}P < 0.05$ versus metformin + HG + H/R.



(a) (b)

Figure 6: Protection of JNK inhibitor SP600125 against HG + H/R induced loss of cell viability and proinflammatory cytokines release. (a) Protective effects of SP600125 on HG + H/R induced loss of cell viability. (b) Effect of SP600125 on HG + H/R induced proinflammatory cytokine expression. Data are shown as mean ± SD of 3 independent experiments. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus HG + H/R.

with a putative JNK activator, ANISO (Figure 5(b)), indicating that the inhibition of JNK is required for the metformin-induced cytoprotection.

3.6. JNK Inhibition Reduced HG and H/R Injury. To further demonstrate that JNK signal pathway plays an essential role in HG and H/R induced cardiac injury, we performed additional experiments and found that a JNK inhibitor (SP600125) prevented the HG + H/R induced loss of cell viability and suppressed the inflammation cytokines synthesis under HG + H/R (Figure 6).

4. Discussion

The main finding of our study was that metformin protected cardiac cells against HG + H/R injury by a mechanism involving P-AMPK, ROS, and JNK signaling pathways. Patients with diabetes mellitus are at higher risk of cardiovascular events compared with nondiabetic individuals as evidenced by numerous clinical studies [19, 20]. For instance, diabetic patients are more vulnerable to ischemic heart diseases [3, 4]. Unfortunately, many therapeutic strategies that have been shown to be effective in the protection of nondiabetic hearts

against I/R injury often lose their effectiveness in diabetic states [21, 22]. Although metformin has been reported to protect diabetic mouse heart against I/R injury [23, 24], its underlying mechanisms remain largely unknown. Our current study showed that metformin at a low concentration (5 mM) reduced the cardiac cell death caused by HG + H/R injury and metformin alone had no effect on cell viability. However, a higher concentration of metformin (10 mM) dramatically decreased cell viability. This cytotoxic effect of higher dose of metformin may result from the excessive enhancement of AMPK activity that led to suppression of platelet-derived growth factor receptor (PDGFR) as previously reported in H9C2 cells [25].

ROS plays a key role in HG + H/R induced cardiac injury and most of the ROS are generated within the impaired mitochondria. Metformin has been shown to reduce HG-induced ROS generation and oxidative stress in endothelial cells [26, 27]. Therefore, we investigated whether metformin had a suppressive effect against HG + H/R induced ROS overgeneration in cardiomyocytes. We first found that mitochondrial transmembrane potential ($\Delta\psi m$) was diffused after HG + H/R treatment indicating mitochondrial dysfunction and this effect was reversed by metformin (5 mM) coincubation (Figure 3(a)). In line with the result of mitochondrial function, metformin administration reduced the increase in ROS generation stimulated by HG + H/R (Figures 3(b)-3(c)). We subsequently examined whether the ROS was produced at the site of mitochondrion. As expected, the activity of mitochondrial electron transport chain complex I and complex III was reduced by HG + H/R injury and metformin attenuated this effect (Figure 3(d)). Overall, our data suggested that metformin protected heart cells against HG + H/R injury by inhibiting overproduction of ROS derived most likely from mitochondria.

Oxidative stress links multiple risk factors to disease and one of the main possible underlying mechanisms is that overproduction of ROS stimulates inflammatory response [28]. We found here that HG + H/R induced cardiac cell injury was associated with activation of inflammatory response as evidenced by the significant increase in mRNA of proinflammatory cytokines (TNF-α, IL-1α, and IL-6) and metformin mitigated these increases (Figures 4(a)–4(c)). Previous studies reported that exogenous ROS could stimulate JNK in H9C2 cells [29, 30]. Consistent with these previous studies, our present research also demonstrated that HG + H/R injury induced cardiac ROS overproduction was associated with an increase in P-JNK expression, which was inhibited by metformin administration (Figures 6(a)-6(b)).

Previous rat study reported that metformin activated AMPK pathway and protected against myocardial I/R injury [31]. To investigate whether metformin protected H9C2 cells against HG + H/R injury also through activation of AMPK, we used AMPK inhibitor compound C and found that the protective effect of metformin against HG + H/R injury was abolished by compound C treatment (Figure 5(b)). We further uncovered that the AMPK activation and cytoprotective effect of metformin were interconnected with inhibition on P-JNK and compound C restored the metformin-induced suppression of JNK, indicating that AMPK activation was an upstream event of JNK inhibition caused by metformin (Figures 6(a)-6(b)). Furthermore, the JNK activator anisomycin (ANISO) antagonized the inhibitory effects of metformin on HG + H/R induced JNK activation and also blocked cytoprotection of metformin (Figure 5(b)). Finally, our results provided additional evidence for a detrimental role played by JNK in HG + H/R injury, because a JNK inhibitor (SP600125) reduced the cell injury (Figure 6(a)) and proinflammatory cytokine response (Figures 6(a)-6(b)) caused by HG + H/R.

5. Conclusion

The current study has revealed for the first time that the in vitro direct treatment of metformin (5 mM) in H9C2 cardiomyoblasts attenuated HG and H/R induced cell injury, mitochondrial dysfunction, ROS overgeneration, and inflammatory response through an AMPK/JNK-dependent signaling pathway. These results from cultured cardiac cells may also implicate an important mechanism by which metformin antagonizes myocardial I/R injury in vivo. Future studies are needed to further validate the role of this signaling mechanism in mediating metformin-induced cardioprotection in various animal species including human.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

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Review Article

A Molecular and Whole Body Insight of the Mechanisms Surrounding Glucose Disposal and Insulin Resistance with Hypoxic Treatment in Skeletal Muscle

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Although the mechanisms are largely unidentified, the chronic or intermittent hypoxic patterns occurring with respiratory diseases, such as chronic pulmonary disease or obstructive sleep apnea (OSA) and obesity, are commonly associated with glucose intolerance. Indeed, hypoxia has been widely implicated in the development of insulin resistance either via the direct action on insulin receptor substrate (IRS) and protein kinase B (PKB/Akt) or indirectly through adipose tissue expansion and systemic inflammation. Yet hypoxia is also known to encourage glucose transport using insulin-dependent mechanisms, largely reliant on the metabolic master switch, 5′ AMP-activated protein kinase (AMPK). In addition, hypoxic exposure has been shown to improve glucose control in type 2 diabetics. The literature surrounding hypoxia-induced changes to glycemic control appears to be confusing and conflicting. How is it that the same stress can seemingly cause insulin resistance while increasing glucose uptake? There is little doubt that acute hypoxia increases glucose metabolism in skeletal muscle and does so using the same pathway as muscle contraction. The purpose of this review paper is to provide an insight into the mechanisms underpinning the observed effects and to open up discussions around the conflicting data surrounding hypoxia and glucose control.

1. Introduction

Type 2 diabetes is a metabolic disease categorized, primarily, by reduced insulin sensitivity, β-cell dysfunction, and elevated hepatic glucose production [1]. Insulin resistance is widely accepted as the starting point for the progression from glucose intolerance to overt type 2 diabetes. Therefore understanding the underlining mechanisms of insulin resistance pathophysiology is of great importance to the development of novel and effective treatments.

Peripheral insulin resistance represents a decrease in insulin-dependent glucose transport in insulin responsive tissues [2], which can be the product of defects at both the insulin receptor and/or postreceptor signaling [3]. Inflammation [4–7], hyperglycemia [8, 9], hyperinsulinemia [10, 11], hyperlipidaemia [12, 13], and hypoxia [14–16] have all been linked to the development of insulin resistance and type 2 diabetes. Indeed, hypoxia has been widely implicated in the development of insulin resistance either via the direct action on insulin receptor substrate (IRS) [17] and protein kinase B (PKB; also known as Akt) [18, 19] or indirectly through adipose tissue expansion [20] and systemic inflammation [21, 22]. However, we demonstrated that acute hypoxic exposure increases two-compartment models of insulin sensitivity ($S_I{}^{2*}$) in human type 2 diabetics [23] with Lecoultre et al. [24] showing that ten nights of moderate hypoxic exposure improved insulin sensitivity in obese males, as measured by the 2-step hyperinsulinemic-euglycemic clamp method. This data highlights some of the controversy over the role hypoxia plays in glucose control and metabolism. Interestingly, the same work showed that muscle expression of Akt and IRS1 was not affected by the hypoxic treatment [24].

The purpose of this review is to consider the literature while providing roles for hypoxia in causing insulin resistance and glucose intolerance. Furthermore, this review will discuss hypoxia's apparent dual ability to increase glucose transport

activity acutely in skeletal muscle, using a pathway independent of insulin, and dissect why hypoxia is also implicated in insulin resistance. Providing a greater understanding of the metabolic responses to hypoxia has genuine clinical relevance and may open up future therapeutic methods in the treatments of glucose intolerance and type 2 diabetes.

2. The Evidence Surrounding Hypoxic-Induced Insulin Resistance

The observation that insulin resistance and glucose intolerance are positively correlated with hypoxia originates from studies by Strohl et al. in 1994 [25]. These authors suggested that sleep apnea was independently associated with body mass index (BMI) and insulin dysregulation [25]. The chronic or intermittent hypoxia patterns occurring with respiratory diseases, such as chronic pulmonary disease or obstructive sleep apnea (OSA), are commonly cited as potential causes of glucose intolerance [26–28], an early indication of a disruption in normal glycemic control. The link between respiratory diseases and insulin resistance is complex. Oltmanns et al. [16] clearly showed that glucose infusion rates are reduced in response to a 30-minute period of sustained hypoxia (oxygen saturations levels ~75%). The authors attribute this to a sympathoadrenal-induced epinephrine release, resulting in increased hepatic glucose production and a reduction in glucose disposal at insulin sensitive peripheral tissue [16]. In addition, epinephrine has been shown to inhibit insulin-stimulated glucose uptake in rat skeletal muscle by reducing glucose phosphorylation [29]. The hypoxic stimulus used in Oltmanns et al. [16] work is likely to have increased hepatic glucose appearance [30] in an attempt to offset a change in peripheral tissue fuel utilization towards glucose metabolism. The consequence would therefore be a reduction in dextrose infusion rates [16] and a decrease in insulin mediated glucose uptake [29]. Indeed, epinephrine causes GLUT-4 translocation yet inhibits insulin mediated glucose disposal skeletal muscle [31]. Thus the responses described might reflect a transient change in metabolic processes and not chronic changes in insulin resistance.

Brooks et al. showed that insulin concentrations were elevated in healthy individuals upon arrival to high altitude (4,300 metres) [32], suggesting that hypoxia may cause glucose intolerance or at the very least disrupt glucose metabolism. However this is hard to conclude without access to c-peptide measures, which were not presented in this work [32]. Furthermore, the rise in blood insulin values may actually be a product of reduced insulin action rather than decreased glucose uptake. In more recent work, Louis and Punjabi [33] demonstrated a reduction in one-compartment models of insulin sensitivity (S_I) and insulin secretion in response to 5 hours of intermittent hypoxia, a treatment used to replicate OSA. In this study it was suggested that increased sympathetic nervous activity, in response to intermittent hypoxia, decreased glycogenesis, increased glycolysis, and diminished the ability of glucose to stimulate its own uptake and disposal [33, 34], which is a very confusing message. Yet if we unpick this, it may provide some clarity to the underlining metabolic response to hypoxia. We suggest that hypoxia stimulates a stress pathway for glycolysis while blocking insulin facilitated glucose uptake, providing a reason for the reduction in glycogenesis seen in the work of Louis and Punjabi [33]. However, this would not explain the decrease in the ability of glucose to stimulate its own transport, measured in Louis and Punjabi [33] work as glucose effectiveness (S_G). However, S_G is a mixed parameter that measures the ability of glucose to affect its own transport by mass action at basal insulin concentrations and is therefore dependent, to an extent, on insulin.

In other reports insulin resistance increases with hypoxia in genetically leptin deficient obese mice [15], respiratory conditions [35], and healthy humans [14, 36]. However, a closer look at the underlining data from these reports is informative and helps unpick more detail around the response of different models of hypoxia. For example, the increase in insulin resistance noted in the Polotsky et al. [15] study, which used obese, leptin deficient mice, was completely abolished by acute leptin replacement. Leptin, an adipose tissue peptide hormone, interacts with skeletal muscle [37], increasing fatty acid oxidation, and reduces intramuscular stores of triglycerides [38] while improving insulin action [39]. There is evidence to show that a decrease in secondary lipid products, ceramide, diglyceride, and long-chain fatty acyl CoA can reduce the inhibitor effect of fats on Akt mediated insulin signaling in skeletal muscle (reviewed [40]).

Using the typically regarded "gold standard" assessment of glucose tolerance (euglycemic-hyperinsulinemic clamp), Larsen et al. [41] found that insulin sensitivity decreased significantly in response to 2 days of altitude exposure (4559 m; ~12% O_2) with a reduction in glucose infusion rates to achieve euglycemia, from 9.8 (1.1) to 4.5 (0.6) $mg \cdot kg^{-1} \cdot min^{-1}$ ($P < 0.05$). The same work did, however, show improvements in insulin action with altitude acclimatisation (7-day exposure) [41] suggesting a haemostatic balance between insulin secretion, insulin action, and glucose disposal rates.

From a cellular mechanistic point of view, hypoxia seems to induce insulin resistance in insulin sensitive tissue through the suppression of total Akt during basal conditions and with IGF-1 stimulation in C2C12 skeletal muscle cells [42]. Low oxygen treatment of C2C12 cells *in vitro* inhibits the PI3-kinase/Akt pathway by reducing IGF-I receptor (IGF-IR) sensitivity to growth factors [19], suggesting that hypoxia may interfere directly with key signaling transduction pathways in skeletal muscle. In addition, the same work showed that pGSK3α^{S21}, pGSK3β^{S9}, total GSK, pAkt^{t308}, pAkt^{s473}, and total Akt were all reduced following 48 hours of differentiation in C2C12 while IRS-1 and IRS-2 were unchanged under severe hypoxic treatment (O_2 ~ 0.5%) [19]. This again suggests that hypoxia alters insulin signaling at a postreceptor-intracellular level and/or via an indirect action on IGF-1 receptor. All of this data taken together demonstrates that hypoxia, at least in *in vitro* models, has the ability to alter insulin signaling of IRS downstream. It is worth mentioning that the relevance of culture models to whole body physiological responses must be read with a degree of caution as the level of hypoxia (i.e., O_2 ~ 0.5–5%) commonly used *in vitro* work is unlikely to be seen at the tissue level of

skeletal muscle in humans subjected to whole body hypoxia (i.e., O_2 ~ 12–15%). However, phosphorylation of Akt^{s473} and GSK-3β^{s9}, obtained from vastus lateralis using standard muscle biopsy techniques, was decreased in chronic obstructive pulmonary disease (COPD) patients presenting with hypoxemia (resting arterial PO_2 = 57.0 (1.0) mmHg) [43]. This evidence suggests that chronic hypoxemia seen in disease conditions may be involved in the progression of insulin resistance. However, these findings were not supported in skeletal muscle extracted from C57BL/6J mice treated with 10% O_2 for 4 weeks [44]. Interpretations from human work are also mixed with Etheridge et al. [45] showing no change in $pAkt^{s473}$ during hypoxia while D'Hulst et al. showed that 11% inspired O_2 reduced $pAkt^{s473}$ [46]. The difference between these studies may be explained by the basal nutritional status of the subjects. Etheridge and colleagues [45] examined their subjects in a fasted state, whereas those in D'Hulst and colleagues study [46] consumed a meal 40 minutes prior to the start of the experimental trial.

Lastly, whole body hypoxic treatment seen in *in vivo* research is likely to affect a variety of tissue types, not just skeletal muscle. Obesity is characterized by adipose tissue expansion that results in pockets of localised tissue hypoxia in the most affected areas. In addition, there is evidence that localised hypoxia seen in adipose tissue may result in systemic metabolic dysfunction seen in different tissue types, further highlighting the complexity of the issue. For this reason the effects of hypoxia on fat tissue have been widely researched. In an attempt to isolate the effects of hypoxia on adipocytes, Regazzetti et al. [47] treated 3T3-L1 cells with 1% O_2 and showed that Akt, pAS160 content, and glucose transport rates were all decreased under these conditions and that this stress further inhibited insulin signaling and glucose uptake in response to insulin treatment [47]. Thus the response of whole body metabolism to hypoxia may be a systemic condition. Nevertheless, hypoxia clearly alters metabolism and affects intracellular signaling of tissues which is likely to serve the goal of reducing energy consuming processes (i.e., glycogen formation and protein synthesis) and upregulate ATP producing (i.e., glycolysis) and cell survival mechanisms.

3. Hypoxia Stimulates Glucose Uptake Independent of the Actions of Insulin

Insulin and contractile activity stimulate glucose disposal in skeletal muscle using separate, independent signaling pathways [48] with insulin mediated via Akt-AS160 and contraction via AMPK-AS160. Hypoxia also activates glucose transport using the same signaling pathway as that of contractile activity [49] (Figure 1). Indeed, glucose transport has been shown to be additive when either hypoxia or contractile activity is coupled with insulin, whereas hypoxia and contractile activity are not [49, 50]. The ability of hypoxia to stimulate glucose disposal, independently of contractile activity, has been documented in both animal [49, 51] and *in vitro* work using isolated human muscle tissue [48, 50].

In 1958 Randle and Smith published data showing that hypoxia, induced via the chemical inhibition of oxidative metabolism, resulted in a loss of cellular potassium (K^+), inhibition of active K^+ uptake, coupled with stimulation of ATP-sensitive K^+ channels, an increase in cellular ATP/AMP ratio, and ultimately an increase in extracellular K^+ levels [52, 53]. These ion changes lead to membrane depolarisation, opening of voltage-gated Ca^{2+} channels, and an increase in SR Ca^{2+} release in a manner similar to muscle contraction. Hypoxia is known to reduce oxygen availability, inhibit mitochondrial respiration [54], and increase AMP : ATP ratio [55], resulting in increased cytolytic AMP availability and greater AMP binding capacity to the γ regulatory subunit, activation of AMPK [56], and stimulation of glucose transport [49].

It is clear that hypoxia (at least during the stress) encourages glucose uptake in skeletal muscle via AMPK and Ca^{2+}-dependent mechanisms. Evidence shows that Ca^{2+} can activate glucose uptake in a calmodulin-dependent protein kinase (CaMKK)/AMPK-dependent manner [57] identical to mechanisms responsible for contraction induced glucose uptake in muscle [48, 49], although elevations in intracellular Ca^{2+} levels may also provoke AMPK-independent glucose transport as glucose transport activity is increased during subcontraction increases in muscle Ca^{2+} when stimulated with caffeine [56]. Activation of glucose uptake with hypoxia is facilitated by an increase in the activation of GLUT-1 preexisting in the cell membrane [54] while stimulating translocation of intracellular GLUT-1 and GLUT-4 to the sarcolemma [49, 54].

AMPK is essential for hypoxia-induced glucose transport [58]. Using AMPKα2 deficient rodents (Tg-KD1), Mu et al. [58] showed that glucose transport was completely blocked under hypoxia when compared to wild-type counterparts. Furthermore, the hypoxia-induced increase in membrane bound GLUT-4 content was reduced in the same Tg-KD1 mice [58]. These findings have been extended into human work, with Wadley et al. [59] showing that AMPKα2 activity and AMPKα Thr^{172} phosphorylation were significantly increased during exercise in hypoxia when compared to the same relative exercise intensity in normoxic conditions. The rate of glucose disappearance was also found to be significantly higher in the hypoxic trial, suggesting that hypoxia, when combined with exercise, has a greater effect on AMPK activity and glucose transport over exercise alone [59] and that hypoxia mediates glucose uptake via a pathway dependent (partly) on AMPK in humans.

Much of the work discussed above is in cell culture models or animal work, yet the findings from a whole body perspective seem to align well with this data. Using isotope methodology Brooks et al. [32] concluded that altitude acclimatisation (4300 m) increased glucose disappearance (R_d) and metabolic clearance rates (MCR) during both exercise and resting states when compared to sea-level values. Interestingly, insulin concentrations were unchanged from prealtitude levels [32], suggesting an increase in contraction-stimulated glucose transport or improved insulin sensitivity at altitude. In support Johnson et al. [61] demonstrated that

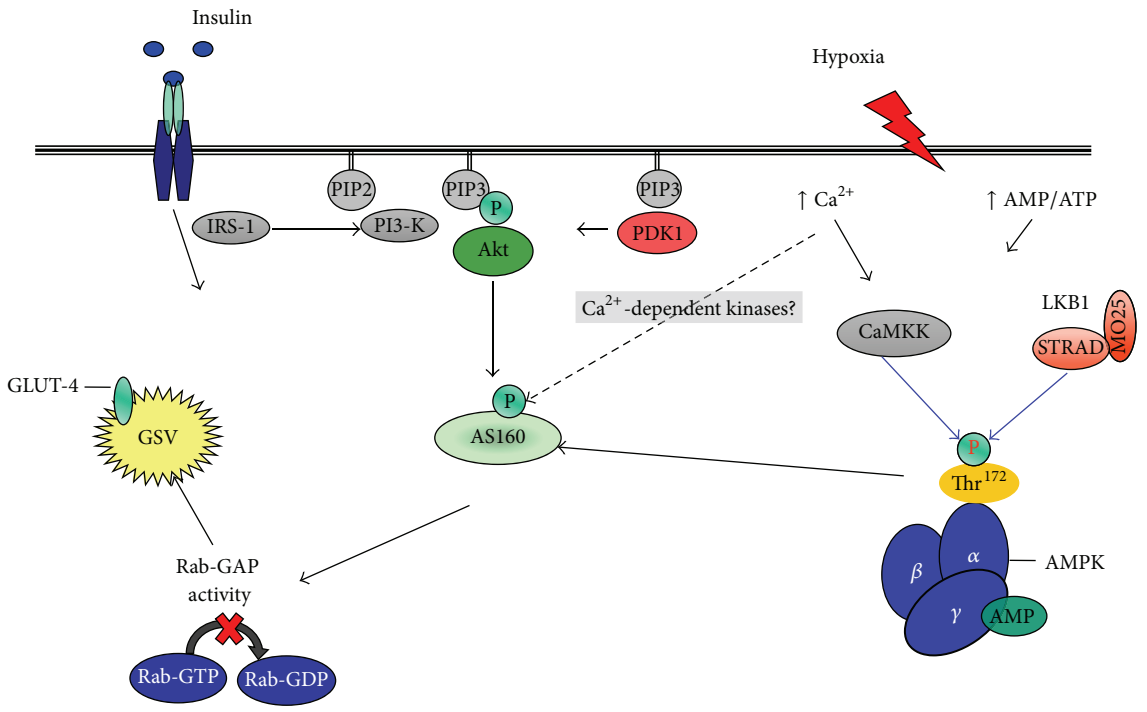

Figure 1: Insulin and contraction signaling pathways during GLUT-4 recruitment and translocation. Adapted from Mackenzie and Elliott [60]. IRS, insulin receptor substrate; PI3-K, class IA phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PDK1, phosphoinositide-dependent protein kinase-1; Akt, serine/threonine protein kinase; AS160, 160 kDa Akt substrate; GLUT-4, glucose transporter 4; GSV, GLUT-4 storage vesicle; Rab-GAP, Rab-GTPase-activating protein; Rab-GDP, guanosine-50-diphosphate-loaded Rab; Rab-GTP, guanosine-50-triphosphate-loaded Rab; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; LKB1, serine/threonine kinase 11; STRAD, putative kinase; MO25, mouse protein 25/scaffold protein; AMPK, 5′-monophosphate-activated protein kinase; Thr^{172}, phosphorylated AMPKα at threonine 172; AMP, adenosine monophosphate; ATP, adenosine triphosphate; P, phosphorylated site.

acute altitude exposure (2–40 hr) resulted in progressive hypoglycemia, which was attributed to increased glucose clearance and oxidation, which was confirmed by Cooper et al. [62]. Using an oral glucose tolerance test, Lee et al. [63] showed that high altitude exposure (3 days) significantly improved glucose tolerance in sea-level natives and in type 2 diabetics. Prior hypoxic exposure is also known to increase two-compartment models of insulin sensitivity [23] with acute intermittent hypoxia shown to improve glucose control in patients with type 2 diabetes [64].

A study by Forbes, in 1936, was one of the first to suggest that altitude could alter the manner in which glucose is handled by health sea-level residents [65]. This work showed that blood glucose clearance was increased during an OGTT administrated at high altitude. Following this work, research has not only confirmed Forbes [65] conclusions but looked to extend them by showing that long-term exposure to simulated or actual altitude results in (1) reduced fasting plasma glucose concentrations [66–69] and (2) elevated glucose clearance rates during an intravenous glucose load [66, 68].

A recent review concluded that long-term exposure to altitude results in improved glycemic control and lower prevalence of obesity and diabetes [70]. It is generally recognised that high altitude natives have a reduced prevalence of type 2 diabetes [67, 71, 72] while the same population also displays lower glucose concentrations (50.6 (3.7) mg/dL) compared to sea-level residents (73.4 (4.0) mg/dL) when monitored during ~12-hour period [73]. Despite having a high prevalence of obesity (BMI $\geq$ 30 kg/m^2) rural Aymara natives (living at altitudes 2050–4250 m) are also known to be at a reduced risk of developing type 2 diabetes [72]. These authors attributed this finding to near normal insulin values (mean (SD); 9.3 (10.2) μU/mL) and low levels of insulin resistance ($HOMA_{IR}$ 1.8 (2.4)) [72].

Studies elsewhere have shown that long-term altitude exposure is linked with low glucose and insulin concentrations [74, 75]. Ge et al. [76] concluded that Tibetan natives exhibit genetic modification (namely, *PPARA*, encoding PPARα) that increases glycolysis and decreases hepatic gluconeogenesis and free fatty acids. The authors further suggest that these adaptations may help to reduce diabetic and obesity risk [76]. Indirect evidence also shows inverse correlations between altitude natives and the risks of diabetes [77].

3.1. Insulin Resistance Seen with Hypoxia May Merely Reflect the Use of a Separate Preferential Pathway for Glucose Uptake. The literature surrounding hypoxic induced changes in glycemic control, insulin resistance, and type 2 diabetes may appear as confusing and conflicting. How is it that hypoxia can seemingly cause insulin resistance while at the

same time stimulating glucose uptake? The answer may be attributed to the duration of stress (minutes versus days), the host (i.e., altitude natives versus obese type 2 diabetics), the nutritional status of the host, the model under investigation (cell culture versus whole body), the degree of adaptation to the conditions, and the measurement methods under use (hyperinsulinemic-euglycemic clamp during hypoxia), if we can put all of these matters to one side for the time being and remember that hypoxia is stress and that, under such conditions, respiring tissue seems to switch to a stress mediated pathway acting independently, in this context, to insulin. This has been demonstrated indirectly through the suppression of insulin action [14, 16] and insulin secretion with hypoxic treatment [78], while, at the same time, encouraging glucose uptake [32, 49]. We suggest that under hypoxic condition a preferential Ca^{2+}/AMPK-dependent pathway may be upregulated to maintain ATP production (glycolysis) and reduce ATP consuming mechanisms (protein and glycogen synthesis). This is partly supported by the literature that shows that hypoxia and exercise stimulate glucose uptake via AMPK-AS160 [58, 79] while insulin acts through Akt-AS160 [80–82] mediated pathways. Comparisons with exercise are used, as this stimulus is known to activate glucose uptake using the same mechanisms associated with hypoxia.

Hypoxia has been shown, in cell lines, to impair IRS-1 [47], Akt, and PI3-kinase activity [19, 42], yet the same stress increases glucose uptake, intracellular Ca^{2+} levels, CaMKK, AMPK-AS160, and GLUT-4 muscle content. In support, whole body experimental work would suggest that hypoxia induces insulin resistance while also stimulating glucose disposal. This review will now try to explain these contrasting views while proposing a working hypothesis as to the role of hypoxia in glucose metabolism.

Firstly, the findings from whole body studies that hypoxia causes insulin resistance may be the product of increased insulin release combined with a decrease in insulin action at the site of insulin sensitive target tissue. Indeed, elevated circulating insulin concentration and perceived insulin resistance with hypoxic treatment [83] are a finding that has some support. In addition, hypoxia is known to encourage insulin synthesis and release by pancreatic β-cells [84], creating an acute or chronic physiological state resulting in blood insulin accumulation and perhaps perceived insulin resistance. This combined with decreased insulin-stimulated glucose disposal and insulin signaling is likely to result in such conclusions. Further, the introduction of exogenous insulin, which is implicit in the hyperinsulinemic-euglycemic clamp method, may further aggravate the problem. Hyperinsulinemia has been demonstrated to inhibit IRS-1/Akt activity *in vitro* [11] and cause insulin resistance *in vivo* [10, 17, 85, 86]. Thus the use of the two-step hyperinsulinemic-euglycemic clamp which delivers low insulin ($20\ mU \cdot m^{-2} \cdot min^{-1}$ for 180 min) followed by high insulin dose ($80\ mU \cdot m^{-2} \cdot min^{-1}$ for 120 min) [24] may be a better approach to assessing insulin sensitivity in insulin resistant/obese populations under additional environmental hypoxia. Importantly, Lecoultre et al. [24] work measured insulin sensitivity under normoxic conditions both before and after the ten-day treatment. Thus the clamp method here was employed following low oxygen treatment, rather than during hypoxic conditions.

Here we suggest that combining hypoxia with insulin accumulation, through its exogenous introduction and/or decreased insulin action, would indicate that the use of clamp methods is not necessarily an appropriate model for assessing insulin sensitivity under nonsteady state conditions such as exercise or hypoxia. Indeed, the introduction of exogenous insulin with the hyperinsulinemic-euglycemic clamp and a decrease in insulin signaling activity [17] due to heavier reliance on contraction-stimulated glucose uptake may merely reflect a shift towards a preferential pathway for glucose uptake resulting in plasma insulin accumulation.

There may also be some intracellular cross-talk between the two prominent regulatory pathways involved in controlling glucose uptake under hypoxic conditions which results in upregulation of AMPK-AS160 that coexists with a reduction in insulin mediated glucose transport. There is certainly some good evidence that hypoxia may cause insulin resistance; however it is proposed that this may be a product of a competition between pathways (i.e., a preferential use of the AMPK pathway under hypoxic stress) with a subsequent decrease in insulin signaling rather than insulin resistance *per se*. This is supported by the direct inhibition of insulin stimulated glucose transport with treatment of the calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) [87]. Calmodulin (CaM) is a calcium-binding protein that modifies target proteins [88] such as AMPK [89]. In addition this Ca^{2+}/calmodulin complex is considered to be involved in hypoxic induced glucose uptake [90] with AMPK activation increased with the overexpression of Ca^{2+}/calmodulin-dependent protein kinase (CaMKK) [89]. In addition, the downregulation of CaMKK using RNA interference inhibits AMPK activity [89], implicating CaMKK in AMPK regulation and glucose uptake [48, 56, 91, 92].

Hypoxia increases intracellular free Ca^{2+} [49] and subsequently CaMKK [90] with the ensuing calcium signaling directly inhibiting insulin-stimulated glucose transport. The proposed mechanisms involved in this inhibition are detailed in Figure 2(a). Youn et al. [87] showed that the effects of W-7 treatment on glucose transport were additive with hypoxia and that the same treatment reduced insulin mediated glucose transport in skeletal muscle. Furthermore, a fivefold increase in insulin concentration was required to produce a half-maximal stimulation in glucose transport [87]. All of this data combined indicates that hypoxia-induced glucose transport is not hindered by W-7 treatment and that free intracellular Ca^{2+} may directly or indirectly inhibit insulin signaling. This data suggests that the upregulation of glucose uptake by hypoxia may result in insulin accumulation systemically, by the effect of reduced processing of insulin into insulin fragments by insulin sensitive tissues. The latter point is important as this may lead to the conclusion that hypoxia causes a reduction in insulin action and results in glucose intolerance due to plasma insulin accumulation.

Further evidence of the regulatory pathways that affect glucose transport work independently of each other, yet

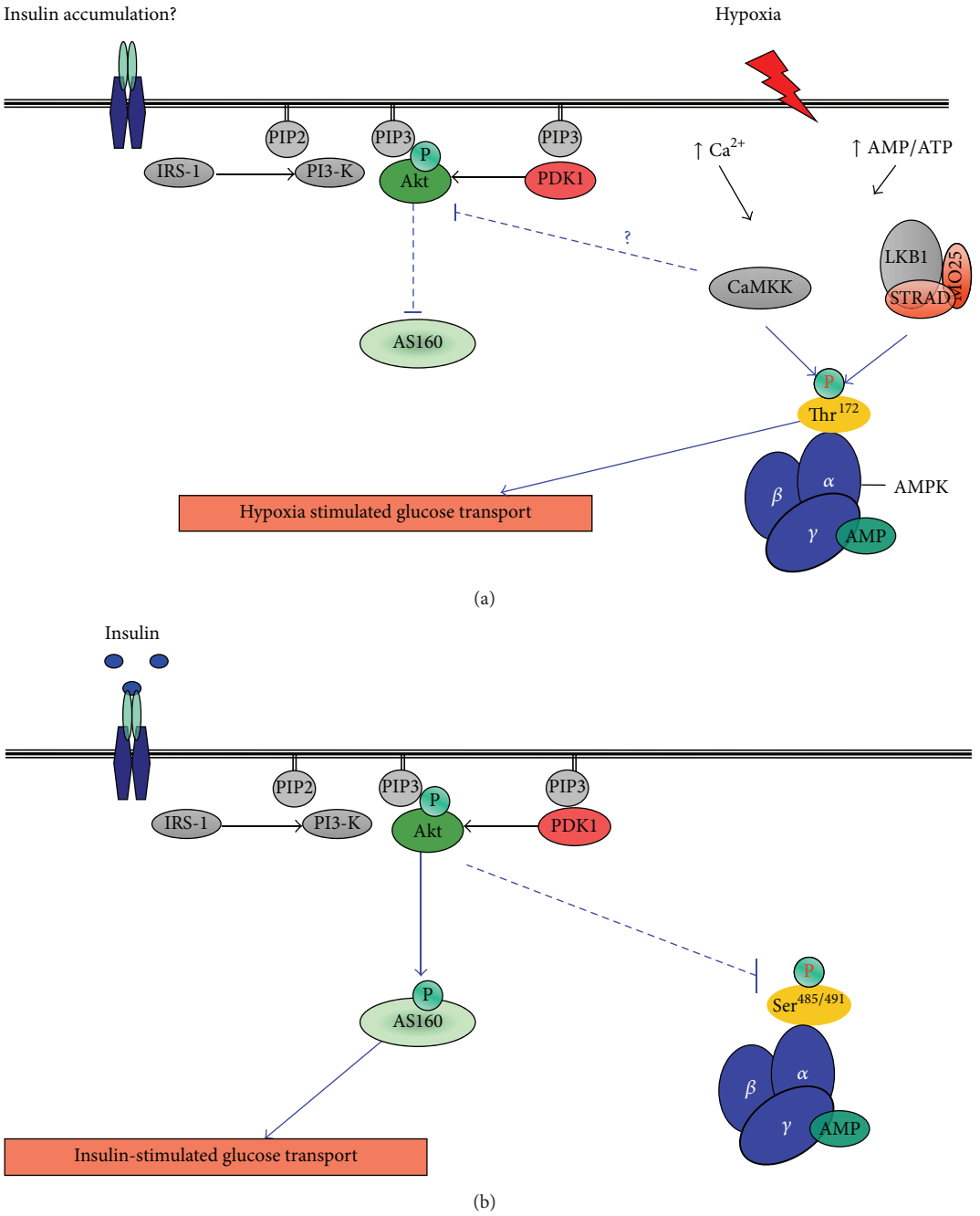

Figure 2: Proposed communication between hypoxia- and insulin-stimulated glucose transport mechanisms. (a) displays the proposed hypoxia-induced Ca^{2+}/CaMKK inhibition on insulin signaling and insulin-stimulated glucose transport. Glucose transport is facilitated via an AMPK-dependent mechanism in response to hypoxia despite decreased Akt activity. (b) Glucose transport mechanisms in response to insulin. (b) also displays Akt mediated AMPK phosphorylation at $Ser^{485/491}$ [93] resulting in AMPK inhibition.

having the ability to communicate with one another, comes from experiments with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which activates AMPK while inhibiting insulin-stimulated glucose transport in 3T3-L1 adipocytes [94]. Incubating C2C12 skeletal muscle cells or rat extensor digitorum longus (EDL) muscle with insulin increases phosphorylation of AMPK at S485/491 [95]. Phosphorylation of AMPK at S485/491 directly inhibits AMPK activity. Indeed, insulin stimulation of Akt results in $pAMPK^{S485/491}$, leading to a reduction in AMPK activity [93] (Figure 2(b)). This phosphorylation can prevent subsequent activation of $AMPK^{Th172}$ by LKB1 and that pAMPK at S485/491 by insulin reduces the interaction between AMPK and LKB1 [96] suggesting that insulin inhibition of AMPK occurs upstream and that insulin, via the activation of Akt, may directly interfere with AMPK phosphorylation and

activity [93], while presumably reducing AMPK-dependent glucose uptake. Collectively, these data again suggest that a potential cross-talk interplay between the Akt and AMPK pathways may exist and that the downregulation of insulin signaling by hypoxia reduces the Akt inhibitory effect on $pAMPK^{S485/491}$.

4. Concluding Remarks

Evidence that hypoxia leads to insulin resistance has been widely published. There seems little doubt that acute hypoxia interferes with insulin signaling/action in skeletal muscle. Yet the same stress is also partnered with increased glucose uptake in a largely insulin independent manner. *In vivo* research presents data which is conflicting, some showing improvements in insulin sensitivity while others suggesting that hypoxia induces insulin resistance. At a cellular level, there seems to be less controversy. Hypoxia increases glucose uptake and activates Ca^{2+}/AMPK mediated pathways in response to low oxygen tension in both cell culture models and *ex vivo* skeletal muscle. Yet, the same stress seems to decrease receptor and postreceptor activity of key insulin signaling intermediates. Firstly, we suggest that hypoxia does indeed downregulate insulin signaling, at least in skeletal muscle, and that a reduction in this pathway ultimately results in insulin accumulation and results in the misleading detection of insulin resistance, making the *in vivo* modelling of insulin sensitivity under hypoxic conditions difficult, particularly with the introduction of high physiological concentrations of exogenous insulin as associated with the hyperinsulinemic clamp approach. It seems clear that the two major pathways involved in glucose transport and metabolism in skeletal muscle, insulin- and contraction-dependent mechanisms, work separately and that upon the application of stress (i.e., hypoxia) cells shift towards a preferred AMPK-dependent mechanism and away from insulin. This is merely a working hypothesis but is not a new notion with Cartee et al. [49] and Azevedo et al. [50] clearly demonstrating that hypoxia activates the contraction-stimulated pathway to facilitate glucose transport and that this occurs independent of the actions of insulin.

It is important that we develop a better understanding of glucose transport mechanisms and the causes of insulin resistance as this has clear clinical applications. Furthermore, it may be that hypoxia has a part to play in the therapeutic treatment of type 2 diabetes rather than being implicated in its progression. Conclusions from *in vitro* work are important but limited in application. At a whole body level, many of the studies that demonstrate a link between insulin resistance and hypoxia have done so while measuring this parameter under low oxygen conditions, which, while being a valid approach in terms of external validity, makes the modelling of insulin sensitivity difficult. Thus developing new *in vivo* approaches to modelling insulin sensitivity, such as the two-step clamp method, may increase our understanding of the role hypoxia plays in glucose transport and glycemic control. Glucose effectiveness, as measured by the labelled intravenous glucose tolerance test (IVGTT), quantifies the ability of glucose to transport itself at basal insulin concentration. This technique also separates glucose control into measures of hepatic glucose production and disposition index and, as such, may provide a useful approach to assessing the true effects of hypoxia on insulin and hypoxic mediated glucose disposal.

The question of whether hypoxia causes insulin resistance, or not, is a complex one. At a cellular level, the evidence clearly shows that hypoxia increases glucose uptake and that this coexists with an inhibition of insulin signaling. The authors propose that under hypoxic conditions, at least acutely, glucose transport is increased using cellular pathways that operate independent of insulin thus given the impression of insulin resistance. Given the current research, the authors conclude that hypoxia may decrease insulin signaling but may not induce whole body insulin resistance.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Prostaglandin E Receptor Subtype 4 Signaling in the Heart: Role in Ischemia/Reperfusion Injury and Cardiac Hypertrophy

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Prostaglandin E_2 (PGE_2) is an endogenous lipid mediator, produced from the metabolism of arachidonic acids, upon the sequential actions of phospholipase A2, cyclooxygenases, and prostaglandin E synthases. The various biological functions governed by PGE_2 are mediated through its four distinct prostaglandin E receptors (EPs), designated as EP1, EP2, EP3, and EP4, among which the EP4 receptor is the one most widely distributed in the heart. The availability of global or cardiac-specific EP4 knockout mice and the development of selective EP4 agonists/antagonists have provided substantial evidence to support the role of EP4 receptor in the heart. However, like any good drama, activation of PGE_2-EP4 signaling exerts both protective and detrimental effects in the ischemic heart disease. Thus, the primary object of this review is to provide a comprehensive overview of the current progress of the PGE_2-EP4 signaling in ischemic heart diseases, including cardiac hypertrophy and myocardial ischemia/reperfusion injury. A better understanding of PGE_2-EP4 signaling should promote the development of more effective therapeutic approaches to treat the ischemic heart diseases without triggering unwanted side effects.

1. Introduction

Ischemia of the heart resulting from the shortage of oxygen supply can lead to the occurrence of myocardial ischemia/reperfusion injury (MI/R), presenting as a leading cause of mortality and morbidity especially in those with preexisting myocardial diseases such as cardiac hypertrophy both in developed and in developing countries [1]. In the general population, the incidence of ischemic heart diseases increases when a person becomes overweight or obese [2, 3], which could be attributed to the alterations in the control of coronary blood flow [4] or the dysregulation of adipocyte-derived hormones (such as adiponectin, leptin) in the obesity [5, 6]. The population of obesity has skyrocketed worldwide over the last three decades [7]. In addition, overweight and obesity were estimated to have caused 3.4 million deaths in 2010, most of which were from cardiovascular causes [8]. Therefore, examining ways to protect the ischemic heart and its associated diseases (like obesity, metabolic syndrome) will be of great clinical value in this industrialized world.

Prostaglandin E_2 (PGE_2) is an endogenous lipid mediator, which belongs to the family of eicosanoids [9]. Upon the action of phospholipase A2, arachidonic acid, the precursor of prostaglandins, is generated from phospholipids in the cell membrane [10]. Arachidonic acid is then metabolized into prostaglandin H2 by cyclooxygenase (COX) enzymes. Prostaglandin H2 is the first intermediate in the biosynthesis of prostaglandins, which requires the action of specific prostaglandin synthases. The specific synthases involved in the formation of PGE_2 are microsomal prostaglandin E synthases-(mPGES-) 1 and mPGES-2 and cytosolic PGE_2 synthases (cPGES) [9, 11]. PGE_2 exerts its diverse effects by activating four subtypes of prostaglandin E receptors (EPs), designated as EP1, EP2, EP3, and EP4 [12]. Of those, the EP4 receptor is

the most widely distributed subtype which exists in almost all tissues, such as the heart, adipose tissue, skeletal muscle, and lung [13–15], and is involved in various pathophysiological processes [16–18]. In particular, mice lacking EP4 exhibited slower weight gain and reduced adiposity upon high fat diet challenge when compared with wild type mice [16]. However, the lean phenotype of EP4 knockout mice is not a beneficial factor. In fact, EP4 knockout mice had a shorter life span than did the wild type mice [16]. In addition, deficiency of EP4 in mice manifests disrupted lipid metabolism due to impaired triglyceride clearance, suggesting a new dimension role of EP4 signaling in controlling lipid homeostasis [16]. Activation of PGE_2-EP4 signaling also can exert multiple biochemical effects on the heart, suggesting the potential wide-ranging use of EP4 in both cardiovascular and metabolic disorders. However, due to the limited reports of EP4 in ischemic heart under complicated disease states, in this review, we thus summarize the current progress regarding the role of the PGE_2-EP4 signaling in ischemic heart diseases, including cardiac hypertrophy and MI/R, which has been obtained from studies using genetic knockout mouse and pharmacological interventions.

2. Prostaglandin E Receptor Subtype 4: Structure and Signaling

As one of the seven-transmembrane G-protein-coupled receptors, EP4 (originally misidentified as EP2 subtype [19]) shares the structure properties of G-protein-coupled receptors. It has an extracellular N-terminus, a seven-transmembrane domain connected by three extracellular loops and three intracellular loops, and an intracellular C-terminus [20]. The N-glycosylation sites in the second extracellular loop of EP4 are important for the ligand binding [21]. EP4 has the longest intracellular C-terminus and third intracellular loop out of the four EP receptors. The human EP4 receptor is comprised of 488 amino acid residues, while the murine EP4 receptor has two isoform variants that consist of 488 and 513 amino acid residues, respectively [22, 23]. The sequence homology of EP4 between these two species reaches up to 88% [21].

The downstream effectors of G-protein-coupled receptors are G-proteins, which consist of α, β, and γ subunits. Upon ligand binding, the conformational change in G-protein-coupled receptors triggers the dissociation of Gα from the G$\beta\gamma$ subunits [22]. EP4 is coupled to stimulated Gα (Gsα), which leads to the production of cyclic adenosine monophosphate (cAMP) in response to PGE_2 [23]. The increased intracellular cAMP level subsequently activates its major target protein kinase A (PKA). PKA then phosphorylates downstream protein, cAMP response element binding protein (CREBP), which is a nuclear transcriptional factor [24]. The activated CREBP then binds to specific sites and regulates the expression of certain genes, such as B-cell lymphoma 2 and tumor necrosis factor α (TNFα), which are involved in development of ischemic heart disease [25]. In addition to PKA, another downstream molecule of Gsα/cAMP is exchange protein directly activated by cAMP (Epac). Epac consists of Epac1 and Epac2. Both Epac isoforms can convert their downstream protein Ras-related protein 1 from inactivated guanosine diphosphate form to activated guanosine triphosphate form, which leads to the initiation of downstream signaling cascades [26]. EP4 is also coupled to the inhibitory Gα (Giα) [27]. In response to PGE_2, Giα inhibits adenylyl cyclase activity leading to the reduced production of cAMP [28]. Furthermore, through activation of Giα, EP4 mediates phosphatidylinositol 3-kinase- (PI3K-) dependent pathway [29]. Activation of PI3K inhibits PKA activity but activates protein kinase B, which also can phosphorylate CREBP [30]. On the other hand, EP4 induces the expression of early growth response factor 1 through the PI3K/extracellular signal-regulated kinase (ERK) signaling pathway, which leads to the expression of PGE_2synthase, suggesting a positive feedback loop between EP4 and PGE_2production [23, 31]. Furthermore, yeast two-hybrid screening of human bone marrow complementary DNA with EP4 protein reveals that a protein named prostaglandin E receptor 4-associated protein (EPRAP) binds to the intracellular C-terminus of EP4 [32]. The interaction of EPRAP and EP4 inhibits stimulus-induced NFκB p105 phosphorylation and thus suppresses activation of NFκB [33] (Figure 1).

Besides EP4 receptor, there are another three GPCRs, EP1, EP2, and EP3, which depend on G-protein to transduce downstream signals and mediate PGE_2 actions [12]. EP1 receptor couples to Gqα protein and thus induces phospholipase C/inositol-1,4,5-trisphosphate signaling and leads to intracellular calcium mobilization [34]. The same as EP4 receptor, EP2 couples to Gsα protein and activates adenylate cyclase to produce cAMP, while EP3 is associated with Giα and inhibits cAMP production [35]. These EP subtypes have their unique expression patterns and associate to distinct downstream G-proteins and thereby lead to PGE_2 being the most versatile prostanoid.

3. Myocardial Ischemia/Reperfusion Injury

Myocardial ischemia caused by partial or complete occlusion of coronary arteries and the subsequent recovery of blood flow induced additional cardiac damage (ischemia/reperfusion injury) are leading causes for death around the world [36]. The underlying pathophysiology of myocardial I/R injury likely involves many factors, such as reactive oxygen species formation [37], altered cardiac energy metabolism [38], activation of cell apoptosis [39], and inflammatory responses [40].

During cardiac ischemia, the PGE_2 level is significantly increased [41], and this increase may be a consequence of hypoxia inducible factor- (HIF-) 1α/COX-2/PGE_2 pathway activation. Under ischemia conditions, the HIF-1α level starts to accumulate in the nucleus, be heterodimerized with β subunit, and initiate the transcription of genes which are involved in cell survival, angiogenesis, apoptosis, vascular remodeling, and glucose metabolism [42]. COX-2 has been proved to be a downstream protein of HIF-1α in various cell types, such as carcinoma cell lines HT29 [43] and human bronchial epithelial BEAS-2B cells [44]. COX-2 has been reported to be induced in the heart during I/R [45] and its expression was positively associated with the expression of HIF-1α at the site of recent acute myocardial infarction

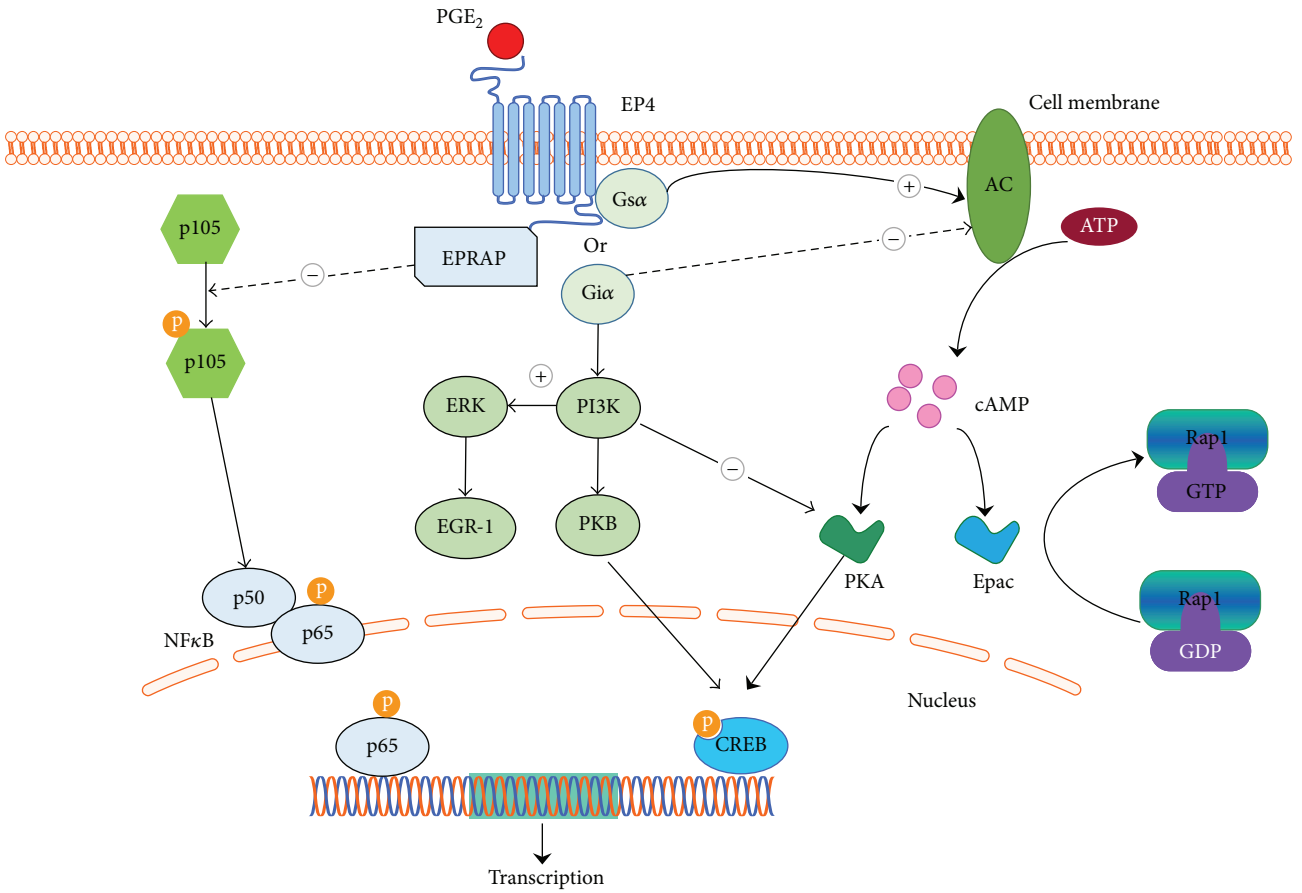

Figure 1: Differential signaling pathway of EP4. In response to PGE_2, activation of EP4 stimulates stimulatory Gα protein (Gsα)/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)/cAMP response element binding protein (CREB) pathway or Gsα/cAMP/exchange protein directly activated by cAMP (Epac) pathway. EP4 is also coupled to inhibitory Gα protein (Giα), which inhibits the cAMP/PKA/CREB pathway. Furthermore, EP4 activates phosphatidylinositol 3-kinase (PI3K) through activation of Giα. Activation of PI3K not only stimulates the protein kinase B (PKB)/CREB pathway but also induces the expression of early growth response factor 1 (EGR-1) through extracellular signal-regulated kinase signaling. Prostaglandin E receptor 4-associated protein (EPRAP) can inhibit phosphorylation of p105 and further suppress the activation of nuclear factor kappa B (NFκB). AC = adenylyl cyclase; ATP = adenosine triphosphate; GDP = guanosine diphosphate; GTP = guanosine triphosphate.

[46], despite the fact that direct evidence regarding whether or not COX-2 was transcriptionally controlled by HIF-1α in cardiomyocytes is lacking. Thus, as the major metabolite of COX-2, cardiac PGE_2 may be produced through HIF-1α/COX-2 axis during cardiac ischemia.

The increased PGE_2 level may play a beneficial role during cardiac I/R through EP4 receptor [15, 47]. Indeed, endogenous PGE_2 protects the heart from I/R injury *in vitro* and *in vivo* [15]. In isolated perfused working hearts, there was a greater degree of functional damage to the myocardium (e.g., decreased developed tension, increased diastolic tension and creatine kinase (marker of myocardial injury) release) in EP4 knockout hearts after global ischemia when compared with wild type hearts [15]. In accordance with this result, mice lacking EP4 developed a greater degree of myocardial infarction size following I/R injury when compared with wild type mice *in vivo* [15]. Likewise, pharmacological intervention with an EP4 agonist significantly reduced infarct size and improved cardiac function, including left ventricular contraction and dilatation when compared with vehicle treated animals [47]. In line with this, the ischemic preconditioning-induced cardioprotection is completely lost in HIF-$1\alpha^{+/-}$ mice [48]. Deficiency of hypoxia inducible transcription factor-prolyl hydroxylase domain-1 (PHD-1) in the mice significantly attenuated MI/R injury through reduced apoptosis by induction of HIF-1α [49], and COX-2 serves a protective role against myocardial I/R injury [50, 51]. These data provide additional evidence that PGE_2-EP4 signaling sourced from HIF-1α/COX-2 axis is cardioprotective in the ischemic heart.

The subsequent downstream signaling of EP4 during the myocardial I/R injury has not been well documented yet. However, there are multiple potential downstream pathways. (1) cAMP-PKA pathway: Through the activation of EP4 receptor on the cell membrane, adenylyl cyclase catalyzes the conversion of adenosine triphosphate to cAMP. There are at least 9 isoforms of adenylyl cyclase; among them, adenylyl

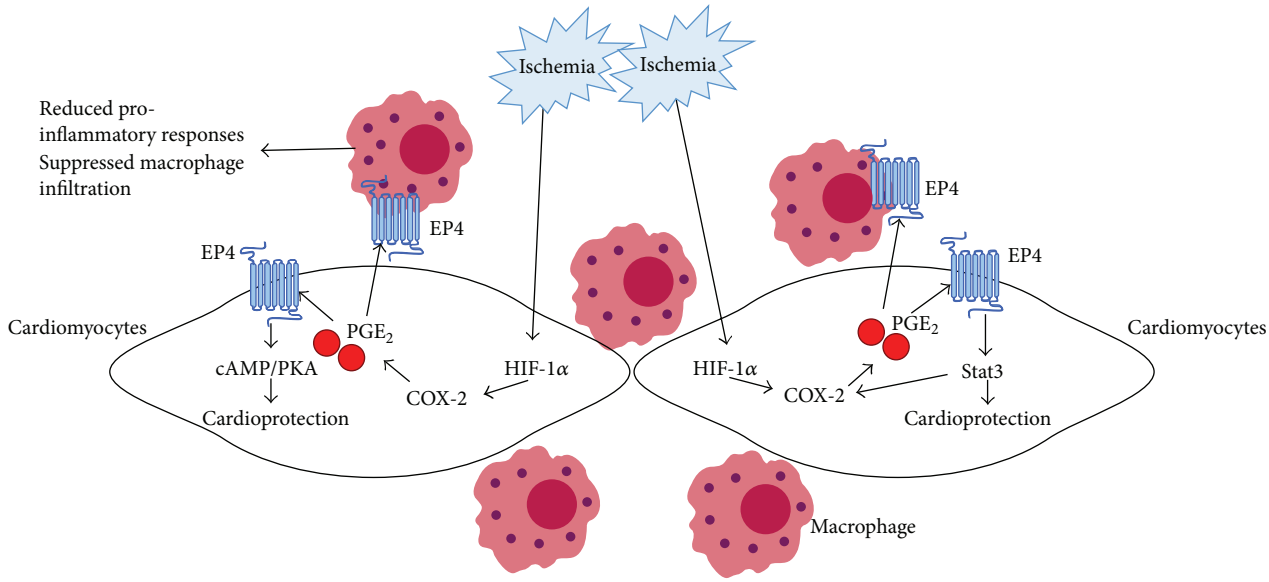

Figure 2: Schematic representation of the proposed role of EP4-mediated signaling under myocardial ischemia. During cardiac ischemia, increased PGE_2 level may be a consequence of increased hypoxia inducible factor- (HIF-) 1α/cyclooxygenase-2 (COX-2) activation. PGE_2 may play a beneficial role in cardiac ischemia/reperfusion injury through EP4/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) or EP4/signal transducer and activator of transcription 3 (Stat3) signaling pathway. In addition, synthesized PGE_2 in cardiomyocytes also diffuses into adjacent infiltrated macrophages or other inflammatory cells, protects the heart from ischemia/reperfusion injury through binding with EP4 receptor, and exerts anti-inflammatory effects.

cyclase V and adenylyl cyclase VI are mainly expressed in the mammalian myocardium [52]. In the mice with cardiac adenylyl cyclase VI overexpression, adverse left ventricular remodeling was attenuated with preserved left ventricular contractile function and reduced mortality after myocardial ischemia [53]. In addition, through activating the downstream protein cAMP, PKA presents a cardioprotective role after ischemia possibly through its negative-inotropic effect during sustained ischemia [54]. In this study, the negative-inotropic effects of postischemic effluent can be significantly suppressed by preincubation with EP2 antagonist (AH6809) or EP4 antagonist (AH23848), indicating a protective role of both EP2 and EP4 receptor during I/R injury. However, unlike EP4 receptor, there is still no direct evidence showing that EP2 receptor can mediate the cardioprotective role of PGE_2 in the postischemic heart during reperfusion *in vivo* and this warrants further investigation. These data suggested that cAMP-PKA may be responsible for the PGE_2-EP4-mediated cardioprotection during myocardial ischemia. (2) Stat3 signaling: In addition to its role in the cardiac hypertrophy, Stat3 signaling activation also plays a causal role in ischemic postconditioning mediated cardioprotection [5]. Multiple lines of evidence suggested that activation of Stat3 signaling exerts cardioprotective effects during I/R injury through scavenging of reactive oxygen species [55] or improving mitochondrial function [56]. In cardiomyocytes, PGE_2 activated Stat3 signaling through EP4 receptor in a concentration- and time-dependent manner [57]. Moreover, MI/R-induced tissue injury involves activation of cell apoptosis [39]. PGE_2 is reported to prevent myocardial apoptosis through activation of Stat3 and ERK1/2 in doxorubicin-induced apoptosis model in neonatal rat ventricular cardiomyocytes [58], suggesting that activation of Stat3 signaling may contribute to PGE_2-EP4-mediated cardioprotective role during I/R injury. In addition, despite the fact that there is no direct evidence showing that activation of EP4 receptor exerts antiapoptotic role in cardiomyocytes during I/R injury, it has been proved that PGE_2 protects normal and transformed intestinal epithelial cells from diverse stimuli-induced apoptosis *via* EP4 receptor [59]. Consistently, another study showed that EP4 agonist (L-902688) significantly inhibits the cell death during *in vivo* focal cerebral ischemia [60]. Taken together, these data suggested that PGE_2-EP4 signaling may mediate cardioprotection through attenuating cell apoptosis during myocardial ischemia.

During ischemia, inflammatory cells like macrophages may migrate into the ischemic myocardium and produce proinflammatory cytokines and chemokines, including TNFα, interleukin 6 (IL6), IL-1β, and monocyte chemotactic protein 1 (MCP-1), which further exacerbate myocardial I/R injury [40]. Thus, it is possible that the anti-inflammatory effect of EP4 on macrophages may contribute to the beneficial role of EP4 in cardiac ischemia. Indeed, EP4 agonist significantly attenuated the production of TNFα, IL-6, IL-1β, and MCP-1 as well as macrophage infiltration in the heart after ischemia [47]. Of note, the EP4-Stat3 signaling may establish a positive feedback loop through controlling the expression of COX-2 in cardiomyocytes, while the newly secreted PGE_2 may not only trigger greater Stat3 activation but also exert anti-inflammatory effect on infiltrated inflammatory cells in a paracrine way (Figure 2). Thus, EP4 receptor may confer protection against I/R injury at multiple levels. EP4 agonist

may provide a novel approach to limit the damage from myocardial I/R injury.

4. Cardiac Hypertrophy

Pathological cardiac hypertrophy is a slow adaptive response to various types of extracellular stressors, including increased hemodynamic load [61], neurohormones [62], growth factors [63], and cytokines [64]. At the early stage, cardiac hypertrophy is compensatory to maintain the circulatory system homeostasis. However, the severe and sustained workload on the heart may trigger the cardiac remodeling process and increase the risk of cardiac dysfunction and, ultimately, the development of heart failure [65] and the underlying mechanism is incompletely understood. A better understanding of the signal transmission from cell surface to the nuclear transcription activities in response to various hypertrophic stimuli may yield novel therapeutic approaches to treat cardiac hypertrophy.

In ventricular myocytes, PGE_2 significantly increased total protein synthesis (measured by [^{3}H]-phenylalanine uptake), cell surface area, and hypertrophic maker genes, including atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), in a dose dependent manner [66–68]. These hypertrophic effects of PGE_2 were conserved *in vivo*. Using a mouse model of myocardial infarction, injection with NS-398 or rofecoxib (COX-2 selective inhibitors) in mice significantly downregulated cardiac PGE_2 production and reduced cardiac hypertrophy as determined by myocyte cross-sectional area when compared with vehicle treated mice [69]. Similarly, mice with global knockout of mPGES-1, which is in charge of the inducible PGE_2 synthase, also exhibited decreased cardiac PGE_2 levels, myocyte cross-sectional area, and cardiomyocyte surface area after myocardial infarction when compared to wild type mice, suggesting that PGE_2 positively regulates cardiac hypertrophy *in vitro* and *in vivo* [70].

Activation of EP4 receptor signaling contributes to the PGE_2-mediated cardiac hypertrophy, since EP4 specific antagonist (L-161982 or ONO-AE3-208) significantly blocked the hypertrophic actions of PGE_2, including the protein synthesis, mRNA expression of ANP and BNP in neonatal cardiac cells [66, 68]. In accordance with these observations, myocyte cross-sectional area was significantly smaller in cardiac-specific EP4 knockout mice after myocardial infarction when compared with wild type mice, suggesting that the lack of EP4 receptor signaling in cardiomyocytes alleviated cardiac hypertrophy after myocardial infarction [71]. By contrast, global EP4 knockout mice did not affect the myocyte cross-sectional area and heart/body weight ratio under basal conditions [15] or pressure overload-induced cardiac hypertrophy through transverse aortic constriction (TAC) treatment [72] as compared with wild type mice. There are several possible explanations regarding the discrepancy among these studies. (1) The development of cardiac hypertrophy is a very slow process, and thus if there are no continuous hypertrophic stimuli, it may take a long time to develop cardiac hypertrophy. Therefore, it may be difficult to detect significant difference in the development of cardiac hypertrophy between EP4 wild type and knockout mice under basal conditions. (2) The wide distribution of EP4 receptor in different kinds of cells indicates its diverse biological functions *in vivo*, such as anti-inflammatory response [73] and energy metabolism [16]. The affected anti-inflammatory pathway or lipid metabolism in global EP4 knockout mice may influence the development of cardiac hypertrophy indirectly. Indeed, inflammatory process and energy metabolism are closely associated with the pathogenesis of cardiac hypertrophy [74, 75]. Thus, under basal condition or TAC-induced cardiac hypertrophy, global knockout of EP4 in mice may affect the pathogenesis of cardiac hypertrophy through other compensatory pathways. (3) Different cardiac hypertrophic stimuli activate diverse signaling cascades and may have distinct cardiomyocyte gene expression pattern [65]. It is possible that PGE_2-EP4 signaling is only involved in myocardial infarction—but not pressure overload-mediated cardiac hypertrophy. (4) In the study of Hara et al. [72], the heart/body weight ratios were only compared between EP4 wild type and knockout mice after 4 weeks of TAC treatment, a time point at which generally the pressure overload-induced cardiac hypertrophy has reached the maximal level. Thus, comparison should be performed at both the early and late phase of the disease to get a solid conclusion. Through these explanations, it is tempting to speculate that EP4 in cardiomyocytes may be the endogenous ligand to mediate the hypertrophic effects of PGE_2 both *in vitro* and *in vivo*, but its definite role in this pathology needs to be confirmed in future studies.

The additional challenge is to identify the subsequent downstream molecules in EP4-mediated cardiac hypertrophy. Mammalian target of rapamycin (mTOR) has emerged as an important regulator of cardiac hypertrophy [76]. There is study that reported that PGE_2 activates the mTOR complex 1 pathway through an EP4/cAMP/PKA mediated mechanism in the human pancreatic carcinoma cell line PANC-1 [77]. Besides PKA, cAMP also exerts its biological functions through Epac. Despite the fact that cAMP-producing β-adrenergic stimulation induces cardiac hypertrophy [78], cAMP/PKA or cAMP/Epac signaling is not likely to mediate the hypertrophic effects of PGE_2 in cardiomyocytes, since all the treatment like cAMP activator (forskolin), cAMP inhibitor (SQ-22536), PKA inhibitor (H89), and Epac activator (8-CPT-2Me-cAMP) at different concentrations had no effect on PGE_2-induced protein synthesis in ventricular myocytes [57, 68, 79]. Thus, mTOR may not be able to mediate the hypertrophic effects of PGE_2/EP4 in cardiomyocytes.

It is well known that mitogen-activated protein kinases (MAPKs) signaling cascade is a prominent player involved in the hypertrophic reaction with its four best characterized subfamilies, extracellular signal-regulated kinases (ERK1/2 or P42/P44 MAPK), c-Jun N-terminal kinase (JNK), p38 MAPK, and ERK5 [80]. The ERK1/2 inhibitor, U0126, significantly reduced the hypertrophic effect of PGE_2 in ventricular myocytes, whereas the p38 MAPK blocker (SB203580) and JNK inhibitor (SP600125) have no such effect [68]. In addition, activation of ERK1/2 by PGE_2 was strongly suppressed by EP4 antagonist ONO-208 compound, indicating that ERK1/2 signaling cascade was involved in

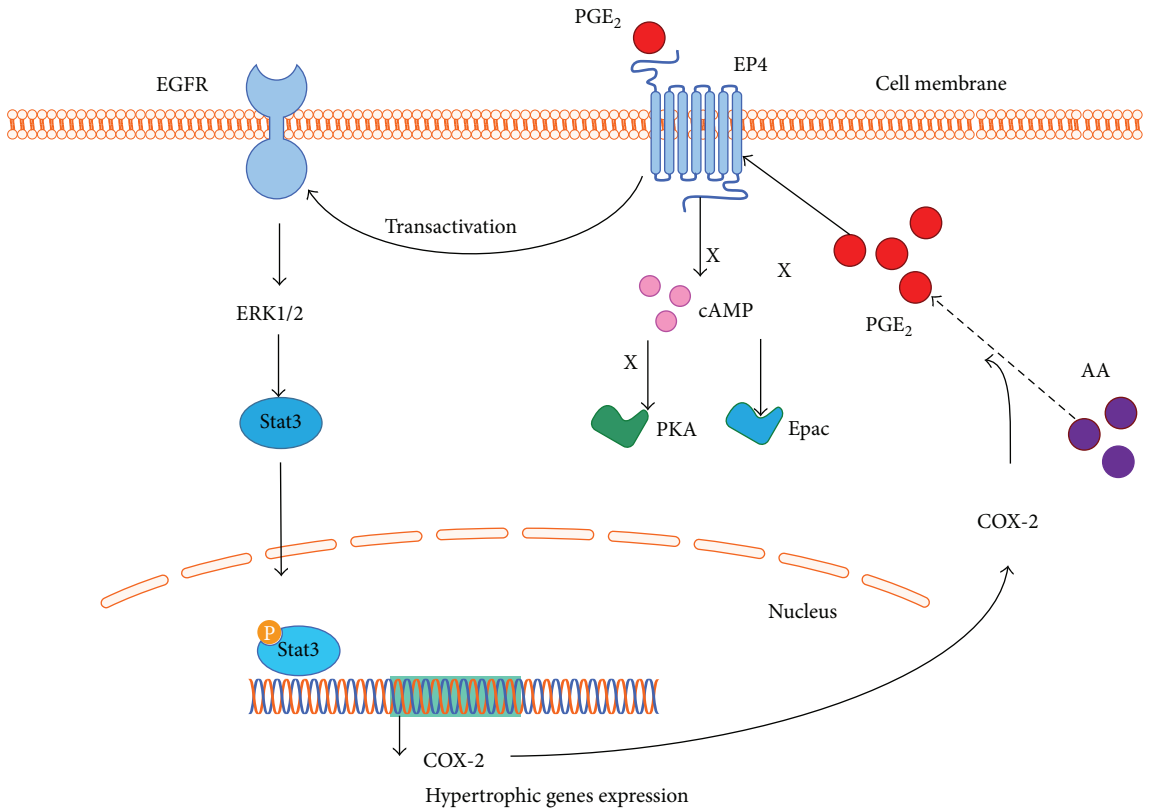

Figure 3: Simplified scheme of the EP4 signaling in PGE_2-mediated hypertrophic actions. In response to PGE_2, activation of EP4 transactivates epidermal growth factor receptors (EGFR), which can result in the activation of extracellular signal-regulated kinases (ERK1/2). ERK1/2 in turn activated signal transducer and activator of transcription 3 (Stat3) pathway. Once Stat3 signaling is activated in the nucleus, the hypertrophic genes will be expressed to mediate the protein synthesis. In addition, cyclooxygenase-2 (COX-2) expression is also stimulated by Stat3 signaling, which will further metabolize the formation of PGE_2 from arachidonic acid (AA) and trigger the greater activation of Stat3 signaling in a positive feedback loop. Cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) or cAMP/exchange protein directly activated by cAMP (Epac) pathways are not involved in the PGE_2-EP4-mediated hypertrophic actions in ventricular myocytes.

PGE_2-EP4-mediated cardiac hypertrophy [57, 68]. However, the involvement of ERK5 on PGE_2-induced protein synthesis remains undefined.

Activation of G-protein-coupled receptors has been shown to transactivate epidermal growth factor receptors (EGFR), which can result in the activation of ERK1/2 signaling [81, 82]. Indeed, EP4 antagonist L-161982 significantly inhibited the phosphorylation of EGFR by PGE_2 in ventricular myocytes. Furthermore, EGFR inhibitor AG-1478 totally blocked activation of ERK1/2 by PGE_2 and PGE_2-EP4-mediated protein synthesis, suggesting that activation of EGFR bridges PGE_2/EP4 and ERK1/2 signaling and also is responsible for PGE_2-induced protein synthesis in cardiomyocytes [79].

As reported in several studies, signal transducer and activator of transcription 3 (Stat3) pathway is another signaling cascade that plays a major role in the development of cardiac hypertrophy. Multiple *in vitro* and *in vivo* studies suggested that activation of Stat3 promotes cardiomyocyte hypertrophy in response to various stimuli through transcriptional control of hypertrophic genes expression [83, 84]. PGE_2 induced Stat3 activation in cardiomyocytes in a concentration- and time-dependent manner, while ERK1/2 inhibitor (U0126) and EP4 antagonists (GW627368X or AH23848B) significantly suppressed PGE_2-induced Stat3 activation [57]. In Stat3-silenced cardiomyocytes, the PGE_2-mediated protein synthesis was dramatically inhibited [57], suggesting that activation of Stat3 works downstream of PGE_2-EP4-ERK1/2-mediated cardiac hypertrophy *in vitro*. Accordingly, myocardial infarction induced cardiac hypertrophy was accompanied with significantly increased Stat-3 phosphorylation in wild type controls, but the increase of Stat-3 phosphorylation was absent in the heart from cardiomyocyte-specific EP4 knockout mouse, suggesting that EP4-Stat3 signaling is conserved *in vivo* and responsible for cardiac hypertrophy [71]. Furthermore, once Stat3 signaling is activated in the nucleus, the gene expression of COX-2 will be stimulated, which will metabolize the first step in the formation of PGE_2 from arachidonic acid and trigger the greater activation of Stat3 signaling in a positive feedback loop [85]. Thus, these findings together demonstrated the pathophysiological importance of PGE_2 signaling through EP4-EGFR-ERK1/2-Stat3 cascade that was involved in the development of cardiac hypertrophy (Figure 3).

5. Conclusion

Cardiac hypertrophy is not only a risk factor for myocardial ischemia, but also part of the response of MI/R-induced tissue injury, which further promotes the extent of ischemia. It is very interesting that activation of PGE_2-EP4 signaling may impact on ischemic heart events through totally opposite pathways. EP4 may mediate the cardiac hypertrophy through EGFR/ERK1/2/Stat3 signaling, whereas PGE_2-EP4 signaling may protect the heart from MI/R injury through cAMP/PKA or Stat3 pathway. Despite the fact that there is still no direct evidence showing the role of Stat3 signaling in EP4-mediated cardioprotection, the *in vivo* model, cardiac-specific EP4 knockout mice with myocardial infarction, exhibited decreased hypertrophic changes but worsened cardiac function, suggesting that activation of EP4 signaling may contribute to the compensatory survival of cardiomyocytes for maintaining the normal cardiac function. In the future, with regard to the potential detrimental effects of EP4, special caution is needed when evaluating how EP4 can be preferably activated in the heart without triggering unwanted side effects. In addition, the role of PGE_2-EP4 signaling pathway in the heart should be further explored in detail, with the aim of developing therapeutic approaches to treat the patients with ischemic heart disease and its associated diseases.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Lei Pang and Yin Cai contributed equally.

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Research Article

Impaired Thermogenesis and a Molecular Signature for Brown Adipose Tissue in *Id2* Null Mice

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Inhibitor of DNA binding 2 (ID2) is a helix-loop-helix transcriptional repressor rhythmically expressed in many adult tissues. Our previous studies have demonstrated that *Id2* null mice have sex-specific elevated glucose uptake in brown adipose tissue (BAT). Here we further explored the role of *Id2* in the regulation of core body temperature over the circadian cycle and the impact of *Id2* deficiency on genes involved in insulin signaling and adipogenesis in BAT. We discovered a reduced core body temperature in *Id2*−/− mice. Moreover, in *Id2*−/− BAT, 30 genes including *Irs1*, *PPAR*s, and *PGC-1*s were identified as differentially expressed in a sex-specific pattern. These data provide valuable insights into the impact of *Id2* deficiency on energy homeostasis of mice in a sex-specific manner.

1. Introduction

The circadian clock is an autoregulatory network that regulates behavioral and metabolic programing in the context of a 24 h light-dark (LD) cycle [1]. Body temperature is one of the representing benchmarks of circadian patterning, which peaks in animals while awake and troughs while asleep [2]. Brown adipose tissue (BAT) is a major site for rodent thermogenesis, due to its involvement in controlling circadian thermogenic rhythms and influencing adaptability to environmental temperature challenges [3]. A previous study has revealed rhythmic expression patterns of over 5,000 genes in murine BAT, including genes associated with the circadian clock, adipose function, and metabolism [4]. Moreover, glucose uptake in BAT exhibits a diurnal rhythm [5].

The *Inhibitor of DNA binding 2* (*Id2*) gene encodes a helix-loop-helix (HLH) transcriptional regulator, which is rhythmically expressed in many mammalian tissues and involved in the input pathway, core clock function, and output pathways of the circadian clock [6–9]. Our previous studies have shown that *Id2*−/− mice exhibit lower levels of locomotor activity, extended nighttime activity patterns of feeding and locomotor activity, and sex- and age-dependent enhanced glucose tolerance and insulin sensitivity [10]. Moreover, an energy-rich diet is able to rescue the disturbances to metabolic homeostasis and survival in the *Id2*−/− mice sex-specifically [11]. Importantly, *Id2*−/− mice show a sex-dependent elevated glucose uptake in interscapular BAT (iBAT) [10]. *Id2* also plays a role in white adipose tissue (WAT) adipogenesis [10–12]. However, the role of *Id2* on temperature homeostasis regulation and its influence on BAT physiology remain unknown. Therefore, we investigated the function of *Id2* in the regulation of temperature rhythms under normal and thermoneutral conditions in a sex-specific manner and also profiled the expression of genes involved in insulin signaling and adipogenesis in BAT of *Id2*−/− mice, sex-specifically.

2. Materials and Methods

2.1. Animals. The generation of *Id2*−/− mice and genotype determination were performed as described previously [7, 10, 11]. Mice were maintained on a regular chow diet and sterile water containing antibiotic *ad libitum* [7, 10, 11]. All mice were housed in laboratory cages at normal temperature (21°C ± 1°C) and humidity of 50–65% under a 12 : 12 light : dark (LD) cycle with lights on at Zeitgeber time (ZT) 0 and

lights off at ZT12. Controls were age- and sex-matched WT littermate mice. Animal experiments were approved by the University of Notre Dame Animal Care and Use Committee (Protocol number 14-02-1559) and performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

2.2. Temperature Measurement. Temperature measurements were carried out on 2-month–1.5-year- (5.5-month median) old male and female *Id2*−/− mice and WT littermates, housed individually in a climate-controlled room set to either normal (21°C ± 1°C) or thermoneutral (30°C ± 1°C) temperature. Body temperature sampling was conducted at 3 h intervals over the 24 h LD cycle. For thermoneutral conditions measurement, all WT and *Id2*−/− mice used in the studies were allowed to acclimate to thermoneutral temperature for 1 week before temperature measurement. Core body temperature was measured using subcutaneously surgically implanted telemetric transmitters positioned proximal to the iBAT (IPTT 300 transponders, Bio Medic Data Systems, Seaford, DE) following isoflurane anesthetization [3]. After a week of recuperation, core temperatures were recorded over a 24 h period.

2.3. iBAT PCR Array Preparation and Analysis. iBAT tissue was harvested at ZT16 (*Id2* mRNA circadian rhythm in iBAT has a broad peak phase between ZT8 and ZT16) [4]. *Id2*−/− and WT male (WT = 8, *Id2*−/− = 6) and female (WT = 6, *Id2*−/− = 4) mice from 3–9 months (6.1-month median) were sacrificed and iBAT tissue was frozen in liquid nitrogen and stored at −80°C until analyzed. RNA extraction was performed as described previously [7, 13]. We also measured iBAT weight of these and additional mice (3–10-month-old *Id2*−/− mice and WT littermates; 6.3-month median; male, WT = 15, *Id2*−/− = 7; female, WT = 10, *Id2*−/− = 9) as described previously [10, 11]. Total RNA was purified following a Trizol extraction and sodium acetate/ethanol treatment. RNA integrity was assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). RNA was subjected to a DNASe I treatment, and cDNA was synthesized by RT^2 First Strand Kit (SABiosciences). Relative mRNA expression of 168 genes involved in insulin signaling and adipogenesis pathways was determined by using the mouse PCR arrays (PAMM-030ZC-24 and PAMM-049ZC-24, SABiosciences). Quantitative real-time PCR was performed using an Applied Biosystems 7500 system with RT2 SYBR green ROX qPCR master mix reagent (Qiagen). PCR array data were calculated by the comparative cycle threshold method and analyzed by Web-based free PCR array data analysis software provided by SABiosciences. Normalization of expression was to housekeeping genes provided on each array (*Actb*, *B2m*, *Gapdh*, *Gusb*, and *Hsp90ab1*). Clock controlled genes (CCGs) were identified from the CIRCA database of Mouse 1.OST Brown Adipose (Affymetrix) (http://bioinf.itmat.upenn.edu/circa/) where we defined CCGs as a JTK_CYCLE algorithm determined $q < 0.1$ value and a period length of 20–28 h as described previously [13–15]. Circadian phase was determined from the Lomb-Scargle phase values within CIRCA.

2.4. Statistics. Data were analyzed using Sigma Plot 12.0 software to run two-factor ANOVA. Where necessary, data were ranks transformed to correct for nonnormal distributions. The linear regression of iBAT temperature-body weight relationship was generated and analyzed using Prism 5.0 Graphpad software. PCR array data were analyzed using the Web-based free PCR array data analysis software provided by SABiosciences (Student's t-test).

3. Results

3.1. Loss of Id2 Results in a Reduced Core Body Temperature in Male and Female Mice. The discovery of a diurnal rhythm of glucose uptake in mice iBAT and a sex-dependent elevated glucose uptake in iBAT of *Id2*−/− mice prompted us to investigate whether *Id2* contributes to thermoregulation [5, 10]. At normal ambient temperature conditions (21°C), ablation of *Id2* reduced core body temperature across the 24 h day, in both male and female mice (Figure 1(a)) (males, wild types (WTs) = 14, *Id2*−/− = 14, ANOVA, time (T), $p < 0.001$, genotype (G) $p < 0.001$, interaction (I), n.s.; females, WTs = 18, *Id2*−/− = 17, ANOVA, T, $p < 0.001$, G, $p < 0.05$, I, n.s.). Considering the possibility of any confounding genetic background contribution and partial stimulation of BAT activity occurring under normal temperature conditions, *Id2*−/− mice core body temperature was also measured under thermoneutral conditions (30°C) [3, 16]. Consistently, at thermoneutrality, *Id2*−/− mice displayed a reduced core body temperature (Figure 1(a)) (males, WTs = 19, *Id2*−/− = 20, ANOVA, T, $p < 0.001$, G, $p < 0.01$, I, n.s.; females, WTs = 18, *Id2*−/− = 17, ANOVA, T, $p < 0.001$, G, $p < 0.01$, I, n.s.). Under both conditions and in both sexes, no interaction between time and genotype was discovered, suggesting a generalized effect of the null mutation on core body temperature rather than a time-of-day specific contribution of the gene deletion. Regression analysis of time-of-day representative core body temperatures (day or night) revealed no significant relationships between temperature and body mass for either *Id2*−/− or WT mice. However, *Id2*−/− mice of both sexes showed consistently lower y-intercept lines compared to WT mice when examined during either the daytime (ZT5.5) or nighttime (ZT17.5 or ZT20.5), thus confirming the consistently lower temperature of the *Id2* null mice (Figure 1(b); Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/6785948). Lastly, *Id2*−/− mice exhibited no statistically significant difference in iBAT weight and iBAT to body weight ratio compared to WT controls (Figures 2(a) and 2(b)) (two-factor ANOVAs, iBAT weight, G, n.s., sex (S), $p < 0.01$, I, n.s.; iBAT/body weight ratio, G, n.s., S, n.s., I, n.s.). The mean and SEM body mass of WT and *Id2*−/− mice for both iBAT weight and body temperature experiments are shown in Supplementary Table 2: note that both male and female *Id2*−/− mice had on average a lower body mass compared to WT counterparts (two-factor ANOVA, G, $p < 0.001$, S, $p < 0.001$, I, n.s.).

3.2. Sex-Specific Differential Gene Expression Associated with Insulin Signaling and Adipogenesis in iBAT of Id2−/− Mice. Our previous results showed sex-dependent enhanced

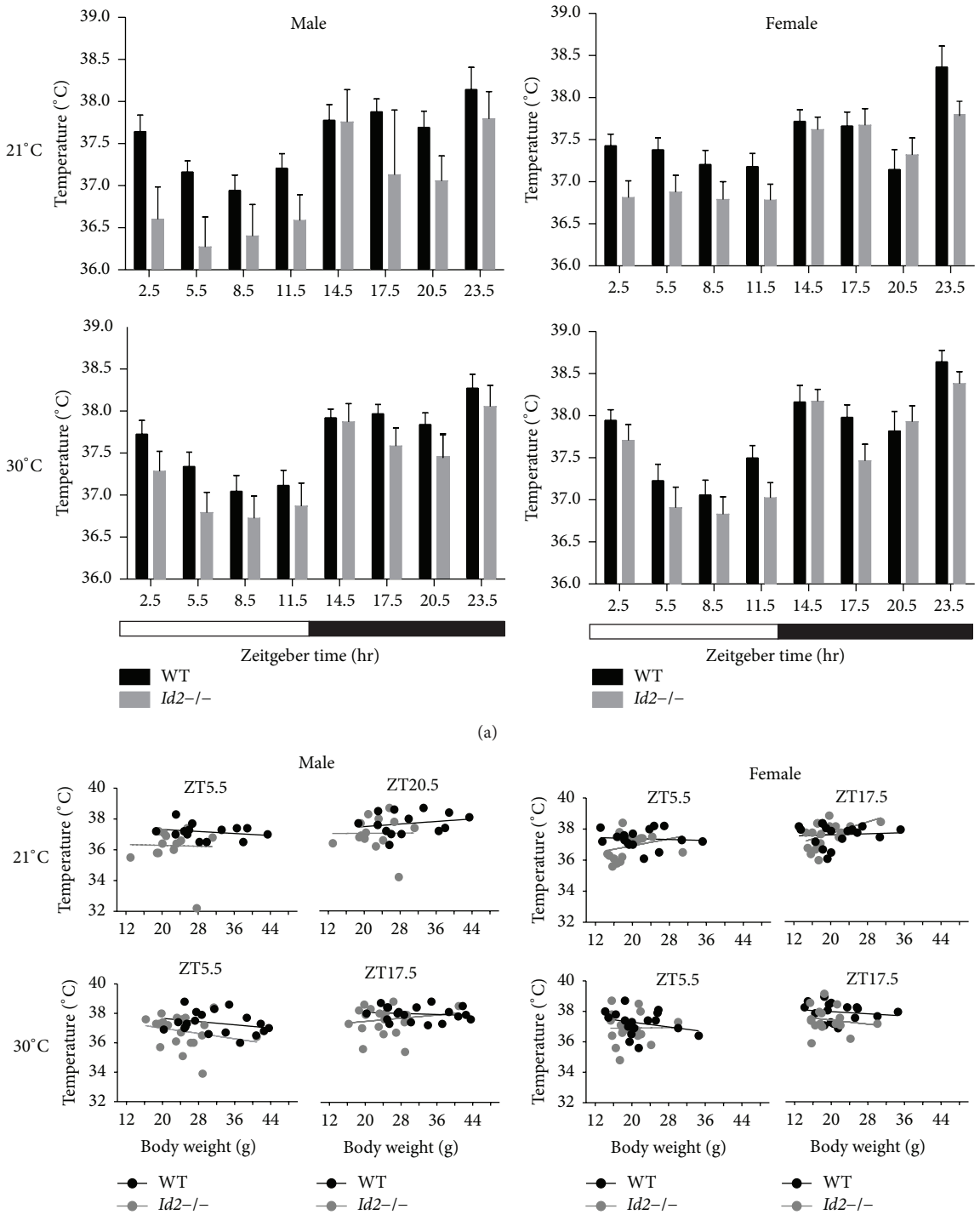

Figure 1: Sex-specific regulation of body temperature in *Id2*−/− mice. (a) Body temperature measurements of WT and *Id2*−/− male (left) and female (right) mice under normal temperature (upper panel) or thermoneutral temperature (lower panel) over 24 hrs. Values are mean ± SEM. Two-factor ANOVA was performed. ANOVAs revealed significantly lower body temperatures for both male and female *Id2*−/− mice compared to WT mice. The genotypic effect was independent of the prevailing ambient temperature. (b) Upper: regression analysis of body weight to body temperature of WT and *Id2*−/− mice under normal ambient temperature (left: male at ZT5.5 and ZT20.5; right: female at ZT5.5 and ZT17.5). Lower: regression analysis of body weight to body temperature of WT and *Id2*−/− mice under thermoneutral temperature conditions (left: male at ZT5.5 and ZT17.5; right: female at ZT5.5 and ZT17.5). Values are individual animal body temperatures and their respective measures of body mass. Note that no linear regression was found to be significant (n.s.), indicating that body mass does not predict body temperature for any group analyzed, examined under either 21°C or 30°C environmental temperatures.

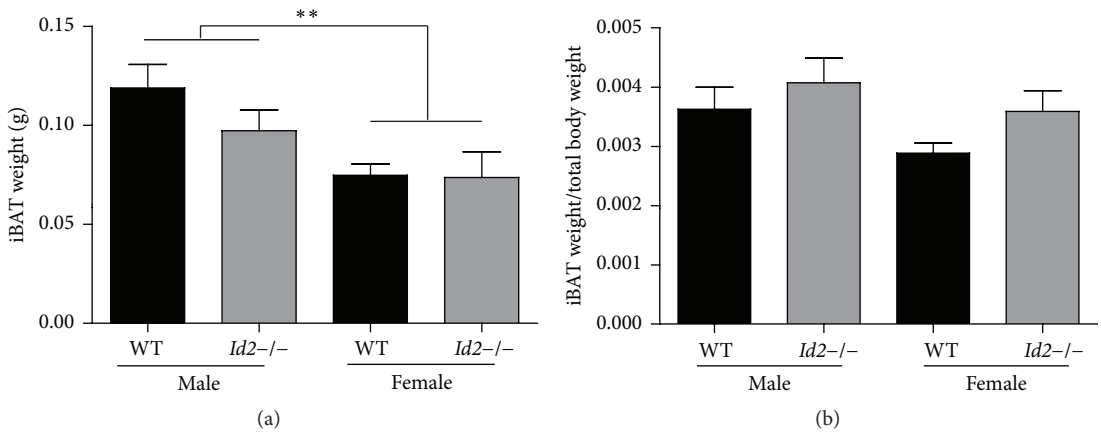

Figure 2: Brown Adipose tissue weight in *Id2*−/− mice. (a) Interscapular brown adipose tissue (iBAT) mass (g) from WT and *Id2*−/− mice. (b) Ratio of weight of iBAT tissue to total body mass from WT and *Id2*−/− mice. Values are mean ± SEM. Two-factor ANOVAs were performed followed by Tukey's post hoc tests, $^{**}p < 0.01$. No significant differences were observed between groups in the iBAT mass/body mass analysis.

insulin sensitivity and glucose uptake in iBAT of *Id2*−/− mice [10]. In the current study we observed a decreased core body temperature in *Id2*−/− mice as described above. To fully evaluate the impact of ablation of *Id2* on BAT gene-regulation, we performed a gene expression analysis using RT^2 Profiler PCR Arrays of BAT derived from *Id2*−/− mice and their WT littermates collected at the same time of the 24 h day (specifically ZT16). Deferentially regulated genes involved in insulin signaling and adipogenesis are shown in Tables 1 and 2, respectively. Thirty of 168 genes examined were identified as differentially expressed when analyzed as a cohort or as individual sex-specific groups. Using the CIRCA database as a resource [14], six genes were identified as clock controlled genes (CCGs), of which four oscillate in proximal phase with the rhythm of *Peroxisome proliferator activated receptor alpha* (*Pparα*), peaking during the middle of the day (~circadian time (CT) 6; CT12 = onset of night in prior LD cycle). Of importance for insulin signaling, *glucose-6-phosphatase*, *catalytic* (*G6pc*), was upregulated in *Id2*−/− females and the related G6pc family member *G6pc2* downregulated in *Id2*−/− males ($p = 0.079$, approaching significance) compared to WTs. *Insulin receptor substrate 1* (*Irs1*) was upregulated in both male and female *Id2*−/− mice. *Protein Kinase C*, *iota* (*Prkcι*), was downregulated in *Id2*−/− males. *Insulin-like growth factor 2* (*Igf2*) was downregulated in female *Id2*−/− mice, while *Fbp1*, a rate-limiting enzyme in gluconeogenesis, and *Shc1*, a component in the IGF-1-regulated pathway, were upregulated. For adipogenesis, *bone morphogenetic protein 4* (*Bmp4*) was elevated 1.7-fold (n.s.) in male and 1.6-fold in female *Id2*−/− mice. Consistent with the insulin signaling array, *Irs1* was elevated in *Id2*−/− mice. *Nuclear receptor coactivator 2* (*Ncoa2*), *PR domain containing 16* (*Prdm16*), *Pparα*, and *twist homolog 1* (*twist1*) were downregulated, in grouped analysis of male and female *Id2*−/− mice. *Fatty acid synthase* (*Fasn*), *lipase*, *hormone sensitive* (*Lipe*), and *Peroxisome proliferative activated receptor*, *gamma*, *coactivator 1 beta* (*Ppargc1β*/*PGC-1β*) were all downregulated in male *Id2*−/− mice. Female *Id2*−/− mice displayed a downregulation of *proliferative activated receptor*, *gamma*, *coactivator 1 alpha* (*Ppargc1α*/*PGC-1α*). A small 1.2-fold downregulation of *Peroxisome proliferator activated receptor gamma* (*Pparγ*) was detected in *Id2*−/− males, where the p value was approaching significance ($p = 0.061$). Note that the thermogenic protein, *uncoupling protein 1* (*ucp1*), was present on both the insulin signaling and adipogenesis arrays, but its levels of expression were not significantly altered in the *Id2*−/− mice.

4. Discussion

In the present study, we discovered a reduced core body temperature in *Id2*−/− mice, and this effect was not found to be dependent upon the time-of-day. Moreover, from the iBAT of *Id2*−/− mice, genes involved in insulin signaling and adipogenesis were differentially regulated in a sex-dependent manner. These results reveal a role of *Id2* in the regulation of thermogenesis and BAT metabolic functions.

Our previous study revealed that *Id2*−/− mice exhibit less activity as demonstrated by daily counts of general activity and the wheel running activity, which could partially explain the reduced core body temperature, since less physical activity would generate less heat [10]. Moreover, *Id2*−/− mice show a reduced body mass and less gonadal adipose deposits [6, 10, 11]. As the subcutaneous and intradermal fat functions as thermal insulation for mice to preserve heat loss, *Id2*−/− mice with low fat content might tend to lose heat more readily than WT mice. Furthermore, the reduced body temperature associated with lower fat content might contribute to the high death rate observed previously (mice housed under normal temperature), which was rescued by a high fat diet that resulted in increased total body fat [11]. Specifically in male *Id2*−/− mice iBAT we observed increased glucose uptake and reduced TG accumulation [10], suggesting alterations in its metabolic programing. Additionally, both male and female *Id2*−/− mice exhibited an increased activated iBAT volume [10]. Interestingly, our results suggest that the role of *Id2* in thermoregulation is opposite to the function of another member of this HLH family, *Id1*, whose deficiency results

Table 1: Differentially expressed genes from insulin signaling pathway of *Id2*−/− mice. Genes with significant differences ($p < 0.05$) are shown in bold. Peak phase value (determined by Lomb-Scargle phase values within CIRCA) in circadian time (CT) is provided where gene was identified as a clock control gene. Rhythmic genes identified from CIRCA database of Mouse 1.OST Brown Adipose (Affymetrix).

Insulin signaling array	Symbol	Refseq	All		Male		Female		Clock control gene-peak phase
			Fold change	*p* value	Fold change	*p* value	Fold change	*p* value	
Gene name									
AE binding protein 1	**Aebp1**	NM_009636	1.9	0.09			**1.6**	**0.03**	—
Complement factor D (adipsin)	**Cfd**	NM_013459	**2.4**	**0.01**	**3.6**	**0.03**			—
Fructose bisphosphatase 1	**Fbp1**	NM_019395	1.3	0.22			**1.6**	**0.05**	—
Glucose-6-phosphatase, catalytic	**G6pc**	NM_008061	1.5	0.28	1.5	0.33	**1.6**	**0.02**	—
Growth factor receptor bound protein 10	**Grb10**	NM_010345	1.5	0.08					—
Insulin receptor substrate 1	**Irs1**	NM_010570	**1.4**	**0.01**	1.3	0.07	**1.4**	**0.05**	—
Protein tyrosine phosphatase, receptor type, F	**Ptprf**	NM_011213	**1.7**	**0.05**			**2.6**	**0.05**	—
Scr homology 2 domain containing transforming protein C1	**Shc1**	NM_011368					**1.25**	**0.01**	—
Glucose-6-phosphatase, catalytic, 2	**G6pc2**	NM_021331			−3.2	0.08			—
Insulin-like growth factor 2	**Igf2**	NM_010514	−1.2	0.21			**−1.4**	**0.04**	—
V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	**Kras**	NM_021284	−1.1	0.09	**−1.25**	**0.03**			—
Protein kinase C, iota	**Prkci**	NM_008857	**−1.2**	**0.02**	**−1.3**	**0.02**			CT0
Thyroglobulin	**Tg**	NM_009375	−1.8	0.29	**−3.5**	**0.02**			—

Table 2: Differentially expressed genes from adipogenesis pathway of *Id2*−/− mice. Genes with significant differences ($p < 0.05$) are shown in bold.

Adipogenesis array	Symbol	Refseq	All		Male		Female		Clock control gene-peak phase
			Fold change	p value	Fold change	p value	Fold change	p value	
Gene name									
Bone morphogenetic protein 4	**Bmp4**	NM_007554	**1.6**	**0.04**	1.7	0.12	**1.6**	**0.01**	—
Complement factor D (adipsin)	**Cfd**	NM_013459	**2.5**	**0.02**	**4.3**	**0.03**			—
Insulin receptor substrate 1	**Irs1**	NM_010570	**1.2**	**0.03**	1.1	0.29	**1.4**	**0.02**	—
TSC22 domain family, member 3	**Tsc22d3**	NM_010286	**1.6**	**0.04**	1.9	0.09			CT16
Axin 1	**Axin1**	NM_009733	−1.2	0.07	**−1.3**	**0.05**			—
Cyclin-dependent kinase 4	**Cdk4**	NM_009870	**−1.4**	**0.003**	**−1.7**	**0.001**			CT6
Delta-like 1 homolog (Drosophila)	**Dlk1**	NM_010052	**−1.9**	**0.01**	**−2.2**	**0.01**			—
Fatty acid synthase	**Fasn**	NM_007988	−1.2	0.06	**−1.4**	**0.02**			—
Lipase, hormone sensitive	**Lipe**	NM_010719	−1.2	0.06	**−1.5**	**0.02**			—
Nuclear receptor coactivator 2	**Ncoa2**	NM_008678	−1.2	0.07	−1.3	0.07			—
Peroxisome proliferator activated receptor alpha	**Ppara**	NM_011144	**−2.1**	**0.01**	−2.3	0.06	−1.7	0.09	CT6
Peroxisome proliferator activated receptor gamma	**Pparg**	NM_011146			−1.2	0.06			
Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	**Ppargc1a (PGC-1α)**	NM_008904	−1.2	0.09			**−1.5**	**0.004**	CT8
Peroxisome proliferative activated receptor, gamma, coactivator 1 beta	**Ppargc1b (PGC-1β)**	NM_133249	**−1.4**	**0.01**	**−1.4**	**0.04**			CT5
PR domain containing 16	**Prdm16**	NM_027504	**−1.3**	**0.03**	**−1.5**	**0.03**			—
Twist homolog 1 (Drosophila)	**Twist1**	NM_011658	**−1.8**	**0.01**	−2.0	0.06	**−1.5**	**0.04**	—
Actin, beta	**Actb**	NM_007393	**−1.2**	**0.02**	**−1.4**	**0.004**			—

in higher thermogenesis and an elevated BAT expression of thermogenic proteins [17]. Notably, *Id1* has a distinct and opposite function in WAT adipogenesis compared to *Id2*, despite both *Id1* and *Id2* null mice exhibiting reduced adiposity [10–12, 17]. Lastly, we examined the relationship between body mass and body temperature in *Id2*−/− mice by regression analysis and revealed a limited relationship between the two variables [18]. No significant relationship was observed between body mass and body temperature at any time of the day or in the two sexes. However, as can be seen with the y-intercept of the regression lines, both *Id2*−/− male and female mice expressed a consistently lower temperature compared to WT controls, irrespective of body mass, and this feature was observed during both the day and night phases of the LD cycle. These results suggest a role for *Id2* in the regulation of core body temperature.

In this study we also measured iBAT mass and iBAT/body mass ratio. While there was a tendency for higher iBAT/body mass in both *Id2*−/− male and female mice, this was not determined to be a significant difference. Note that the average body mass of *Id2*−/− mice used for both the iBAT weight and body temperature experiments was found to be significantly lower, consistent with our previous studies [7, 10]. Important is the fact that a lower body mass, found for some of the *Id2*−/− mice and for males in particular, does not correlate with a lower body temperature, and body mass in this situation is therefore an independent factor when predicting core body temperature.

It is important to note that while the objective of examining body temperature using the implanted thermometers was to record "core" body temperature, the position of the implants may not give an *exact* measure of true core body temperature. However, in a comparable study of mouse body temperatures, temperature measurements were similar whether derived from similarly subcutaneously implanted thermometers in the interscapular region of WT and *Rev-erbα* mutant mice or as determined using dataloggers that were implanted within the abdomen [3].

Id2 is rhythmically expressed in BAT [4, 15] amongst other tissues [6, 7]. ID2 protein has also been observed to be rhythmic in its abundance over the 24 hr diurnal/circadian cycle within the liver and heart [6] (Ward, Fernando, Hou, and Duffield, unpublished data). A role for ID2 has been established as a mediator of circadian clock output and control of expression patterns of clock controlled genes (CCGs) within the liver [6]. CCGs encompass ~10% transcriptome in individual tissues [19]. It is for this reason that we examined whether any of the genes identified as differentially expressed in iBAT were in fact known CCGs. Using the CIRCA database [14, 15], 5 of the 17 differentially genes associated with adipogenesis were found to be CCGs (e.g., *Pparα* and *PGC-1α*), and so a possible role for ID2 is in mediating circadian regulatory effects on these genes within BAT. However, further investigation would be required to test this hypothesis. The observation that few of the differentially regulated genes involved in insulin signaling are CCGs (1 out of 13 genes) suggests that the contribution of ID2 to insulin signaling intrinsic to BAT is independent of the role of ID2 in mediating circadian clock output [6].

In order to explain how *Id2* deficiency has an impact on BAT insulin signaling and adipogenesis, we propose a network model (Figure 3). The nuclear receptor PPARs are fundamentally important for energy homeostasis and *Id2* plays a role in interfacing with the molecular pathways upstream or downstream of these transcriptional factors. Expression of two members of the PPAR subfamily of ligand-activated nuclear receptors, *PPARα* and *PPARγ*, was downregulated in our study. PPARα is highly expressed in BAT and considered a marker of BAT; it also plays an important role in the overall regulation of lipid metabolism; and its target genes are involved in mitochondrial and peroxisomal β-oxidation of fatty acids (FAs) [20–22]. Moreover, PPARα regulates the expression of uncoupling protein 1 (*ucp1*), which confers on BAT its thermogenic capacity [23]. *PGC-1α* (downregulated in our study) is a transcriptional coactivator involved in the control of energy metabolism and critical for BAT thermogenesis and enhancing overall mitochondrial oxidative activity [24]. PPARα can induce *PGC-1α* gene expression and contributes to the thermogenic activation of brown fat [25]. PRDM16 exhibits a brown fat selective expression pattern and regulates the thermogenic gene program in brown and beige adipocytes [26]. The observation of reduced *Prdm16* expression in *Id2*−/− mice is consistent with the role of PRDM16 as a transcriptional regulator of PGC-1α [27]. Likewise, studies have demonstrated the linkage between *Id2* and PPARα [28]. PPARγ is essential for adipocyte differentiation, and PPARγ alone generates a fat phenotype that is common to both WAT and BAT. The CCAAT enhancer binding protein beta (C/EBPβ) and PGC-1α are critical for controlling PPARγ expression in BAT and for determining BAT-specific programs [29, 30]. The PPARγ thermogenic effect in BAT is mediated by PGC-1α [24]. It has been observed that overexpression of *Id2* associates with *PPARγ* expression, ID2 acts upstream of PPARγ, and C/EBPβ induces *Id2* expression during the adipogenesis process [12, 31]. Cofactors such as NCoA2 (downregulated in our study) can interact directly with PPARγ to initiate its own transactivation [32]. Moreover, LIPE (downregulated in our study) could modulate adipose metabolism by reducing the availability of ligands for PPARγ, since gene knockout of LIPE in mice attenuates activation of PPARγ [33]. LIPE is also able to hydrolyze stored TGs in adipose tissue and to mobilize free FA from adipose tissue [34]. Furthermore, PPARγ is a direct target of the transcription factor sterol response element binding protein 1 (SREBP1), whose transcriptional activity is modulated by ID2 and which regulates downstream lipid metabolism genes such as *lipe* and *Fasn* [35, 36]. Additionally, *Irs1* (upregulated in our study) plays essential roles in the differentiation of brown adipocytes and expression of PPARγ [37, 38]. Previous studies have revealed IRS1-regulated *Id2* gene expression [39], although in the current study it is unclear whether this is a direct effect or a feedback response. As for the mechanism by which IRS1 is elevated in *Id2*−/− iBAT, it is unclear and warrants further investigation. BMP4 (upregulated in our study) is able to induce the white to brown transition of adipose cells, which could indirectly regulate PPARγ activation [40, 41]. The elevated BMP4 expression in the context of reduced PPARs is surprising

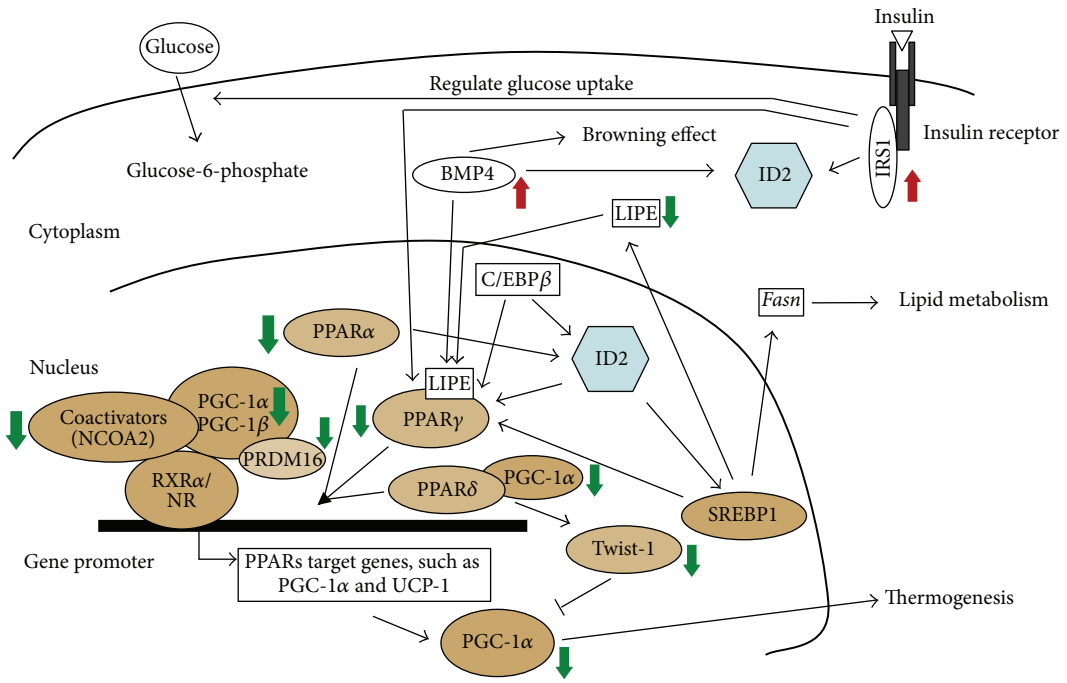

FIGURE 3: *Id2* network model in insulin signaling, thermogenesis, and adipogenesis pathways. A schematized and partial view of the signaling pathway indicating its major downstream targets and factors susceptible to interfering with signaling. Function of ID2 is not necessarily limited to the nucleus [8]. ↑ or ↓, up- or downregulation of gene expression, respectively, as determined by PCR array analysis in the current study.

since BMP4 upregulation is associated with increased BAT adipogenesis and the WAT browning effect [42]. Interestingly, the Id2 gene promoter has BMP-response elements and has been shown to be a target of BMP signaling [43]. PPARδ plays an integral role in transcriptional network regulation of fat-burning genes and brown fat metabolism. PGC-1α/PPARδ could regulate brown fat metabolism through Twist-1 tuning [44]. *Twist-1* (downregulation in our study) encodes a basic HLH transcription factor, and overexpression of *Twist-1* is associated with *Id2* expression [45].

Note that the gene encoding the thermogenic protein, UCP1, was present on the PCR arrays, but no difference in its expression was observed between genotypes of either sex. Interestingly, in the *Id1* null mouse, *ucp1* gene expression is elevated in iBAT, and this is associated with an *increased* core body temperature phenotype [17]. Thus, it is surprising that in the *Id2*−/− mouse that exhibits a *reduced* body temperature phenotype we do not observe a reduction in *ucp1* gene expression. Of course, this does not exclude, however unlikely, the possibility of an altered UCP1 protein abundance through a posttranscriptional/posttranslational process.

Hypoxia, while not a focus of the current study, is known to reduce body temperature in mammals and contribute to the thermogenic activity of BAT [46]. It is noteworthy that in a recent study of human glioblastoma cells/tissue, an important role was established for ID2 in modulating the cellular effects of hypoxia and its activation of the HIF2α pathway [47]. The Id2 gene is also a target for HIF1α and HIF2α [47, 48], making it part of a positive feedback loop mechanism, at least in models of brain tumor. It is plausible that the hypoxic effects on BAT function might also include a contribution from ID2, and this would be an important pathway to examine in future experiments in this context.

It is a somewhat contradictory finding that elements of the thermogenic pathway are reduced (e.g., *PGC1-α*) or unaltered (*ucp1*) in *Id2*−/− mice but that *Id2*−/− male iBAT exhibits increased glucose uptake (PET-FDG) coupled with reduced iBAT triglyceride levels and a systemwide enhanced insulin sensitivity [10, 11] and that core body temperature is consistently reduced in both *Id2*−/− male and female mice. *Id2*−/− mice also exhibit an altered 24 hr locomotor activity and feeding profile and an overall reduction in nocturnal locomotor activity [10], the latter of which suggests a reduction in energy expenditure/increased energy conservation. Clearly the BAT adipogenic program is altered in both male and female *Id2*−/− mice, as is WAT adipogenesis [10–12], and it is likely that a change in BAT function contributes to the reduced temperature phenotype. Due to the nature of whole body knockout of ID2, it is possible that the temperature phenotype and iBAT gene changes observed are secondary to whole body metabolic changes. Clearly additional experiments are required to elucidate the relative contributions of these and other potential components in generating the altered core body temperature phenotype.

Our previous study showed enhanced glucose uptake in iBAT of *Id2*−/− mice [10], and the present study reveals a reduced core body temperature in *Id2*−/− mice. This discrepancy could partially be explained by the differential regulation of *Irs1*, *Lipe*, *PPAR*s, and *PGC-1*s. It has been reported that degradation of IRS1 leads to impaired glucose

uptake in adipose tissue [49]. Therefore, upregulation of IRS1 might explain the increased glucose uptake we observed before [10], whereas downregulation of LIPE, PPARs, and PGC-1s might contribute to reduced FA oxidation, impaired adipogenesis, and a lower body temperature. Inactivation of PPARs is associated with insulin resistance [50, 51], yet paradoxically *Id2*−/− mice show enhanced insulin sensitivity with downregulated PPARs [10, 11]. It has been suggested that mice that lack one allele of the PPARγ gene are more sensitive to insulin, which could partially explain the enhanced insulin sensitivity we observe in *Id2* null mice [10, 51]. Furthermore, the differential regulation of genes specifically in female mutant mice, such as *Fbp1*, a rate-limiting enzyme in gluconeogenesis, *Shc1*, a component in the IGF-1-regulated pathway, and *Igf2*, suggests a sex-specific physiological program for ID2 in BAT.

5. Conclusion

Inhibitor of DNA binding 2 is rhythmically expressed in BAT [4, 15], and the observation that few of the differentially regulated genes involved in insulin signaling are CCGs suggests that the contribution of ID2 to insulin signaling intrinsic to BAT is independent of the role of ID2 in mediating circadian clock output [6]. Overall, ID2 seems to be an important coordinator of energy homeostasis including insulin signaling, adipogenic programing, and thermoregulation. In conclusion, our finding that ID2 contributes to the regulation of body temperature and energy homeostasis presents the possibility that ID2 could be a potential therapeutic target for metabolic disease. Further, these data emphasize the influence of *Id2* on BAT molecular signaling and physiology in a sex-specific manner.

Additional Points

Supplementary Table 1 summarizes the regression analysis of time-of-day representative core body temperatures (day and night). Supplementary Table 2 shows the mean ± SEM body mass of wild type and *Id2*−/− mice.

Disclosure

The present address of Peng Zhou is Division of Endocrinology, Diabetes, and Metabolism, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Hypoxia Alters the Expression of Dipeptidyl Peptidase 4 and Induces Developmental Remodeling of Human Preadipocytes

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Dipeptidyl peptidase 4 (DPP4), a transmembrane protein, has been identified in human adipose tissue and is considered to be associated with obesity-related type 2 diabetes. Since adipose tissue is relatively hypoxic in obese participants, we investigated the expression of DPP4 in human preadipocytes (hPA) and adipocytes in hypoxia, during differentiation and upon insulin stimulation. The results show that DPP4 is abundantly expressed in hPA but very sparsely in adipocytes. During differentiation *in vitro*, the expression of DPP4 in hPA is reduced on the addition of differentiation medium, indicating that this protein can be hPA marker. Long term hypoxia altered the expression of DPP4 in hPA. In *in vitro* hypoxic conditions the protease activity of shed DPP4 is reduced; however, in the presence of insulin, the increase in DPP4 expression is potentiated by hypoxia.

1. Introduction

Dipeptidyl peptidase 4 (DPP4) is a 110 kDa transmembrane glycoprotein. A soluble form of DPP4 (sDPP4) in the circulation is the result of proteolytic cleavage of the membrane bound form [1]. DPP4 has a rare protease activity, cleaving N-terminal x-Pro dipeptide from selected proteins. Besides enzymatic inactivation of incretins, DPP4 also mediates degradation of several growth factors, neuropeptides, chemokines, and vasoactive peptides, which results in alterations in their biological activity, often by altering their receptor specificity [2].

Altered DPP4 activity has been reported in a number of diseases, including type 2 diabetes [3, 4] and tumor biology [2, 5–7]. It is thought to be associated with sensitivity to anticancer agents in haematologic malignancies and is involved in the development of various chronic liver diseases [8]. DPP4 was considered as a therapeutic target for type 2 diabetes as it degrades incretins: glucagon-like peptide- (GLP-) 1 and gastric inhibitory peptide (GIP). Both hormones cause an increase in insulin secretion. DPP4 inhibitors target the enzyme activity of DPP4, thus prolonging the insulinotropic effects of incretins [9].

In patients with type 2 diabetes acute hypoxia increases the glucose uptake into the tissue [10, 11]; however, prolonged exposure to hypoxia has been associated with induction of insulin resistance in adipose tissue [12, 13]. More specifically, adipose tissue hypoxia, which develops with the onset of obesity [14, 15], has been linked to the development of insulin resistance and type 2 diabetes by decreasing insulin signaling pathways [13]. It was reported that DPP4 is expressed in preadipocytes and in adipocytes [16], indicating that adipose tissue might be a major source of circulating DPP4. Therefore, an altered expression of adipose tissue DPP4 could be linked to the development of type 2 diabetes; however the factors that alter the expression of DPP4 are poorly understood.

In the present study we developed a confocal microscopy assay to study the expression of DPP4 in human preadipocytes (hPA). We evaluated DPP4 expression during the differentiation of human preadipocytes into adipocytes

and studied how insulin affects the expression and activity of released DPP4. The results show that in hypoxic conditions the protease activity of shed DPP4 is reduced. Interestingly, insulin increases DPP4 expression and this is potentiated by hypoxia.

2. Experimental Procedures

2.1. Chemicals. Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12), l-glutamine, dexamethasone, isobutylmethylxanthine (IBMX), biotin, d-pantothenic acid hemicalcium salt, phosphate-buffered saline (PBS), bovine serum albumin (BSA), trypsin-EDTA, Trypan Blue, and goat serum were purchased from Sigma (St. Louis, MO). Insulin was purchased from Novo Nordisk (Bagsvaerd, Denmark). Fetal bovine serum (FBS) was obtained from Biochrom (Berlin, Germany). Rosiglitazone maleate was obtained from GlaxoSmithKline (Worthing, UK). Dimethyl sulfoxide (DMSO) was purchased from Merck Schuchardt (Hohenbrunn, Germany). Antibiotic-antimycotic mixture was purchased from Gibco (Invitrogen Corporation, NY). Paraformaldehyde was purchased from Thermo Scientific, USA.

2.2. Primary Preadipocyte Maintenance and Differentiation Procedure. Preadipocytes (human subcutaneous) were purchased from ZenBio, Inc. (Research Triangle Park, NC). Cells were cultured under standard conditions (at 37°C, humidified atmosphere, 5% CO_2) in PM medium. For each set of experiments, cells were seeded on coverslips in uniform density, which was provided by counting cells before seeding. For differentiation into adipocytes we used the protocol from ZenBio. The start of the differentiating procedure was marked as day 0. On indicated days the 16 h conditioned 1% BSA/PBS medium was collected, filtered (0.2 μm), and analyzed for enzymatic activity and quantification of sDPP4. On day 21 60–70% of cells were fully differentiated, indicated by the accumulation of lipid droplets (not shown). The cells were than subjected to immunolabeling protocol or trypsinized and counted using a hemocytometer (improved Neubauer type). Three separate samples were prepared for each time point.

2.3. Hypoxia Treatment In Vitro. For the study of DPP4 expression under hypoxic conditions, preadipocytes were cultured in a hypoxic chamber (Billups-Rothenberg, Dell Mar, CA), flushed twice at a 2 h interval for 4 min with a gas mixture consisting of 1% O_2, 5% CO_2, and 94% N_2, and incubated for the indicated time at 37°C in a humidified atmosphere.

2.4. Immunocytochemistry. To identify the expression of HIF-1α and DPP4 in the preadipocytes, primary monoclonal mouse anti-HIF-1α antibodies and primary monoclonal mouse anti-DPP4 antibodies (both Abcam, Cambridge, UK; ab8366 and ab3154) were used and secondary goat anti-mouse antibody conjugated to Alexa Fluor 546 and to Alexa Fluor 488, respectively (A11003 and A11001, Molecular Probes). Cells were washed with PBS and fixed in 2% paraformaldehyde for 20 min, which was sufficient to permeabilise the cell membrane and allow binding of antibody to a total cell protein. Cells were incubated at 37°C in blocking buffer (3% BSA, 10% goat serum in PBS) for 1 h, with primary antibodies for 2 h and with secondary antibodies for 45 min. Subsequently, they were mounted using a Light Antifade kit (Invitrogen).

2.5. Confocal Microscopy. Z-stacks of immunolabeled cells were acquired using a Zeiss LSM 510 confocal microscope through a Plan Apochromatic oil-immersion objective (63x, NA = 1.4), excited by the 488 nm argon laser line and filtered with the 505–560 nm low-pass emission filter and excited by the 543 nm He/Ne laser line and filtered with the 560 nm low-pass emission filter. Images were analyzed quantitatively using LSM 510 software (Carl Zeiss). Eight to 15 markers were manually set to the cell perimeter, and the software interpolated the curve between them. The area above the threshold (20% of the maximal fluorescence intensity) fluorescence intensity relative to the cell cross-sectional area was determined.

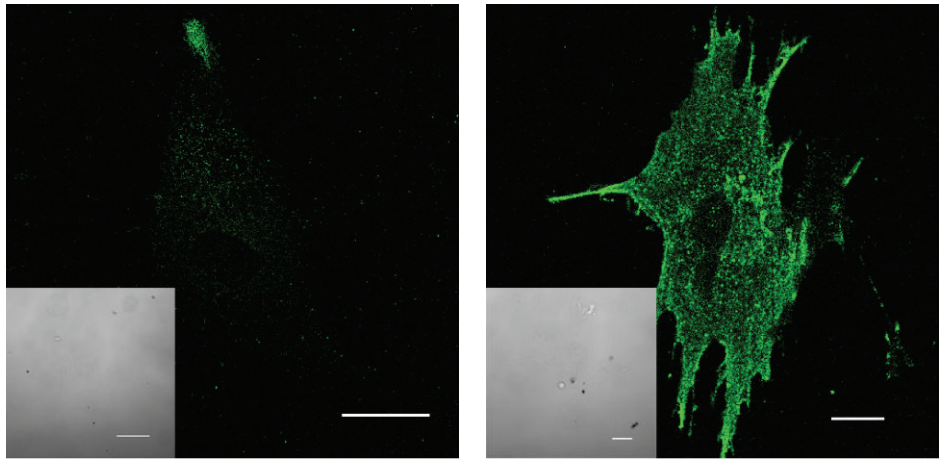
2.6. Enzymatic Activity. Peptidase activity of the sDPP4 released from nonpermeabilised cells from the cell surface was determined using the DPPIV/CD26 assay kit for biological samples (Enzo Life Sciences, Plymouth Meeting, PA) according to the manufacturer instructions. The relative fluorescence units for each sample were calculated by plotting the linear region of the change in fluorescence over time and calculating the slope of the line. This was then used with the conversion factor to calculate the activity expressed as pmol/min and divided by the number of cells in individual samples to obtain the values expressed as pmol/min/cell. Data are presented as means ± s.e.m. of all tests ($n = 9$).

2.7. ELISA. The amount of sDPP4 released by the cell at different stages of differentiation and at different oxygenation of the cell atmosphere was quantified by a Human DPPIV/CD26 Quantikine ELISA kit (R&D Systems, Minneapolis MN) following the manufacturer's recommendations.

3. Results

3.1. Hypoxia-Mediated Reduction in DPP4 Expression in Single hPA. hPA were cultured in a normoxic chamber (18% pO_2) and in a hypoxic chamber (1% pO_2). To confirm that these hPA responded to hypoxia, cells were immunolabeled with antibodies against hypoxia-inducible factor-1α (HIF1-α). The results show that exposure to 1% pO_2 induced a massive expression of transcriptional factor HIF1-α (Figure 1(b)) and are consistent with previously published data [17]. In cells cultured in 18% pO_2, the expression of HIF1-α was negligible (Figure 1(a)). Thus hPA incubated under hypoxic conditions responded physiologically to the lowered pO_2.

To examine the expression of DPP4 in a hypoxic environment *in vitro* and to get insights into short and long term effects of hypoxia, cells were incubated at 1% pO_2 for 2 and 9 days and labeled with the anti-DPP4 antibody. The images of the largest optical slice of hPA (Figure 2(a)) were analyzed by determining the percent area of DPP4-labelled



(a) (b)

Figure 1: Expression of HIF-1α, a hypoxia marker, in human preadipocytes cultured *in vitro*. Confocal images and transmission light images (insets) of human preadipocytes immunolabeled with antibody against HIF-1α. Preadipocytes were cultured for 2 days in normoxic conditions (a, 18% O_2) or hypoxic conditions (b, 1% O_2). Cells were fixed and immunolabeled with an antibody against HIF-1α. Inserts show transmission light microscopy images of the same cells as in the confocal fluorescent images. Scale bars indicate 20 μm.

part relative to the area of the entire optical slice of a cell. Note that the staining pattern of cells in Figure 2(a) is evenly distributed through the entire cell area. The 2-day exposure to hypoxia significantly reduced the expression density of DPP4 to 25.7 ± 2.7% compared with cells in normoxia (37.0 ± 2.9%; $P < 0.001$; Figure 2(b)). Prolonged incubation (9 days) had no further effect on DPP4 expression density in a hypoxic (28.3 ± 2.5%) or normoxic environment (41.8 ± 2.7%).

3.2. Time-Dependent Increase in the Protease Activity of sDPP4 Is Reduced by Hypoxia. DPP4 is a transmembrane protein; however, it is also active in its soluble form, after shedding the extracellular domain of the protein from the cell's surface. We investigated the protease activity of sDPP4 in conditioned medium of cells incubated in different oxygenation environments. Significant differences were detected between samples incubated in normoxia for 2 versus 9 days (Figure 2(c)). After 2 days, the activity of DPP4 was 2.0 ± 0.6 fmol/min/cell, increasing to 4.4 ± 0.7 fmol/min/cell after 9 days ($P < 0.05$). In samples incubated in hypoxia, the activity also appeared to increase from day 2 to day 9, from 0.9 ± 0.2 fmol/min/cell to 2.1 ± 0.3 fmol/min/cell ($P = 0.08$). In hypoxia on day 2, the DPP4 activity was similar to that in normoxia. However, in the 9-day samples, the activity of DPP4 significantly decreased with hypoxia ($P < 0.05$). We conclude that although the amount of DPP4 protein that is shed from the cell surface of hPA is insensitive to 2 or 9 days of culture under normoxic and hypoxic conditions, we detected a significant time-dependent increase in DPP4 protease activity in normoxic controls; the increase was relatively reduced by hypoxia.

3.3. Differentiation-Mediated Decrease in DPP4 Expression in Adipocytes Is Potentiated by Hypoxia. We studied the influence of the state of differentiation of adipocytes on the DPP4 expression pattern. In normoxia DPP4 expression is variable but tends to decline as a function of time (Figure 3(a)), especially at the induction of differentiation, and is barely detectable on mature adipocytes on day 21 (Figure 3(a)). In hypoxia (Figure 3(b)), the expression of DPP4 also decreased continuously. Comparison of differentiation-dependent DPP4 expression between both oxygenation conditions revealed that, with the exception of day 21, DPP4 expression density is significantly lower under hypoxic conditions at all stages of differentiation ($P < 0.05$), consistent with data in Figure 2.

We also studied the concentration of sDPP4 in conditioned medium of cells, which tended to decrease as a function of differentiation (Figure 4(a)). With only a few exceptions (see # on Figure 4(a)), the concentration of sDPP4 seemed to decrease at a similar rate under normoxic and hypoxic conditions during differentiation.

Investigation of the protease activity of sDPP4 in the culture medium revealed that as in Figure 4(a) a similar trend of decrease during differentiation was found (Figure 4(b)). To confirm that the protease activity of sDPP4 is significantly lower under hypoxic versus normoxic conditions, we correlated the concentration of sDPP4 protein and its protease activity in normoxic and hypoxic conditions (Figure 4(c)). The slopes of the regression lines are significantly different, indicating that hypoxic conditions significantly enhance the differentiation-dependent reduction of sDPP4 protease activity, relative to the protein content of DPP4.

3.4. Insulin Enhances the Shedding and Protease Activity of sDPP4 in Normoxic and Hypoxic Preadipocytes. Preadipocytes incubated for 2 days at 18% and 1% pO_2 were stimulated with insulin (100 nM) for 30 min. In normoxia, insulin significantly increased DPP4 expression in preadipocytes (42.2 ± 2.5%; Figure 5(a)) versus stimulation with vehicle only (31.3 ± 4.7%; $P < 0.05$). A similar increase was observed

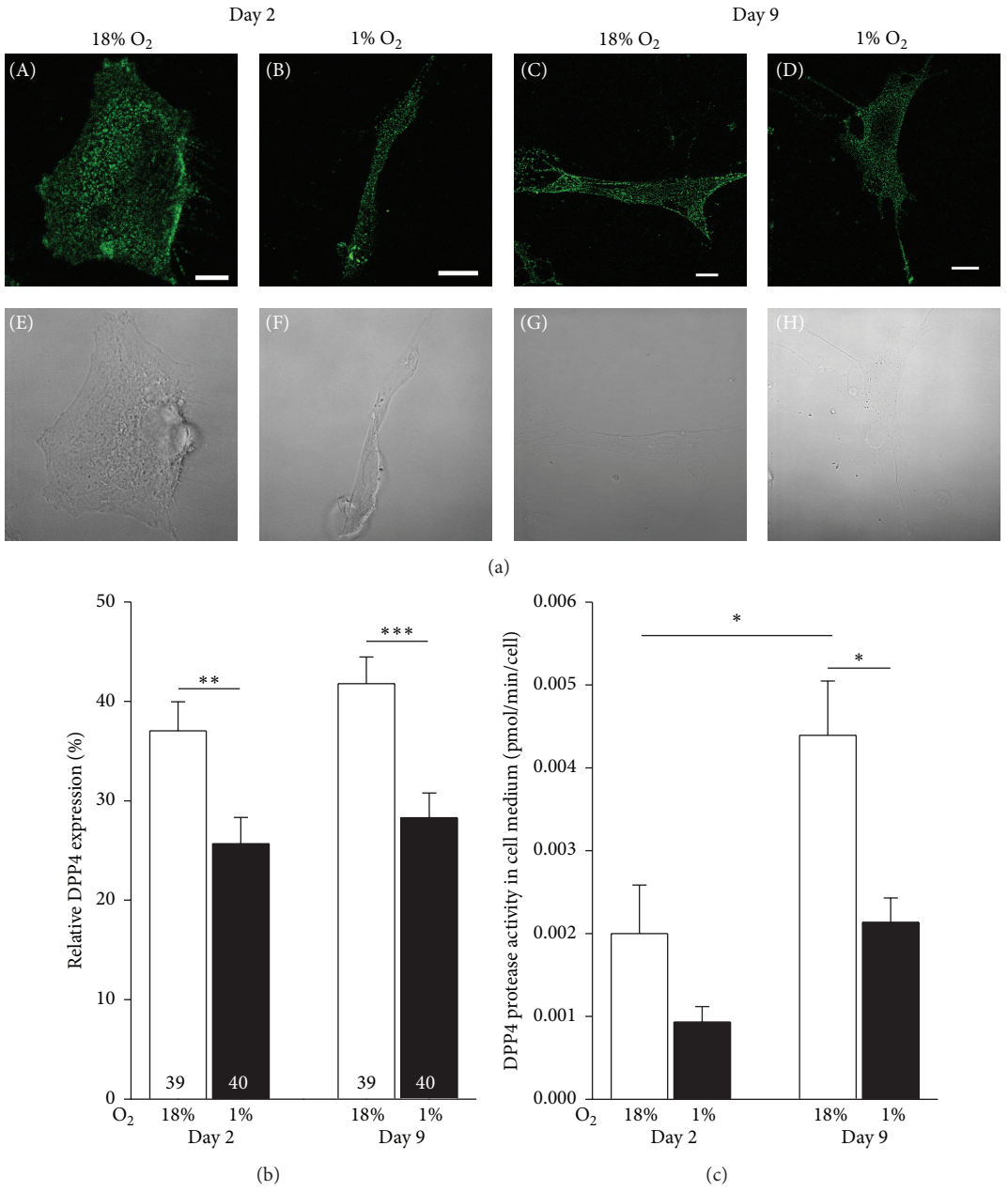


Figure 2: Oxygenation-dependent expression density and shedding of DPP4 in human preadipocytes cultured *in vitro*. (a) Confocal images (upper panels) and transmission light microscopy images (lower panels) of human preadipocytes (subconfluent cultures) immunolabeled with antibody against DPP4. Each panel shows representative confocal images of a cell incubated for 2 days (A, B, E, and F) and 9 days (C, D, G, and H) under normoxic (A, C, E, and G) and hypoxic (B, D, F, and H) environmental conditions. Scale bars indicate 10 μm. (b) Relative expression density of DPP4 in human preadipocytes cultured for 2 and 9 days in normoxic or hypoxic conditions. Numbers denote the number of cells imaged. (c) Normalized DPP4 protease activity in conditioned medium. Asterisks denote statistically significant difference ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, two-way ANOVA).

in hypoxia; insulin significantly increased DPP4 expression (41.9 ± 2.7%) versus controls (23.2 ± 4.2%; $P < 0.001$).

To study shedding, we also examined the concentration of sDPP4 in the conditioned medium (Figure 5(b)) of cells stimulated with insulin. Hypoxia did not influence the sDPP4 concentration in controls or insulin-stimulated cells. However, insulin treatment significantly increased DPP4 protein shedding in normoxia ($P < 0.001$) and in hypoxia ($P < 0.01$). Under both oxygenation conditions, we recorded a pronounced effect of insulin treatment on sDPP4 protease activity in the culture medium (Figure 5(c)). In normoxia, insulin significantly increased protease activity (8.0 ± 1.4 fmol/min/cell) compared to nonstimulated cells (2.0 ± 0.6 fmol/min/cell; $P < 0.05$). In hypoxia, insulin stimulation also increased the activity of shed protein (13.1 ± 3.0 fmol/min/cell) compared to controls (0.9 ±

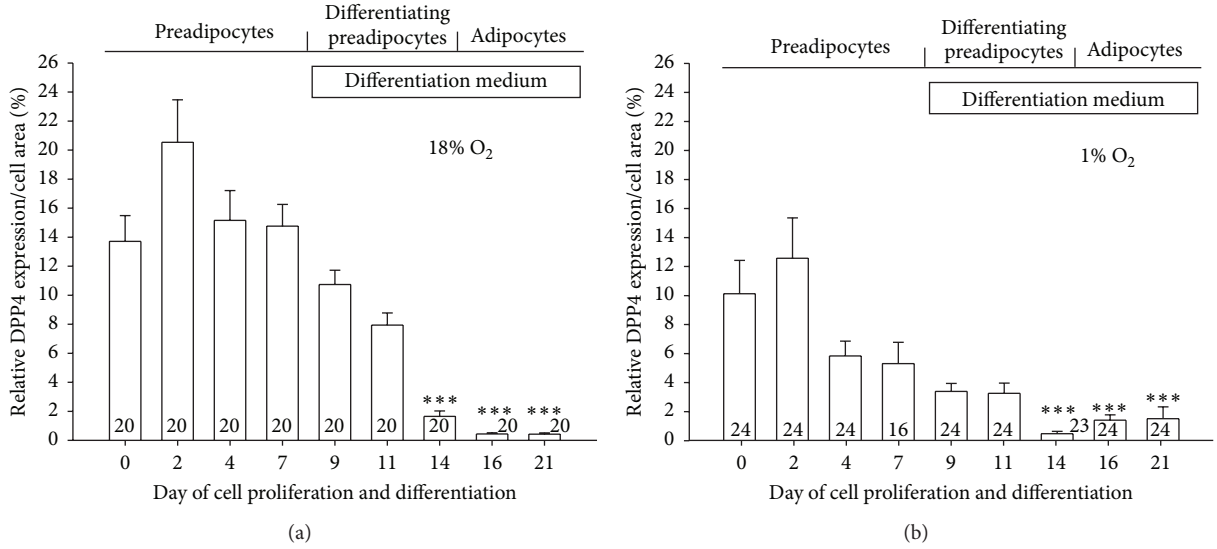


Figure 3: Differentiation-dependent expression density of membrane DPP4 of human preadipocytes and adipocytes cultured *in vitro* under different environmental conditions. Relative expression density of DPP4 in human preadipocytes in the course of differentiation to mature adipocytes cultured in normoxia (a) and hypoxia (b). Data are presented as mean values ± SE. Numbers denote the number of cells imaged ($^{***}P \leq 0.001$ versus preadipocytes on day 0; one-way ANOVA on ranks). On day 0, cells were already confluent.

0.2 fmol/min/cell; $P < 0.001$). These results indicate that hypoxic conditions increase insulin-mediated expression, shedding, and protease activity of DPP4 in preadipocytes.

4. Discussion

4.1. Is DPP4 a Marker for Differentiation Status of Adipocytes? DPP4 is abundantly expressed in hPA, while in mature adipocytes we found very modest, if any, expression of DPP4 (Figure 3). The significantly lower expression of DPP4 in mature adipocytes could not be explained with differences in cell size between both stages of differentiation, since the difference is not statistically significant (not shown). These results suggest that during differentiation from hPA into adipocytes cells gradually suppress the DPP4 expression. If this reduction in DPP4 in hPA is associated with differentiation into adipocytes *in vivo*, then DPP4 may well represent a marker for differentiation status. Therefore, like Pref-1, a stemness marker for preadipocytes, DPP4 is also robustly expressed in hPA: the higher the expression of DPP4 in preadipocytes, the higher the stem-like character of these cells. In support of this, DPP4 is abundantly expressed in cultured hPA (Figure 2), and during differentiation DPP4 expression declines (Figure 3). It is unlikely that DPP4 downexpression during differentiation is associated with a response to some of the factors in the differentiation medium added, as the DPP4 downexpression started prior to the addition of differentiation medium (Figures 3 and 4), at a stage when cells reached confluence. Although these results contrast with the report where cancer cells were studied by Abe et al. [18], the most probable explanation for the reduction of DPP4 in the (pre)adipocytes is the cell-to-cell contact-induced differentiation process into mature adipocytes that contain very modest amounts of DPP4.

DPP4 was found to be expressed in a variety of cell types [19, 20], from which it is also shed and sDPP4 may influence the shedding of membrane bound DPP4 from preadipocytes that were investigated in this study.

Current experiments revealed that the stage of differentiation of hPA into adipocytes can be assessed by monitoring the DPP4 density in cells, which can be considered as a developmental or differentiation marker, reporting the relatively undifferentiated stage of these cells. Consistent with this, it has recently been demonstrated that hypoxia inhibits adipogenesis through the HIF1α pathway [21, 22]. Hypoxia arrests preadipocytes in an undifferentiated state, thereby maintaining their stemness [23, 24].

The reduction in DPP4 in single differentiating cells is further supported by the determination of the concentration of sDPP4 and its enzymatic activity. Both parameters exhibit a differentiation-dependent decrease (Figures 4(a) and 4(b)) and this is consistent with the view that the soluble protein arises from the shedding of DPP4 from the plasma membrane. DPP4 has been shown to move from the cytoplasm to the cell surface rapidly and consequently the protein amount on the cell membrane is steadily proportional to the total cell protein [25]. The correlation between the concentration of sDPP4 and its activity (Figure 4(c)) shows that in hypoxia the activity is reduced by almost a factor of three relative to the concentration of soluble protein. One possible explanation would be that hypoxia lowers pH of cells. DPP4 enzymatic activity is pH dependent with the optimum at 7.8 [26]. A lower pH in hypoxic conditions would result in lower DPP4 activity. However, the activity assay was performed with buffered solution maintaining constant pH during the assay; therefore we can exclude the pH influence at the time of measurements of enzymatic activity. Although pH was not quantitatively measured during hypoxia it was observed qualitatively with pH indicator in the cell medium with no obvious changes during incubation in hypoxia, indicating that pH of the cell medium did not change significantly.

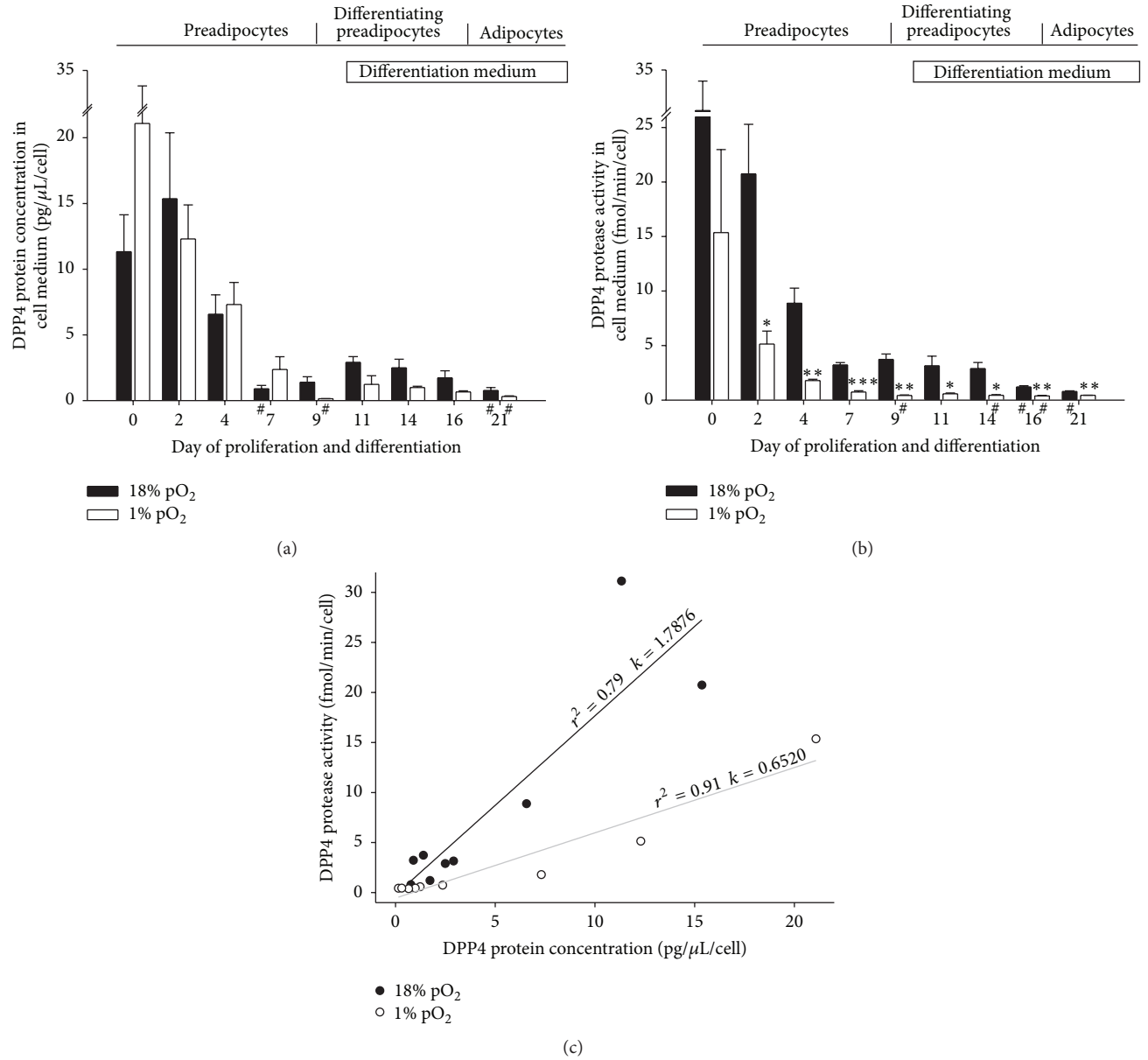


Figure 4: Differentiation and hypoxia reduce the shedding of DPP4 from human preadipocytes cultured *in vitro*. (a) Normalized DPP4 protein concentration in conditioned medium of cells at different stages of differentiation from preadipocytes to mature adipocytes cultured under normoxic (black bars) and hypoxic (white bars) environmental conditions. (b) Normalized DPP4 protease activity in conditioned medium of cells at different stages of differentiation from human preadipocytes to mature adipocytes cultured in normoxic (black bars) and hypoxic (white bars) environmental conditions. Data are normalized to the total cell number in the sample and presented as mean values ± SE. Asterisks above the white bars denote significant difference between hypoxic versus normoxic condition on the same differentiation day (Student's t-test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$); # under the bars denotes significant difference compared with day 0 for respective oxygenation condition (one-way ANOVA; $^{\#}P \leq 0.01$). (c) Correlation between the concentration of DPP4 in the conditioned media and its activity under normoxic (black dots) and hypoxic (open dots) conditions (k, slope coefficient; r^2, correlation coefficient). The equation of regression lines is as follows: DPP4 activity [fmol/min/cell] = (1.79 ± 0.35) × DPP4 concentration [pg/μL/cell] − (0.2 ± 2.4) [fmol/min/cell] for the correlation in normoxia and DPP4 activity [fmol/min/cell] = (0.65 ± 0.08) × DPP4 concentration [pg/μL/cell] − (0.6 ± 0.7) [fmol/min/cell] for the correlation in hypoxia. The slopes are significantly different ($P < 0.001$), whereas the intercepts are similar and not different from 0.

Hypoxia-related increase of preadipocyte stemness could be an additional mechanism in the development of insulin resistance. The results imply that in obesity-related hypoxia DPP4 is abundantly expressed, contributing to the reduction in insulin activity and thereby to the onset of insulin resistance in these subjects.

4.2. Insulin Increases DPP4 Expression Density in hPA. Most gastrointestinal (GI) hormones involved in satiety regulation and glucose metabolism, especially through regulation of insulin secretion, are hydrolyzed and inactivated by DPP4 [27]. Thus DPP4 action reduces the amount of insulin secretion. This mechanism of action is well exploited by

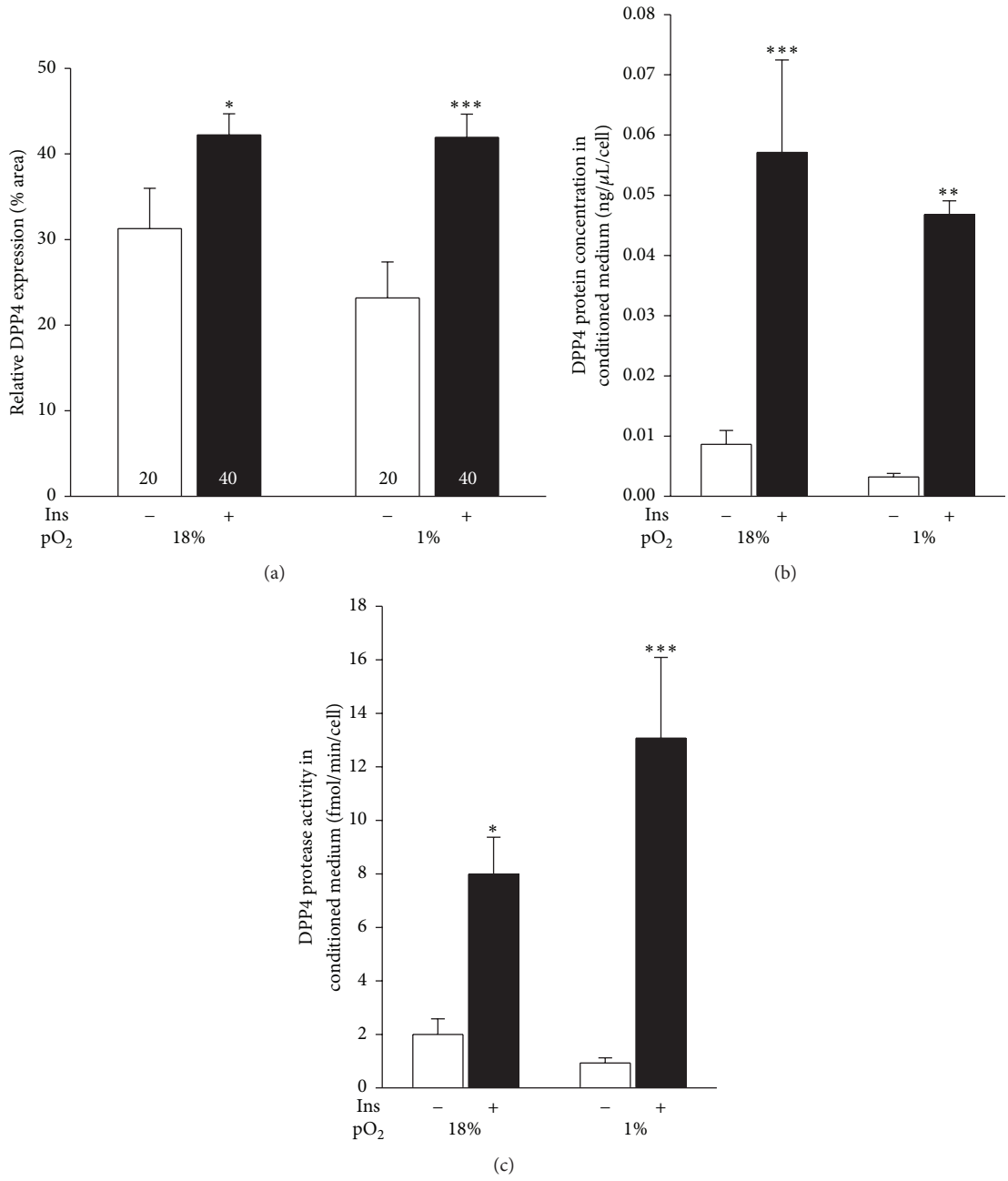


Figure 5: The effect of insulin on DPP4 expression and activity in human preadipocytes. (a) Human preadipocytes cultured for 2 days under normoxic (18% pO_2) and hypoxic (1% pO_2) conditions were treated for 30 min with 100 nM insulin (black bars). Control cells were treated with vehicle only for 30 min (white bars). Subsequently, the cells were immunolabeled with an antibody against DPP4 and imaged on a confocal microscope. The expression density of immunolabeled DPP4 was analyzed. Data are presented as means ± SE. Numbers denote the number of cells analyzed. The conditioned media were collected from cells treated for 30 min with insulin (Ins) and the protein concentration (b) and protease activity (c) of DPP4 were analyzed. Data are normalized to the number of cells in the sample and presented as means ± SE. Asterisks denote statistically significant differences compared with controls treated with vehicle only, as denoted ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ANOVA).

the development of a class of antidiabetic drugs that inhibit DPP4. But little is known about how insulin influences the expression of DPP4. The results of this study demonstrate that at single-cell level insulin significantly increases DPP4 expression in hPA in a rapid manner (30 min) by about 35%. Under hypoxic conditions, this increase was 63% relative to control (Figure 5(a)). These results indicate that DPP4 is also regulated by insulin but unlike hypoxia, which renders DPP4 expression, the rapid effect of insulin is most likely posttranscriptional.

Insulin induces DPP4 expression on cells which in turn deactivated GI hormones, thereby inhibiting the stimulation

of insulin secretion. In agreement with this, incubation of cells with insulin for 5 and 24 h resulted in decreased DPP4 expression to baseline level (not shown). This is probably due to insulin receptor internalization and deactivation of insulin signaling. As expected, sDPP4 in culture medium and its protease activity also significantly increased after 30 min of insulin action in both oxygenation environments.

5. Conclusions

This study shows that human preadipocytes express DPP4 abundantly and this expression decreases in the course of differentiation into mature adipocytes. Therefore, DPP4 can be considered a differentiation marker highlighting the stemness properties of preadipocytes. The strong inhibition of DPP4 protease activity by hypoxia and the insulin-mediated increase in DPP4 indicate that DPP4 represents an important marker for early detection of insulin resistance.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Free Fatty Acids Activate Renin-Angiotensin System in 3T3-L1 Adipocytes through Nuclear Factor-kappa B Pathway

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The activity of a local renin-angiotensin system (RAS) in the adipose tissue is closely associated with obesity-related diseases. However, the mechanism of RAS activation in adipose tissue is still unknown. In the current study, we found that palmitic acid (PA), one kind of free fatty acid, induced the activity of RAS in 3T3-L1 adipocytes. In the presence of fetuin A (Fet A), PA upregulated the expression of angiotensinogen (AGT) and angiotensin type 1 receptor (AT_1R) and stimulated the secretion of angiotensin II (ANG II) in 3T3-L1 adipocytes. Moreover, the activation of RAS in 3T3-L1 adipocytes was blocked when we blocked Toll-like receptor 4 (TLR4) signaling pathway using TAK242 or NF-κB signaling pathway using BAY117082. Together, our results have identified critical molecular mechanisms linking PA/TLR4/NF-κB signaling pathway to the activity of the local renin-angiotensin system in adipose tissue.

1. Introduction

Activation of the renin-angiotensin system (RAS) is instrumental in regulating blood pressure and fluid balance. RAS activation is also associated with impaired differentiation of preadipocytes [1] and increased lipolysis and enhanced oxidative stress and inflammatory response [2–8]. Defects in the system are associated with obesity, type 2 diabetes, and cardiovascular diseases. RAS are found in a number of tissues, including kidneys, heart, and nervous and immune systems. Components of RAS, including renin, angiotensinogen (AGT), angiotensin-converting enzyme (ACE), and angiotensins I, II, and III (ANG I, ANG II, and ANG III), have also been found in adipose tissue [9, 10]. It is well established that free fatty acids (FFAs) are activators of RAS in leukocytes [11, 12]. However, whether FFAs play a role in the activation of RAS in adipocytes is unclear. It has been shown that the levels of FFAs originating from lipolysis in adipocytes are significantly increased in peripheral circulation as well as local tissues in obese humans and animals [13, 14]. It has been hypothesized that RAS could regulate adipocyte differentiation through Ang II and the adipocyte AT_1R in mice [15]. Therefore, FFAs may directly regulate RAS activation in adipose tissue which might be a trigging mechanism of glucose and lipid metabolism disorder and obesity-related diseases. FFA components such as palmitic acid (PA) and lauric acid can bind to Toll-like receptor 4 (TLR4) with the assistance of the endogenous ligand, fetuin A (Fet A), thus mediating the activation of TLR4 and NF-κB pathways and leading to the inflammatory cascade [14]. TLR4 is a member of the family of Toll-like receptors (TLRs), which can activate mitogen-activated protein kinase and nuclear factor

κB (NF-κB) to regulate inflammatory and immune responses after binding to ligands [16]. Moreover, a recent study has demonstrated that active TLR4 can induce the activation of RAS in hepatocytes [17] and cardiac muscle cells [18]. Therefore, we hypothesize that palmitic acid (PA) triggers the TLR4 signaling pathway, leading to RAS activation in adipocytes.

2. Material and Methods

2.1. Reagents. We purchased 3T3-L1 preadipocyte line from ATCC (CL-173); Dulbecco's Modified Eagle Medium (DMEM) with 25 mM D-glucose from HyClone (USA); Dexamethasone (DXM), Isobutylmethylxanthine (IBMX), 4% paraformaldehyde, Oil Red O, Irbesartan, and Captopril from Sigma (Sigma-Aldrich, St. Louis, USA); dimethyl sulfoxide (DMSO) from Invitrogen (USA); Ang II Enzyme-linked immunosorbent assay kit from Cusabio (Wuhan, China); Anti-TLR4 antibody from Abnova (Taiwan, China); anti-AGT antibody from Merck (Merck Millipore, Darmstadt, Germany); anti-AT_1R antibody and anti-GAPDH antibody from Santa Cruz (CA, USA); Anti-α-tubulin antibody from Cell Signaling Technology (Beverly, MA, USA); and horseradish peroxidase-linked goat-anti-rabbit antibody from KPL (Gaithersburg, MD, USA).

2.2. Cell Culture, Differentiation, and Identification. 3T3-L1 preadipocytes were subcultured with 25 mM D-glucose DMEM. Two days after confluence, the cells were differentiated to adipocytes using the same medium containing 10% fetal bovine serum (FBS, HyClone), supplemented with 10 μg/mL insulin, 1 μM DXM, and 0.5 mM IBMX. This medium was replaced with a fresh medium containing insulin 48 hours later, after which the medium was replaced every other day. Approximately 90%–95% of cells differentiated into mature adipocytes on days 8–10 of culture, which were used in the experiments. After the 3T3-L1 preadipocytes were completely differentiated, 4% paraformaldehyde was added to the culture dish and maintained for 10 min, stained with Oil Red O staining for 30 min, followed by stain extraction and observation using a microscope.

2.3. Preparation of Palmitic Acid. Palmitic acid (PA) and bovine serum albumin (BSA) were purchased from Sigma (Sigma-Aldrich, St. Louis, USA). PA were dissolved completely in 0.1 M NaOH at 70°C and then complexed with 9.5 mL 10% BSA at 55°C for 10 min such that a final PA concentration of 5 mM was achieved. Stock solutions were stored at 4°C after filtration or diluted with DMEM to one-tenth (500 μM PA) or one-twentieth (250 μM PA) that were prepared fresh before experiments.

2.4. Enzyme-Linked Immunosorbent Assay. 3T3-L1 adipocytes were treated, respectively, with DMEM, DMEM + 0.1% BSA, DMEM + 0.1% BSA + 10 μg/mL Fet A, or DMEM + 0.1% BSA + 500 μM PA for 3 hours or treated in 500 μM PA + Fet A condition for 1, 3, or 5 hours. In other experiments, 3T3-L1 adipocytes were treated with 250 μM PA + 10 μg/mL Fet A or 500 μM PA + 10 μg/mL Fet A in the same vehicle, containing DMEM and 0.1% BSA, for 3 hours. The supernatants were collected and the concentrations of ANG II were determined by double antibody sandwich method, using Ang II *Enzyme-linked immunosorbent assay* kit. The OD values were measured by a microplate reader, and then ANG II concentration was calculated.

2.5. Quantitative RT-PCR. Amplification and detection of RNA were performed in an ABI Prism 7300 Sequence Detection System using SYBR Green (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Primers for quantitative RT-PCR were designed based on sequences from the GenBank, as follows. The relative mRNA expression level was calculated using the comparative expression level $2^{-\Delta\Delta CT}$ method:

TLR4: F: 5′-GCATCATCTTCATTGTCCTTGA-3′,
R: 5′-CTTGTTCTTCCTCTGCTGTTTG-3′;
AGT: F: 5′-CCTTCCATCTCCTTTACCACAA-3′,
R: 5′-GCAGGGTCTTCTCATTCACAG-3′;
AT_1R: F: 5′-TGCCATGCCCATAACCATCTG-3′,
R: 5′-CGTGCTCATTTTCGTAGACAGG-3′;
GAPDH: F: 5′-GGAAGCCCATCACCATCTT-3′,
R: 5′-GGTTCACACCCATCACAAACAT- 3′.

2.6. Western Blotting Analysis. Protein extract was separated on a 15% SDS-polyacrylamide gel and electrophoretically transferred onto a PDVF membrane (Millipore, Etten-Leur, The Netherlands). Membranes were blocked overnight with 5% nonfat dried milk and incubated for 2 h after washing with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20), and the membranes were incubated for 1 h with horseradish peroxidase-linked goat-anti-rabbit antibody. The membranes were washed again with TBST, and the proteins were visualized using ECL chemiluminescence.

2.7. Immunofluorescence Double Staining. 3T3-L1 adipocytes were treated with 500 μM PA after pretreatment with two kinds of RAS blocking agents, respectively; one is an angiotensin receptor blocking agent (ARB), Irbesartan, with 10 μM concentration; the other is an inhibitor of ACE (ACEI), Captopril, with 10 μM concentration. Cells were then fixed by paraformaldehyde after the supernatant was removed. FITC + DAPI double staining method was used to detect nuclear translocation of the p65 subunit of NF-κB.

2.8. Statistical Analysis. All statistical analyses were performed using SPSS version 16.0 software. Results were presented as means ± SD. Student's t-test was used to compare the means between two samples and statistical comparisons of more than two groups were performed using one-way analysis of variance (ANOVA); post hoc tests were performed using LSD test or Tamhane's T2 test. P values < 0.05 were considered statistically significant.

3. Results

3.1. Combined Fet A and PA Upregulated the Expressions of AGT, AT_1R, and TLR4 and Stimulated the Secretion of ANG II in 3T3-L1 Adipocytes. To investigate whether the involvement of Fet A has an effect on the components of RAS

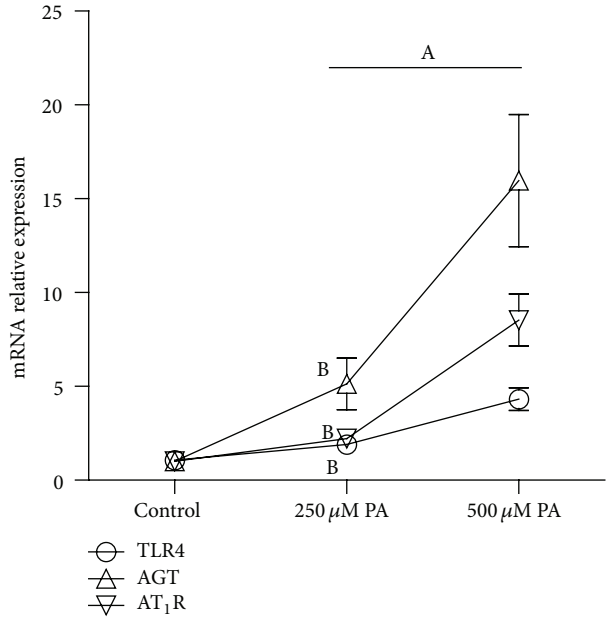

Figure 1: mRNA expression of TLR4, AGT, and AT_1R in 3T3-L1 adipocytes induced by PA. Data are presented as mean ± SD. (A) $P < 0.05$ versus control, (B) $P < 0.05$ versus 500 μM PA ($\overline{x} \pm s$, $n = 9$).

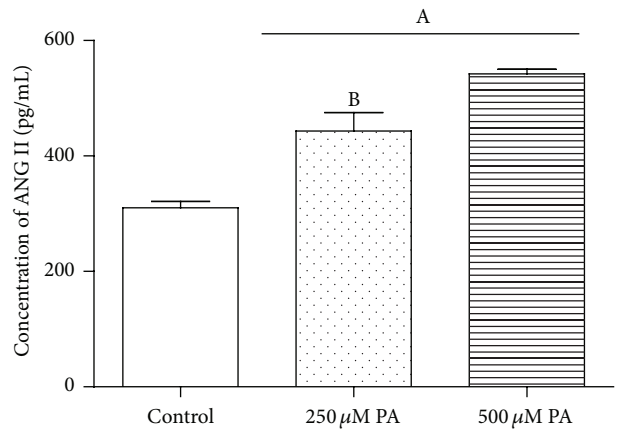

Figure 2: Concentrations of ANG II secreted by 3T3-L1 adipocytes after being treated by different concentrations of PA. Data are presented as mean ± SD. (A) $P < 0.05$ versus control, (B) $P < 0.05$ versus 500 μM PA ($\overline{x} \pm s$, $n = 6$).

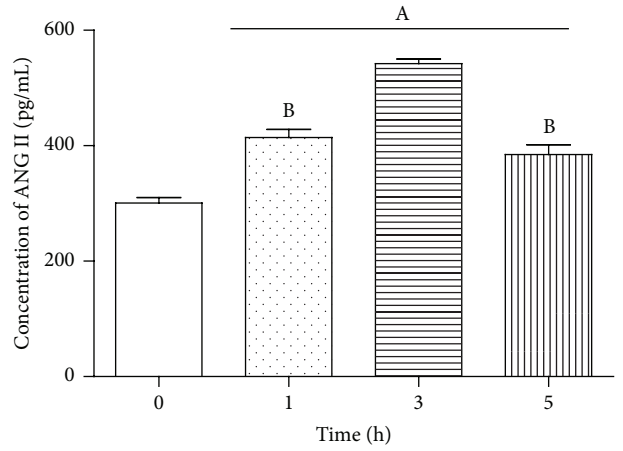

Figure 3: Concentrations of ANG II secreted by 3T3-L1 adipocytes after being treated with PA + Fet A at different time. Data are presented as mean ± SD. (A) $P < 0.05$ versus control, (B) $P < 0.05$ versus 3 h ($\overline{x} \pm s$, $n = 6$).

induced by PA in adipocytes, we conducted the following experiments. 3T3-L1 adipocytes were treated with DMEM, DMEM + 0.1% BSA, DMEM + 0.1% BSA + 10 μg/mL Fet A, or DMEM + 0.1% BSA + 500 μM PA for 3 hours, respectively. We found that there were no significant differences in mRNA expressions of AGT, AT_1R and secretion of ANG II between groups. In particular, treatment group of PA or Fet A alone has no significant effect in mRNA expressions of RAS components ($P > 0.05$) (data not shown). In contrast, when 3T3-L1 adipocytes were treated with DMEM + 0.1% BSA + 250 μM PA + 10 μg/mL Fet A or DMEM + 0.1% BSA + 500 μM PA + 10 μg/mL Fet A for 3 hours, PA increased the mRNA expressions of TLR4, AGT, and AT_1R (Figure 1) and the secretion of ANG II (Figure 2). We also test the optimal Fet A and PA treatment time for the secretion of ANG II. We found that the optimal time was 3 hours and time longer than 3 hours caused unspecific effect (Figure 3).

3.2. Combination of Fet A + PA Totally Lost the Effect on the Expressions of AGT and AT_1R in the 3T3-L1 Adipocytes When Blocking TLR4 Beforehand. To investigate whether TLR4 is the medium of PA affecting RAS component expression, we pretreated 3T3-L1 adipocytes with 5 μM TLR4 inhibitor-TAK242 for 1 hour and then treated with DMEM + 0.1% BSA or DMEM + 0.1% BSA + 500 μM PA + 10 μg/mL Fet for 3 hours, respectively. Compared with the Fet A + PA alone group, TLR4 inhibitor-TAK242 completely blocked the expressions of AGT and AT_1R in the mRNA level (Figure 4) and protein level (Figure 6).

3.3. Combination of Fet A + PA Only Partly Lost the Effect on Expressions of AGT and AT_1R in the 3T3-L1 Adipocytes When Blocking NF-κB Beforehand. To investigate whether NF-κB is the medium of PA affecting the expression of RAS components, we pretreated 3T3-L1 adipocytes with 1 μM NF-κB inhibitor-BAY117082 for 1 hour and then treated with DMEM + 0.1% BSA or DMEM + 0.1% BSA + 500 μM PA + 10 μg/mL Fet for 3 hours, respectively. Compared with the Fet A + PA alone group, NF-κB inhibitor-BAY117082 only partly blocked the expressions of AGT and AT_1R in the mRNA level (Figure 5) and protein level (Figure 6).

3.4. Combination of Fet A + PA Enabled the Translocation of p65 Subunit of NF-κB to the Nucleus in the 3T3-L1 Adipocytes, and the Effect Was Blocked by RAS Inhibitors. 3T3-L1 adipocytes were treated with DMEM + 0.1% BSA (group 1) or DMEM + 0.1% BSA + 500 μM PA + 10 μg/mL Fet A (group 2) for 3 hours or pretreated with DMEM + 10 μM Irbesartan for 1 hour followed by DMEM + 0.1% BSA + 500 μM PA + 10 μg/mL Fet A for 3 hours (group 3) or pretreated with DMEM + 10 μM Captopril for 1 hour followed

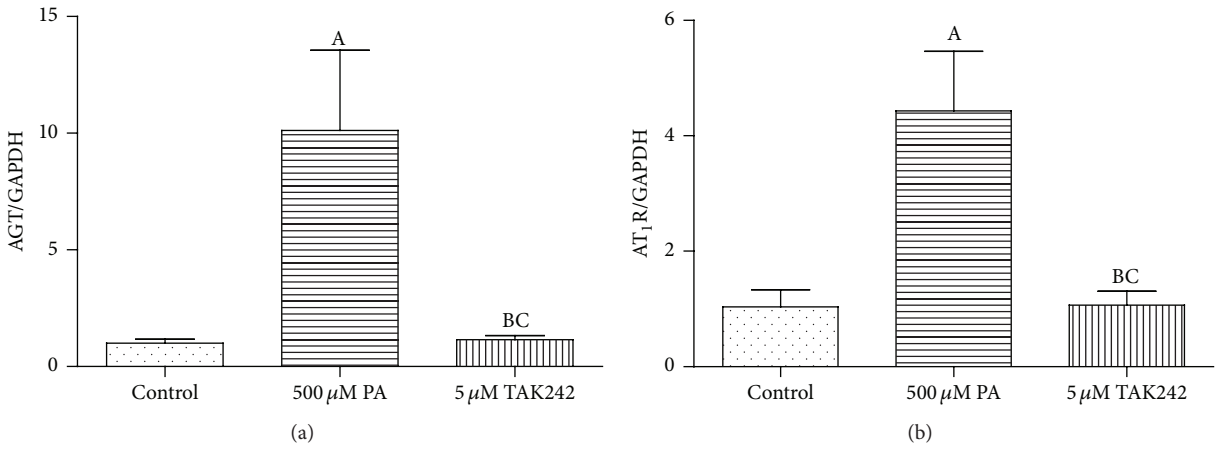

Figure 4: TAK242 pretreatment prevented upregulation of AGT and AT_1R mRNA expressions. Data are presented as mean ± SD. (A) $P < 0.05$ versus control, (B) $P < 0.05$ versus PA group, and (C) $P > 0.05$ versus control ($\overline{x} \pm s$, $n = 9$).

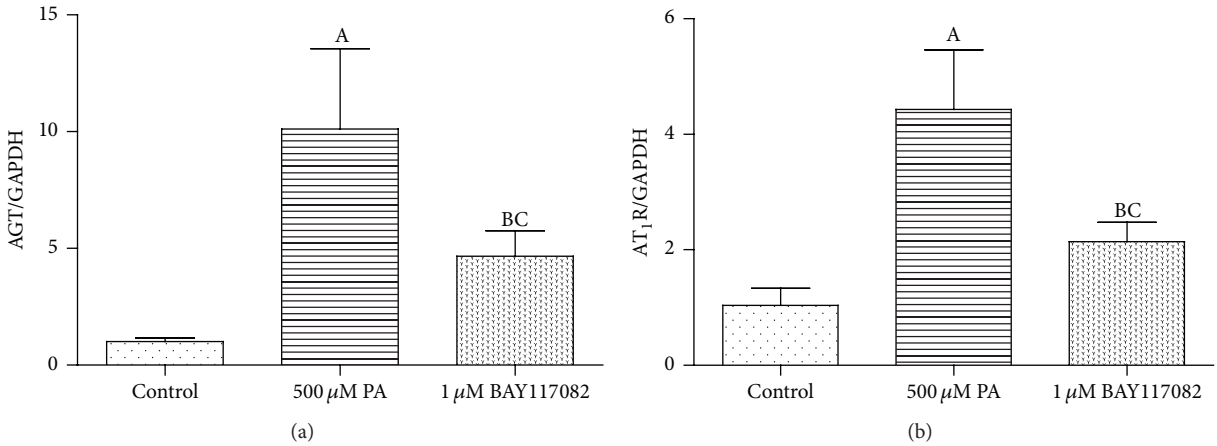

Figure 5: BAY117082 pretreatment partly prevented upregulation of AGT and AT_1R mRNA expressions. Data are presented as mean ± SD. (A) $P < 0.05$ versus control, (B) $P < 0.05$ versus PA group, and (C) $P > 0.05$ versus control ($\overline{x} \pm s$, $n = 9$).

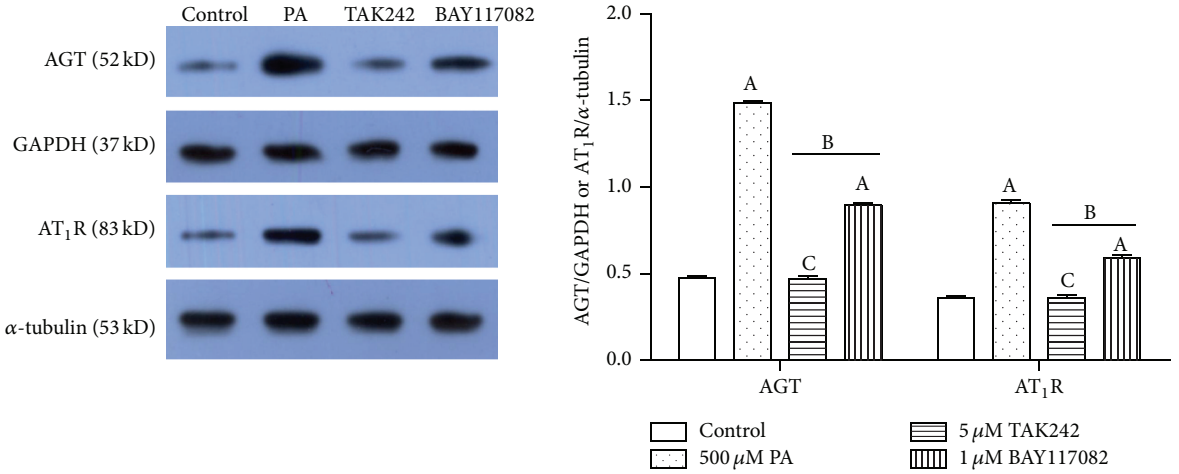

Figure 6: Effects of PA with or without TLR4/NF-κB inhibitors on AGT and AT_1R protein expression in the 3T3-L1 adipocytes. Data are presented as mean ± SD. (A) $P < 0.05$ versus control, (B) $P < 0.05$ versus 500 μM PA, and (C) $P > 0.05$ versus control ($\overline{x} \pm s$, $n = 3$).

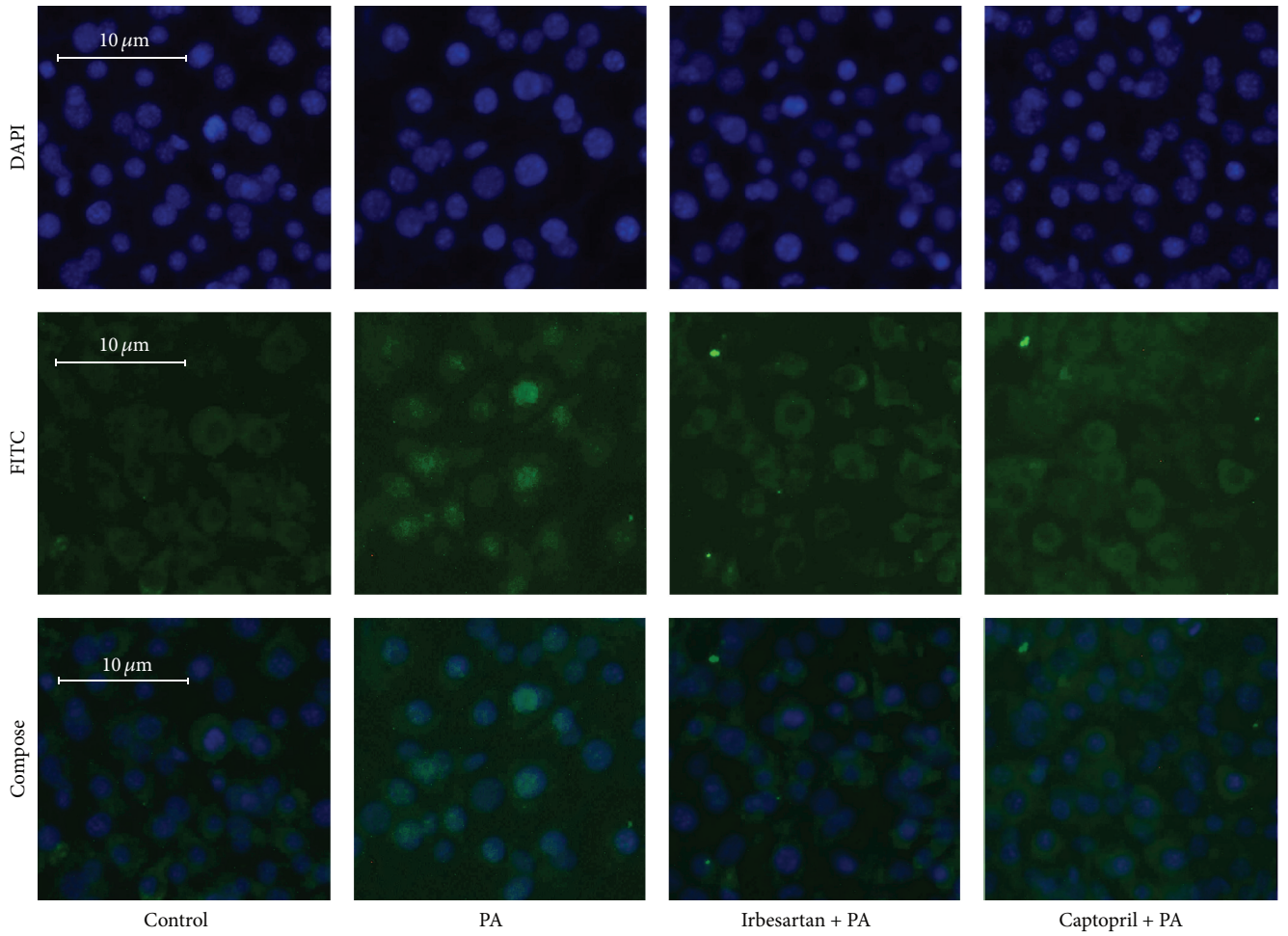

Figure 7: Effect of PA with or without Irbesartan or Captopril pretreatment on NF-κB p65 subunit translocation in 3T3-L1 adipocytes. DAPI: DAPI staining of nucleus; FITC: FITC staining of NF-κB p65 subunit; Compose: composite images of DAPI and FITC.

by DMEM + 0.1% BSA + 500 μM PA + 10 μg/mL Fet A (group 4) for 3 hours. The intensity of green fluorescence of FITC in the nucleus of group 2 was stronger, and the cytoplasm of group 2 was weaker than the other 3 groups. The intensity of green fluorescence of FITC in the nucleus and cytoplasm was almost similar in the control (group 1), Irbesartan pretreatment (group 3), and Captopril pretreatment (group 4) groups (Figure 7).

In summary, we found that PA upregulated the expressions of AGT and AT_1R in both gene and protein level, as well as the gene expression of TLR4 in 3T3-L1 adipocytes. The PA-induced enhancement of AGT resulted in increasing secretion of ANG II, which is believed to be a crucial early step in the development of adipocytes inflammation. Moreover, we found that RAS activation mediated by PA in adipocytes needs to act through TLR4 signaling pathway but not entirely to be dependent on TLR4 downstream NF-κB pathway.

4. Discussion

AGT, the precursor of ANG II, is mainly expressed in adipocytes [19], which is an important component of RAS in adipose tissue. AGT gets converted to ANG II after being catalyzed by the components of RAS-renin and ACE and the increased generation of ANG II responses to upregulation of AGT expression [4]. Therefore, AGT expression is the symbol of local RAS activation [2, 8, 10]. In addition to the elevation of AGT expression, local RAS activation is often accompanied by the increased expression of AT_1R, which is the major ANG II receptor expressed in adipocytes, mediating a series of pathophysiological effects [20]. In current study, we show that PA with Fet A, a liver secretory glycoprotein which exists in blood circulation [21], upregulated the expression of AGT and AT_1R and the secretion ANG II in 3T3-L1 adipocytes. Our results confirm that the activation of RAS in the adipose tissues is mediated by PA.

Next, we try to identify the signaling pathways of activation of local adipose RAS mediated by PA infusion. We found that PA with Fet A induced TLR4 activation in 3T3-L1 adipocytes. To further confirm PA-mediated activation of the adipose RAS through TLR4 signaling pathways, TLR4 blocker TAK242 was used to block TLR4 signal pathway before addition of PA and Fet A. We found that the TLR4 blocker completely prevented elevating expressions of AGT, ANG II, and AT_1R, confirming that activation of adipose RAS depends on the TLR4 signaling pathway.

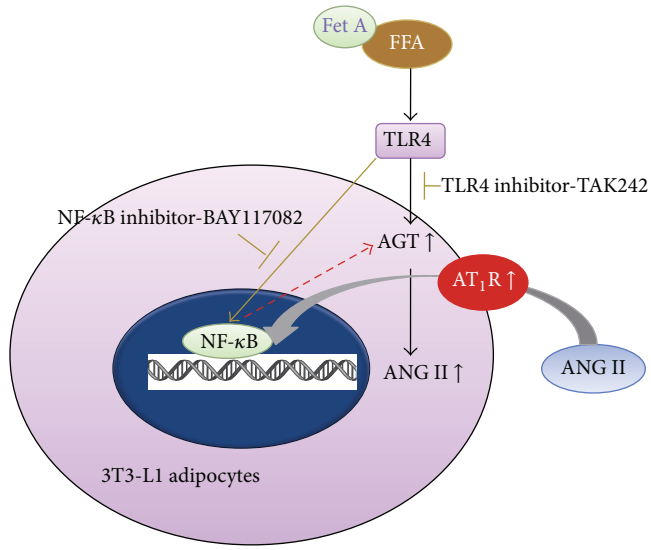

Figure 8: Mechanism of RAS activation induced by FFA (PA) in 3T3-L1 adipocytes.

NF-κB, a nuclear transcription factor [22], also upregulated AGT and AT_1R, the RAS components, in rat vascular smooth muscle cells [23] and preglomerular vascular smooth muscle cells [23]. Previous studies also found that Ang II could activate NF-κB and its downstream inflammatory pathways [20]. To examine whether NF-κB is involved in the activation of adipocyte RAS, we tested the expressions of AGT, ANGII, and AT_1R in PA with Fet A induced RAS activation after using NF-κB inhibitors. We found that PA with Fet A still caused a marginal increase in the expressions of AGT, ANGII, and AT_1R with the pretreatment of NF-κB inhibitors. Together, our results suggest that the activation of adipose RAS completely depends on the TLR4 signaling pathway but only partly depends on NF-κB although NF-κB can be activated by TLR4 signaling pathways.

To further vindicate the PA/TLR4/NF-κB signaling pathway, we examined the effect of PA with Fet A on the nuclear translocation of NF-κB. We found that PA with Fet A stimulated the nuclear translocation of NF-κB P65, resulting in eventually increasing NF-κB activity in 3T3-L1 adipocytes. However, this effect is diminished or partly prevented by ACEI or ARB pretreatment, further indicating that PA-induced activation of adipose RAS is correlated with NF-κB activation.

We present a schematic diagram to explain the pathways of activation of adipose RAS induced by PA/Fed A through TLR4/NF-κB signaling pathway in 3T3-L1 adipocytes (Figure 8). When combining act with Fed A, PA enhances TLR4/NF-κB activity, subsequently upregulating the gene and protein expression of AGT and AT_1R in adipocytes. PA-induced enhancement of AGT results in increasing secretion of ANG II, which is a crucial early step in the development of adipocytes inflammation. Moreover, the adipose RAS activation mediated by PA/TLR4 is not entirely dependent on NF-κB.

Our findings identify a potential mechanism involved in the pathogenesis of obesity-related diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jia Sun and Jinhua Luo contributed equally to this work and they are co-first authors.

Acknowledgments

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