Oxidative Medicine and Cellular Longevity

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Lead Guest Editor: Maria João Martins
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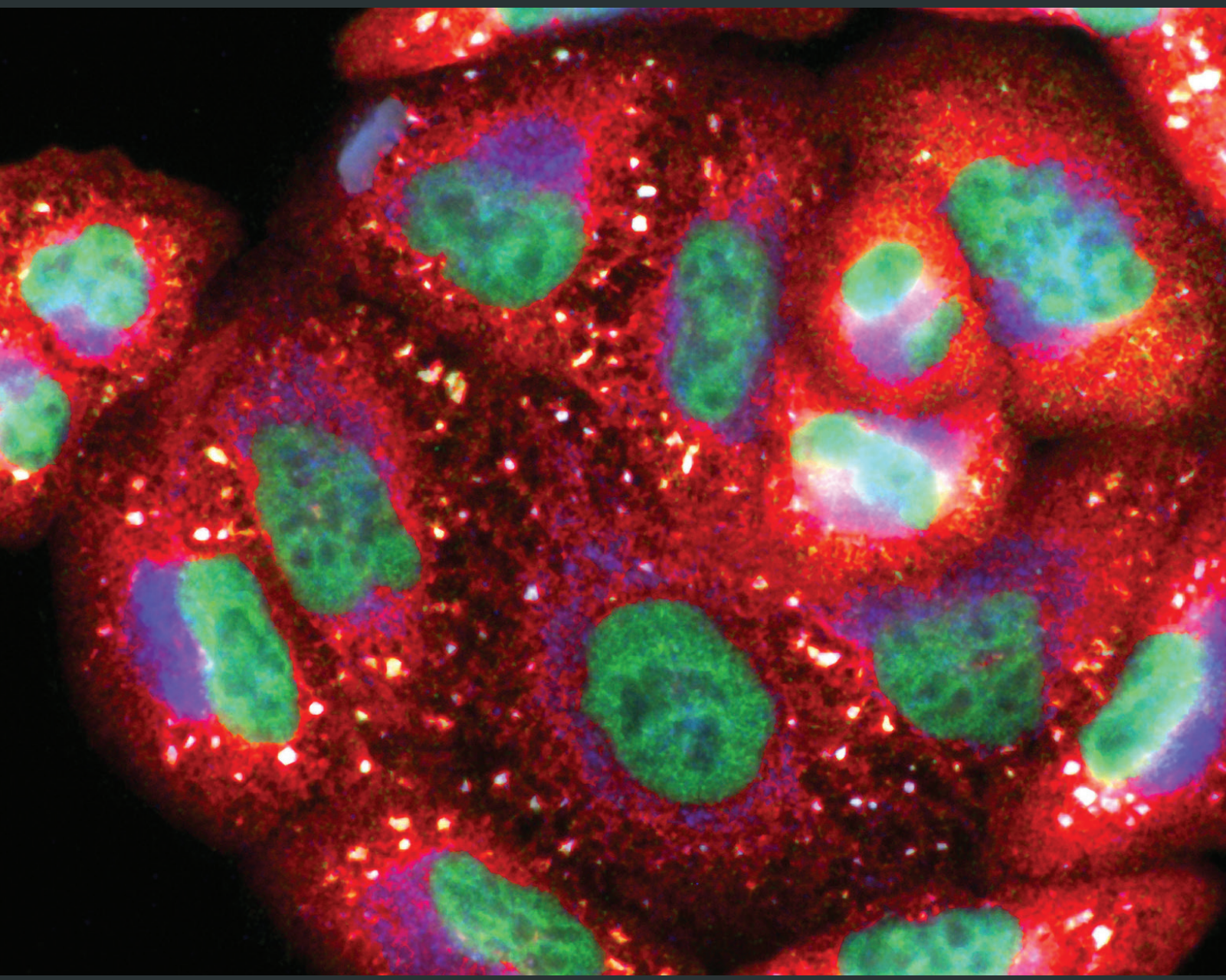

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Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 7280690, 2 pages
https://doi.org/10.1155/2017/7280690

Editorial

Biomarkers of Aging: From Cellular Senescence to Age-Associated Diseases

Maria João Martins,[1,2] Miguel Constância,[3] Delminda Neves,[1,4] and Andreas Simm[5]

[1]*Institute for Research and Innovation in Health (i3s), University of Porto, 4200-135 Porto, Portugal*
[2]*Department of Biomedicine, Unit of Biochemistry, Faculty of Medicine of the University of Porto (FMUP), 4200-319 Porto, Portugal*
[3]*Department of Obstetrics and Gynaecology, University of Cambridge, Cambridge CB2 0SW, UK*
[4]*Department of Biomedicine, Unit of Experimental Biology, Faculty of Medicine of the University of Porto (FMUP), 4200-319 Porto, Portugal*
[5]*Heart Centre of the University Hospital Halle (Saale) of the Martin-Luther-University Halle-Wittenberg, 06120 Halle (Saale), Germany*

Correspondence should be addressed to Maria João Martins; mmartins@med.up.pt

Received 23 February 2017; Accepted 23 February 2017; Published 2 August 2017

The major common risk factor of degenerative diseases is the age of the person. For a long time, the calendrical age was used to calculate the risk to develop a disease or to have a negative outcome. Indeed, calculated by the Gompertz survival function, mortality rate increases exponentially with time. Older patients have a higher risk to get a worse outcome in comparison to younger patients. The plasticity of stem cells is reduced with age. Whereas all these data seem to fit on the basis of cohorts, on the level of individuals, age, as calculated time, is a bad predictor. We all know fit people at the age of 80 and old people at the age of 70. Therefore, one needs a better calculation of the age of a person, the so-called biological age. This age should be analysed using biomarkers of aging. In contrast to the calendrical age, the biological age can be influenced by the environment and our behaviour. The collection of review and primary research papers in this special issue of *Oxidative Medicine and Cellular Longevity* covers a wide range of topics from mechanisms of cellular aging to nutritional and lifestyle interventions in aging and age-associated conditions.

The article by J. Strycharz et al. is an in-depth review of the role played by the pleiotropic P53 gene in metabolic homeostasis regulation and age-associated obesity and diabetes. The authors provide insights into p53-dependent metabolic actions in the adipose tissue, liver, pancreas, and muscle and discuss how p53 activation may contribute to the regulation of insulin resistance across the life course. The papers by S.-T. Oh et al. and J. Mikuła-Pietrasik et al. are focused on vascular endothelial dysfunction, a pathology that is commonly observed in age-related cardiovascular and brain diseases. These studies demonstrate that native low-density lipoprotein (LDL) or serum from varicose patients induces premature senescence of endothelial cells (HUVECs), with both treatments leading to the generation of reactive oxygen species (ROS). In the case of LDL, the downstream mechanisms involve modulation of p53, p21, and p16-pRb signal transduction pathways after LDL receptor-mediated endocytosis of native LDL. In the case of serum from varicose patients, the production of ROS is TGFβ1-dependent. Importantly, this study proposes that varicosity generates local (endothelium-related) and systemic (serum-related) proinflammatory conditions, leading to "inflammaging." Still on the theme of cellular senescence, L. Matos et al. demonstrate that copper-induced fibroblast senescence can be attenuated by resveratrol through positive modulation of protein quality control systems (namely, autophagy upregulation) and, consequently, improvement of cellular proteostasis. Â. Carneiro and J. P. Andrade argue, in a compelling review paper, that lifestyle changes, including nutritional advice, may delay the onset of age-related macular degeneration (where increased

oxidative stress and/or endothelial dysfunction are relevant) and can slow the progression of the disease. Finally and also on lifestyle choices, M. Tolahunase et al. consider in their paper whether yoga and meditation (YMLI) can impact on cellular aging. They show that YMLI reduce the rate of cellular aging, as measured by improvements in both cardinal (DNA damage, telomere attrition, and oxidative stress) and metabotropic (neuroplasticity, stress and inflammatory responses, and longevity) blood biomarkers of cellular aging.

Aging is a major risk factor and the basis for degenerative diseases. Any efforts to reduce the risk for developing those conditions will require a better understanding of the cellular and molecular mechanisms of aging and their effects on homeostasis regulation. Overall, the articles in this series describe common molecular targets involved in cellular aging and provide insights into the plasticity of the aging process that can be "modulated" and potentially "delayed" or "reversed." Much more research is needed to identify how precisely "delaying" or "reversing" aging will impact on the metabolic disease burden. In this regard, a particular interesting avenue of future research is epigenetic reprogramming, since one of the hallmarks of aging is epigenetic drift. In times of rapid dietary and environmental change, understanding the cross-talk between the environment and genome, through epigenetics, is likely to be important not only for the understanding of the aging process and its relationship with degenerative disease risk but also for the immediate health of human populations through the design of therapeutic-targeted approaches.

Acknowledgments

We are grateful to the authors for these six articles and to the anonymous reviewers who provided insightful contributions to this special issue. Finally, we thank the editorial board of *Oxidative Medicine and Cellular Longevity* for giving us the opportunity to coordinate this special issue.

Maria João Martins
Miguel Constância
Delminda Neves
Andreas Simm

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 3793817, 12 pages
https://doi.org/10.1155/2017/3793817

Research Article

Resveratrol Attenuates Copper-Induced Senescence by Improving Cellular Proteostasis

Liliana Matos,[1,2] Alexandra Monteiro Gouveia,[1,2] and Henrique Almeida[1]

[1]*Departamento de Biologia Experimental, Faculdade de Medicina, IBMC, Instituto de Biologia Molecular e Celular and I3S, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal*
[2]*Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal*

Correspondence should be addressed to Liliana Matos; lilianamatos@gmail.com

Received 16 September 2016; Accepted 5 January 2017; Published 9 February 2017

Academic Editor: Miguel Constância

Copper sulfate-induced premature senescence ($CuSO_4$-SIPS) consistently mimetized molecular mechanisms of replicative senescence, particularly at the endoplasmic reticulum proteostasis level. In fact, disruption of protein homeostasis has been associated to age-related cell/tissue dysfunction and human disorders susceptibility. Resveratrol is a polyphenolic compound with proved antiaging properties under particular conditions. In this setting, we aimed to evaluate resveratrol ability to attenuate cellular senescence induction and to unravel related molecular mechanisms. Using $CuSO_4$-SIPS WI-38 fibroblasts, resveratrol is shown to attenuate typical senescence alterations on cell morphology, senescence-associated beta-galactosidase activity, and cell proliferation. The mechanisms implicated in this antisenescence effect seem to be independent of senescence-associated genes and proteins regulation but are reliant on cellular proteostasis improvement. In fact, resveratrol supplementation restores copper-induced increased protein content, attenuates BiP level, and reduces carbonylated and polyubiquitinated proteins by autophagy induction. Our data provide compelling evidence for the beneficial effects of resveratrol by mitigating $CuSO_4$-SIPS stressful consequences by the modulation of protein quality control systems. These findings highlight the importance of a balanced cellular proteostasis and add further knowledge on molecular mechanisms mediating resveratrol antisenescence effects. Moreover, they contribute to identifying specific molecular targets whose modulation will prevent age-associated cell dysfunction and improve human healthspan.

1. Introduction

Normal somatic dividing cells have been proved to be valuable in vitro models to study cellular senescence and unravel molecular mechanisms and pathways implicated in the human aging process. The well-known model of replicative senescence (RS) is achieved when human diploid fibroblasts (HDFs) spontaneously stop dividing after an initial active period of population doublings and become unresponsive to mitogenic stimuli [1]. Besides the irreversible cell cycle arrest, RS fibroblasts exhibit other typical, morphological, and molecular features, such as increased cellular volume, higher senescence-associated beta-galactosidase (SA beta-gal) activity, and increased expression of senescence-associated genes and proteins [2, 3]. A similar senescent phenotype, termed stress-induced premature senescence (SIPS), can be attained by the exposure of HDFs to subcytotoxic doses of oxidative stress inducers such as hydrogen peroxide (H_2O_2-SIPS) [4], tert-butyl hydroperoxide, ultraviolet B radiation [3], or copper sulfate ($CuSO_4$-SIPS) [5]. Recently, the latter was shown to mimic better the RS model compared to the most frequently used H_2O_2-SIPS model [6].

Resveratrol is a natural polyphenolic compound that was shown to increase maximum lifespan of several organisms, such as *Saccharomyces cerevisiae* [7], *Caenorhabditis elegans* [8], *Drosophila melanogaster* [9], and the short-lived fish *Nothobranchius furzeri* [10]. Yet, resveratrol failed to extend longevity in rodent mammals, even though it improved their healthspan, thus evidencing a protective role against age-related deterioration [11].

At the cellular level, resveratrol has also been shown to attenuate senescence features in either RS [12] or H_2O_2-SIPS

[13, 14] cellular models. These antiaging effects have long been associated with resveratrol ability to activate sirtuin 1 deacetylase, Sirt1 [for a review see [15]]. Actually, it was demonstrated that Sirt1 overexpression attenuates senescence and extends replicative lifespan of several cultured cell types [16–18], while its inhibition results in increased cellular senescence [16]. Also, Sirt1 was shown to be downregulated with aging [19] and in cellular senescence models [20, 21] further favoring its preventive role of senescence features. Besides resveratrol ability to modulate signal transduction pathways through the activation of Sirt1 [14, 22], several other biological events were assigned as responsible for its positive effects, including its ability to increase stress resistance [12], to induce telomerase activity [23], to decrease the secretion of senescence-associated proinflammatory proteins [24], and to inhibit the mechanistic target of rapamycin, mTOR [13]. Resveratrol was also found to modulate protein quality control cellular responses, as it was shown to regulate the expression of the heat shock molecular chaperones [25] and to promote cellular protein degradation mechanisms, namely, ubiquitin-proteasome system (UPS) [26, 27] and lysosomal autophagy [28, 29]. Moreover, resveratrol was able to increase *C. elegans* lifespan through the upregulation of *abu11* (activated in blocked unfolded protein response-11), which encodes a protein involved in the endoplasmic reticulum (ER) unfolded protein response (UPR) that protects the organism from damage by improperly folded proteins [8].

In the present study we aimed to evaluate the ability of resveratrol to attenuate the establishment of cellular senescence upon $CuSO_4$ induction, unravelling the molecular mechanisms that might be involved. It was found that resveratrol supplementation was able to reduce the appearance of some senescence-associated features by the improvement of cellular proteostasis probably by protecting proteins from oxidative damage and preventing their accumulation by the induction of protein degradation mechanisms.

2. Material and Methods

2.1. Cell Culture. WI-38 human fetal lung fibroblasts were purchased from The European Collection of Cell Cultures (ECACC) and were cultivated in complete medium composed of Basal Medium Eagle (BME) supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 humidified atmosphere. WI-38 cells are considered to be young below 30 population doublings (PDs) and enter senescence at 45 PDs or above. For the induction of SIPS with copper sulfate ($CuSO_4$-SIPS), subconfluent young WI-38 fibroblasts were exposed to 350 μM $CuSO_4$ (Na_2SO_4 for controls) for 24 h. Then, cells were washed once with phosphate buffered saline (PBS) and replaced with fresh complete medium containing 5 or 10 μM of resveratrol (R5010-Sigma-Aldrich®) for an additional 72 h period. Control cells were submitted to a final concentration of 0.1% DMSO for the same period.

2.2. Cell Morphology and SA Beta-Gal Detection. Cell morphology evaluation was performed 72 h after copper removal by optical inspection using an inverted microscope. To assess the presence of senescent cells, SA beta-gal was detected 72 h after copper removal as already described [5]. The percentage of SA beta-gal positive cells in each condition was determined by microscopically counting 400 total cells/well from at least three independent experiments.

2.3. Cell Proliferation and Total Protein Content. To assess the effect of the different treatments on cell proliferation and total protein content, cell number determination and SRB assay [30] were performed along time after copper removal. Briefly, 3000 cells/well were seeded in 96-well culture plates, treated for 24 h with $CuSO_4$ (or Na_2SO_4 for controls), and then analyzed at different time-points (0, 24, 48, and 72 h) while recovering in the presence or absence of resveratrol. For cell number determination, cells were trypsinized and stained with Trypan Blue, and the viable cells were microscopically counted in a Neubauer chamber. The total number of cells per well for each condition at the different time-points was calculated and plotted, assuming that, at $t = 0$, for each condition, cell number equals 1. For the total protein content determination, cells were treated with 10% trichloroacetic acid (TCA), 1 hour at 4°C. The TCA-precipitated proteins fixed at the bottom of the wells were stained for 30 minutes with 0.057% (w/v) SRB in 1% acetic acid solution and then washed four times with 1% acetic acid. Bound dye was solubilized with 10 mM Tris base solution and the absorbance at 510 nm of each well was recorded using a microplate reader (Infinite 200, TECAN).

2.4. Real Time PCR. Gene expression experiments were performed 72 h after copper sulfate treatment by real time quantitative PCR (qPCR). Total RNA extracted (PureLink® RNA Mini Kit, Ambion) from cells derived from at least three independent cultures from each condition was converted into cDNA by reverse transcription reaction. Amplification reaction assays contained SYBR Green Mastermix (SYBR® Select Master Mix, Applied Biosystems®), 50 ng cDNA and primers (STAB VIDA, Lda.) at optimal concentrations. The primer sequences were p21, 5′-CTGGAGACTCTCAGGGTCGAA-3′ and 5′-CCAGGACTGCAGGCTTCCT-3′; ApoJ, 5′-GGATGAAGGACCAGTGTGACAAG-3′ and 5′-CAGCGACCTGGAGGGATTC-3′; TGFβ1, 5′-AGGGCTACCATGCCAACTTCT-3′ and 5′-CCGGGTTATGCTGGTTGTACA-3′; and TATA box binding protein (TBP), 5′-TCAAACCCAGAATTGTTCTCCTTAT-3′ and 5′-CCTGAATCCCTTTAGAATAGGGTAGA-3′. The protocol used for qPCR was 95°C (3 min); 40 cycles of 95°C (15 sec); and 60°C (1 min). qPCR was performed in the StepOnePlus™ thermal cycler (Applied Biosystems™). TBP was the selected housekeeping gene when calculating relative transcript levels of the target genes.

2.5. Western Blot. Protein levels were assessed 72 h after copper sulfate exposure by western blot analysis. WI-38 cells submitted to the different treatments were washed with PBS and scrapped on ice in a lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) supplemented with protease inhibitors cocktail (Sigma-Aldrich).

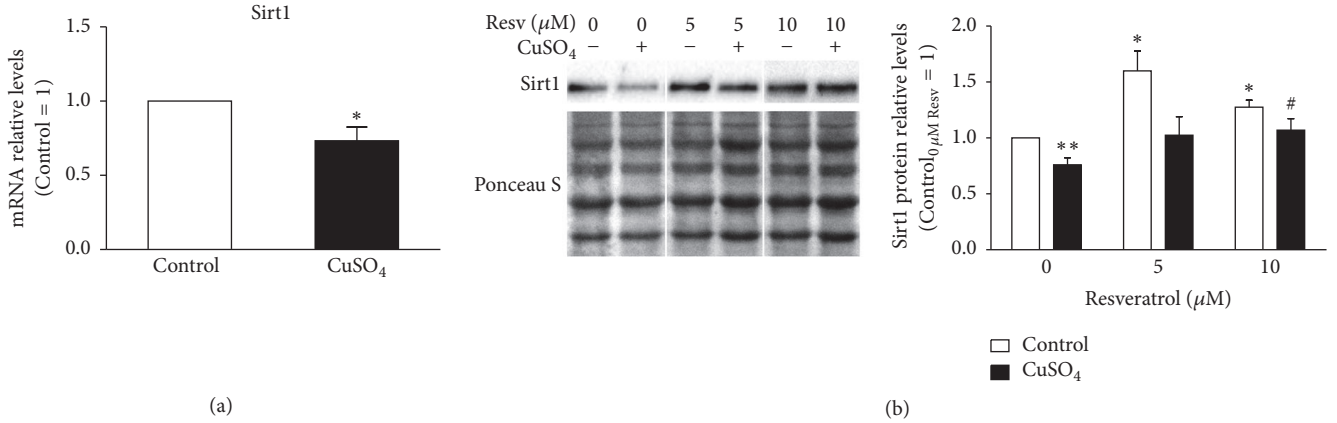

Figure 1: Reduced sirtuin 1 expression in $CuSO_4$-SIPS fibroblasts is restored by the addition of resveratrol. (a-b) WI-38 fibroblasts were incubated with 350 μM $CuSO_4$ (or Na_2SO_4, for controls) for 24 h. Then, media were changed and cells were allowed to recover for an additional 72 h period in the presence of 5 or 10 μM resveratrol (or 0.1% DMSO, for controls). After this recovery period, fibroblasts were processed for different assays. (a) Sirtuin 1 (Sirt1) transcript levels were assessed by qPCR and plotted assuming that mRNA level of controls equals 1. TBP was the selected housekeeping gene. (b) Sirt1 relative protein content was determined by western blot, using Ponceau S staining to normalize protein loading. Depicted blots are representative and densitometric quantification is plotted assuming that control cells in the absence of resveratrol presents a relative protein level of 1. Data represent mean ± SEM of at least three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$, when compared to control cells in the absence of resveratrol; $^{\#}p < 0.05$, relatively to $CuSO_4$-treated cells without resveratrol.

Upon Bradford assay, 20 μg (or 10 μg for the detection of carbonylated or poly-Ub proteins) of protein from each cell extract was resolved by SDS-PAGE. Proteins were blotted into a nitrocellulose membrane and, after blocking with 5% nonfat dry milk diluted in Tris-buffered saline 0.1% tween 20 (TBST), were probed with specific primary antibodies (anti-HSP90 ab13495 and anti-p62 ab109012, Abcam®; anti-LC3 NB100-2220, Novus Biologicals; anti-ubiquitin PW0930, Enzo® Life Sciences; anti-p21 #2946, anti-phospho-eIF2 #3398, anti-HSP70 #4876, and anti-BiP #3177, Cell Signaling Technology®) overnight at predetermined optimal dilutions. For the specific detection of carbonylated proteins, immediately after protein transfer, the nitrocellulose-bound proteins were treated as described elsewhere [31]. Briefly, the membranes were equilibrated in 20% methanol in TBS, washed for 5 min with 10% trifluoroacetic acid (TFA), derivatized with 5 mM 2,4-dinitrophenylhydrazine (DNPH, Sigma-Aldrich) diluted in 10% TFA for 10 min (protected from light), washed with 10% TFA to remove the excess of DNPH, and finally washed with 50% methanol. Following this procedure, the membranes were blocked with 5% bovine serum albumin in TBST and incubated with primary anti-DNP antibody (D9656, Sigma-Aldrich). From here on, the western blot procedure was similar to all antibodies: after TBST washing, immunoblots were incubated with the appropriate peroxidase-conjugated secondary antibodies for 1 h, detected using ECL western blotting substrate (Pierce™, Thermo Scientific) and visualized in ChemiDocTM XRS (BioRad Laboratories). Results were quantified by densitometry using the Image Lab® software. Protein loading was normalized using Ponceau S protein staining, but similar data were also obtained using tubulin detection (data not shown).

2.6. Statistical Analysis. Student's t-test was used to compare the means between two different conditions. A p value lower than 0.05 was considered statistically significant.

3. Results

3.1. Sirtuin 1 Expression Is Diminished in $CuSO_4$-SIPS. It was already demonstrated that Sirt1 expression decreases with increasing population doublings [20] and also in H_2O_2-SIPS cellular models [21]. Here, Sirt1 transcript and protein levels were evaluated by qPCR and western blot, respectively, in $CuSO_4$-induced senescent WI-38 fibroblasts. Similarly to other RS and SIPS models, $CuSO_4$-SIPS fibroblasts also presented decreased expression of both gene (Figure 1(a)) and protein (Figure 1(b)) Sirt1. Namely, mRNA and protein relative level presented a 27% and 23% reduction, respectively, in copper-treated cells when compared to controls ($p = 0.04$ and $p = 0.008$, resp.). The effect of resveratrol (5 or 10 μM), a Sirt1 activator, was evaluated 72 h after the 24 h incubation of cells with $CuSO_4$, which is the usual recovery time that cells need to adapt and develop the senescent phenotype [5]. The addition of 10 μM resveratrol attenuated the copper-induced decrease in Sirt1 protein levels ($p = 0.047$) to values similar to the young control cells. Incubation of non-$CuSO_4$ submitted fibroblasts with 5 and 10 μM resveratrol for 72 h increased Sirt1 protein level by 1.6- and 1.3-fold ($p = 0.008$ and $p = 0.01$), respectively, when compared to young control cells (Figure 1(b)).

3.2. Resveratrol Attenuates the Appearance of Some Typical Senescence-Associated Alterations. Senescent cells usually present typical morphological alterations, increased level of

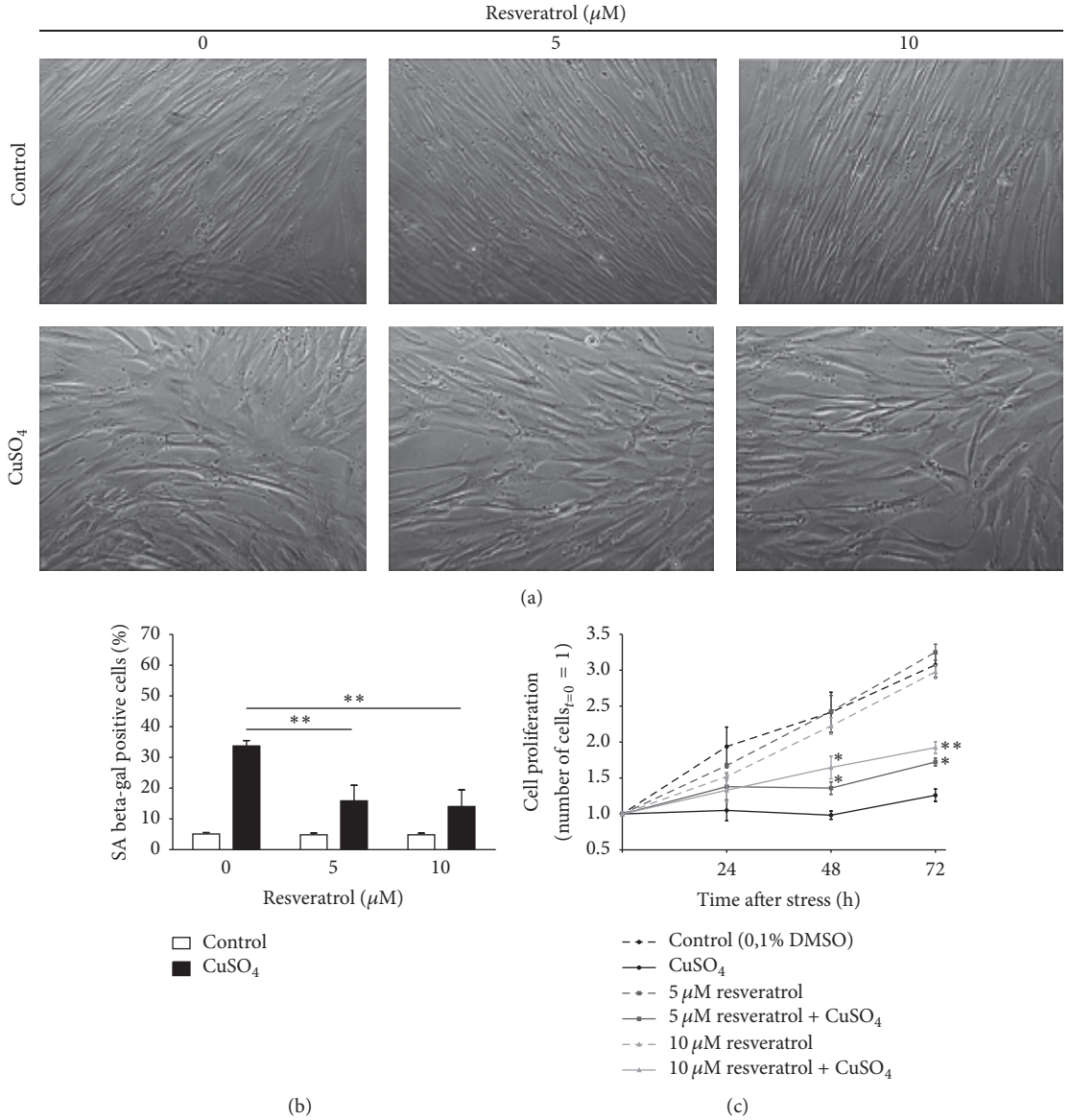

Figure 2: Resveratrol attenuates the appearance of typical senescence-associated features induced by $CuSO_4$. (a) Cell morphology was evaluated 72 h after the removal of 350 μM $CuSO_4$ (or Na_2SO_4, for controls) in fibroblasts that were allowed to recover in the presence or absence of 5 or 10 μM resveratrol. Representative images from the indicated conditions are depicted. (b) Senescence-associated beta-galactosidase (SA beta-gal) activity was detected 72 h after $CuSO_4$ removal and the percentage of positive cells was calculated for each condition after counting a minimum of 400 cells/well. (c) Cell proliferation was assessed by counting the viable cells in a Neubauer chamber at different time-points after $CuSO_4$ treatment (0, 24, 48, and 72 h). To facilitate direct comparison between the indicated conditions along time, the number of viable cells at day 0 was assumed as 1 for all treatments. Data represent mean ± SEM of at least three independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$, when compared to $CuSO_4$-treated cells without resveratrol at the respective time-point.

SA beta-gal, and irreversible inhibition of cell proliferation. Therefore, these three features were evaluated in order to assess the effect of resveratrol in $CuSO_4$-SIPS fibroblasts. Briefly, cell proliferation was assessed by counting the viable cells at 0, 24, 48, and 72 h after copper removal. Then, at the last time-point (72 h), cell morphology was observed and the percentage of SA beta-gal positive cells was quantified for each condition. As shown in Figure 2(a), in the absence of resveratrol, $CuSO_4$-SIPS fibroblasts presented the typical senescent morphology, as they were no longer small and fusiform and became enlarged and flattened. However, copper-treated cells recovering in the presence of resveratrol exhibited less pronounced senescent-like alterations, as they appeared thinner and more elongated, when compared to cells in the absence of resveratrol. This was particularly evident for the highest concentration of resveratrol used (10 μM). It is noteworthy to mention that even cells not submitted to copper exhibited a slightly different aspect in the presence of resveratrol, as they seemed smaller and their cell limits were more clear-cut.

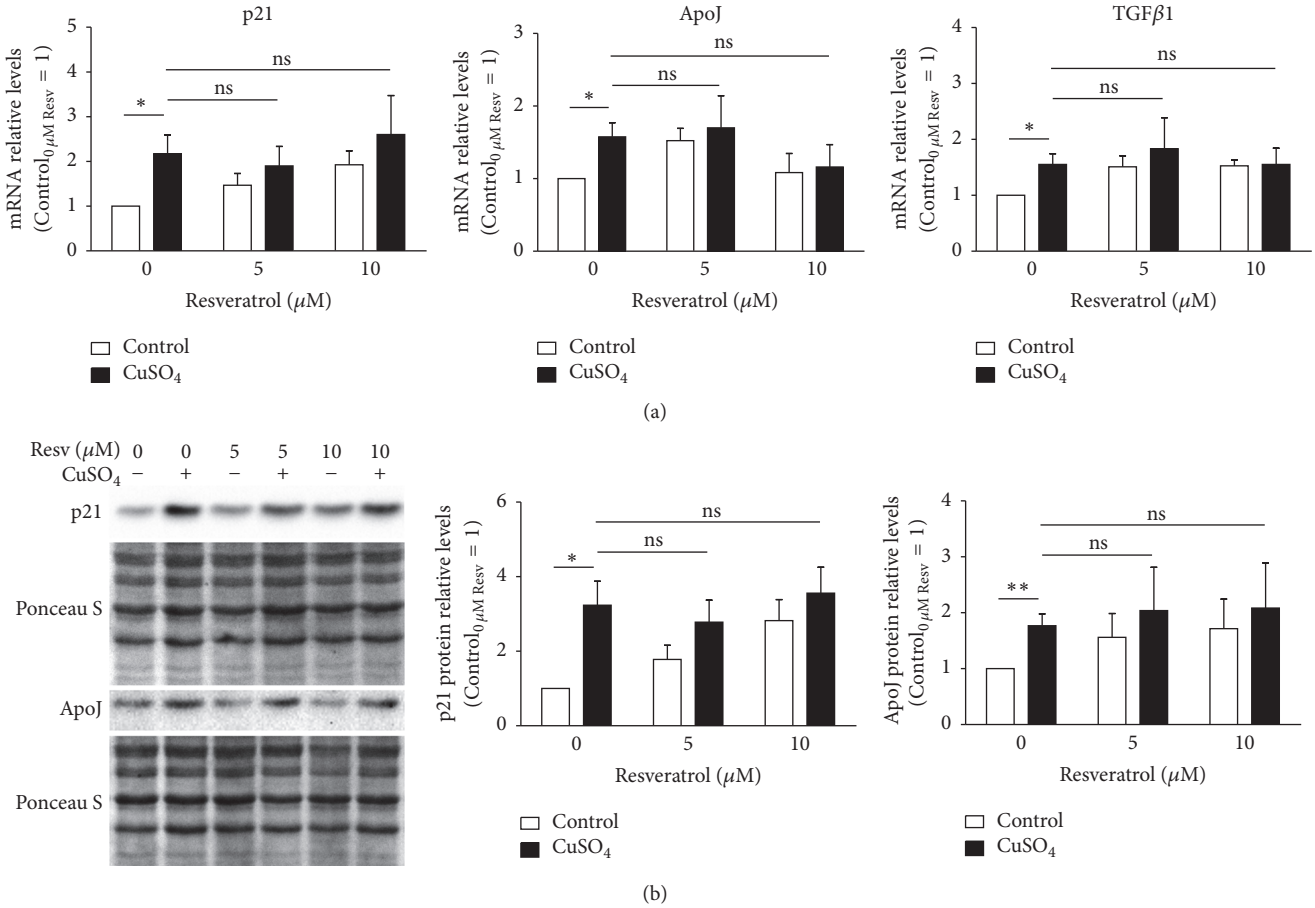

Figure 3: Resveratrol supplementation does not affect copper-induced expression of senescence-associated molecules. (a) Transcript relative levels of cyclin-dependent kinase inhibitor 1A (p21), apolipoprotein J (ApoJ), and transforming growth factor beta 1 (TGFβ1) were assessed by qPCR in 350 μM $CuSO_4$-treated fibroblasts that were allowed to recover in the presence of the indicated doses of resveratrol. (b) Representative blots obtained for the determination of p21 and ApoJ protein level by western blot are depicted; the resulting densitometric analysis, normalized for control cells in the absence of resveratrol, is plotted for each analysed protein. Ponceau S staining was used to control protein loading. Data represent mean ± SEM of at least three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; and ^{ns}non-significant, for the comparisons between the indicated groups.

Similar to previously reported results [6], $CuSO_4$-SIPS cellular model contained 34% of cells positive for SA beta-gal (Figure 2(b)), whereas the controls had only 5% of senescent cells. However, the addition of 5 and 10 μM resveratrol to copper-treated cells resulted in a statistically significant reduction in the number of SA beta-gal positive cells (to 16 and 14%, resp.). The ability of copper sulfate to inhibit cell proliferation had previously been described [6] and herein is again demonstrated (Figure 2(c)), as 3 days after stress, copper-treated cells presented a reduction of 88% in their proliferation when compared to controls. Media supplementation with 5 and 10 μM resveratrol during the recovery period resulted in the attenuation of cell proliferation inhibition by 20 and 34%, respectively. In addition, in the absence of copper, the selected concentrations of resveratrol were not able to affect significantly cell proliferation when compared to control cells. Altogether, these data show that resveratrol can actually attenuate the induction of senescence by copper sulfate in WI-38 fibroblasts.

3.3. Resveratrol Does Not Alter Copper-Induced Upregulation of Senescence-Associated Genes and Proteins. There are several genes and proteins, such as the cyclin-dependent kinase inhibitor 1A (p21), apolipoprotein J (ApoJ), and transforming growth factor beta 1 (TGFβ1), whose overexpression is typical of the senescent phenotype observed in RS and SIPS cellular models. Herein, we evaluated the ability of resveratrol to adjust the levels of p21, ApoJ, and TGFβ1 upon copper treatment, in order to justify its effect in the attenuation of copper-induced senescence. Therefore, the mRNA transcript relative levels of these genes were quantified by qPCR (Figure 3(a)). In accordance with previous publication [5], p21, ApoJ, and TGFβ1 mRNA levels were found upregulated by 2.2-, 1.6-, and 1.6-fold, respectively, in $CuSO_4$-SIPS fibroblasts when compared to control cells. However, the addition of resveratrol (either 5 or 10 μM) immediately after copper sulfate removal did not have any statistically significant effect on the transcript levels of these genes. To validate these results and exclude the occurrence of posttranslational regulation,

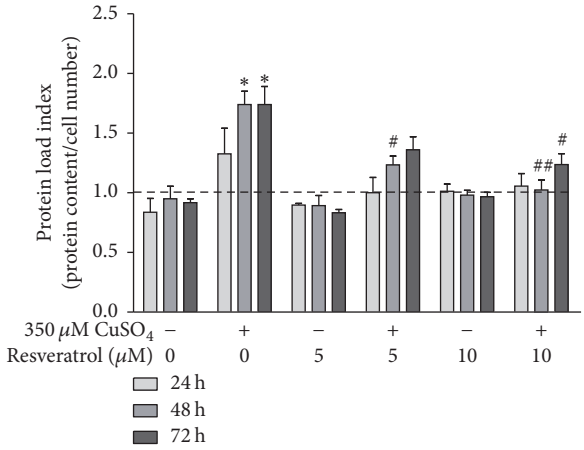

Figure 4: $CuSO_4$-induced proteostasis imbalance is attenuated by resveratrol. Protein load index (PLI), used as a measure of cellular protein accumulation, was calculated as the ratio between total protein content and cell number for each condition, at different time-points after $CuSO_4$ treatment (0, 24, 48, and 72 h). PLI values were normalized for the initial time-point (0 h) and the relative values are plotted for the indicated conditions. Data represent mean ± SEM of at least three independent experiments. $^{*}p < 0.05$, when compared to control cells in the absence of resveratrol; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, relatively to $CuSO_4$-treated cells without resveratrol, at the respective time-points.

the relative protein levels of p21 and ApoJ were evaluated by western blot (Figure 3(b)). At the protein level, p21 and ApoJ presented a 3.2- and 1.8-fold increase in copper-treated cells, when compared to controls, thus confirming the previously noticed trend. In addition, similarly to transcript levels results, resveratrol supplementation did not affect copper-induced augmentation of these proteins. Overall, the effect of resveratrol in the attenuation of copper-induced senescence does not involve the regulation of p21, ApoJ, and TGFβ1 senescence-associated genes.

3.4. $CuSO_4$-Induced Proteostasis Imbalance Is Attenuated by Resveratrol. The occurrence of proteostasis imbalance is a major hallmark of aging [32] and, at the cellular level, may be shown by increased intracellular protein content [33]. To measure cellular protein accumulation for each experimental condition, the ratio between total protein content and cell number, here defined as the protein load index (PLI), was calculated at 0, 24, 48, and 72 h after $CuSO_4$ removal (sodium sulfate for controls). Assuming that immediately after stress removal PLI equals 1, it was shown that it significantly increased 1.7-fold at 48 and 72 h time-points in $CuSO_4$-SIPS cells when compared to the respective control conditions (Figure 4). $CuSO_4$-treated fibroblasts that were allowed to recover in the presence of 5 μM resveratrol exhibited a statistically significant 0.5-fold decrease in PLI at 48 h, when compared with cells without added resveratrol. Moreover, the addition of 10 μM resveratrol after copper removal totally reverted PLI to the level of controls in the absence of copper at 48 h and presented a statistically significant 0.5-fold decrease at 72 h, when compared to copper-treated cells in the absence of resveratrol at the same time-point.

To compensate the altered proteostasis, $CuSO_4$-SIPS cells present higher levels of phosphorylated eukaryotic translation initiation factor 2 (p-eIF2) [6], which inhibits general protein translation and allows cells to restore homeostasis. A possible explanation for the diminished PLI obtained for copper cells recovering in the presence of resveratrol could be an increased inhibition of overall protein synthesis caused by higher p-eIF2. When p-eIF2 was quantified by western blot (Figure 5(a)), as expected it was found increased in $CuSO_4$-treated cells, compared to controls. However, resveratrol supplementation upon copper removal did not result in any additional alteration in p-eIF2 protein level. Next, cell chaperoning ability was evaluated by the quantification of the molecular chaperones immunoglobulin binding protein (BiP), heat shock protein (HSP) 90, and HSP70 by western blot (Figure 5(b)). In fact, the intracellular protein levels of BiP, HSP90, and HSP70 were 1.4-, 1.9-, and 6.3-fold increase in $CuSO_4$-SIPS fibroblasts, when compared to control cells. The presence of resveratrol after copper removal had no effect on HSP90 and HSP70 protein levels comparing to the levels of copper-treated cells without resveratrol. However, BiP protein levels were diminished (to 1.1-fold) in copper-treated cells that were allowed to recover in the presence of 10 μM resveratrol, relatively to the condition without resveratrol, reflecting a lower need to buffer defective or damaged proteins.

3.5. Resveratrol Attenuates $CuSO_4$-Induced Accumulation of Modified Proteins by the Induction of Lysosomal Autophagy. The altered proteostasis observed in $CuSO_4$-SIPS fibroblasts could be a consequence of a progressive accumulation of oxidatively modified proteins. Protein carbonylation is a type of irreversible protein oxidation that is frequently used as an indicator of increased permanent levels of oxidative stress. Actually, cellular senescence models [34] and cells treated with oxidative stress inducers [35] were both shown to exhibit increased levels of carbonylated proteins. Herein, carbonyl protein content was evaluated to infer about cellular oxidative status in the different experimental conditions. $CuSO_4$-SIPS cells presented a statistically significant 13% increase ($p = 0.0017$) in the relative levels of carbonylated proteins, when compared to control cells (Figure 6(a)). The addition of 10 μM resveratrol during cell recovery (but not 5 μM) was able to attenuate such increase in protein oxidation by 34%, a variation that was close to reach statistical significance ($p = 0.054$). These data suggest that resveratrol may be able to prevent or attenuate the accumulation of copper-induced oxidized proteins. This may be achieved either by its well described antioxidant properties that might prevent protein damage or by its ability to modulate protein degradation processes. UPS activity is known to be reduced during aging. The accumulation of polyubiquitinated (poly-Ub) proteins is usually associated with decreased UPS efficiency and, in fact, here a 22% increase in the levels of poly-Ub proteins in $CuSO_4$-SIPS fibroblasts was observed (Figure 6(b)). In addition, resveratrol supplementation (only at 10 μM) immediately after copper sulfate removal showed to be effective on

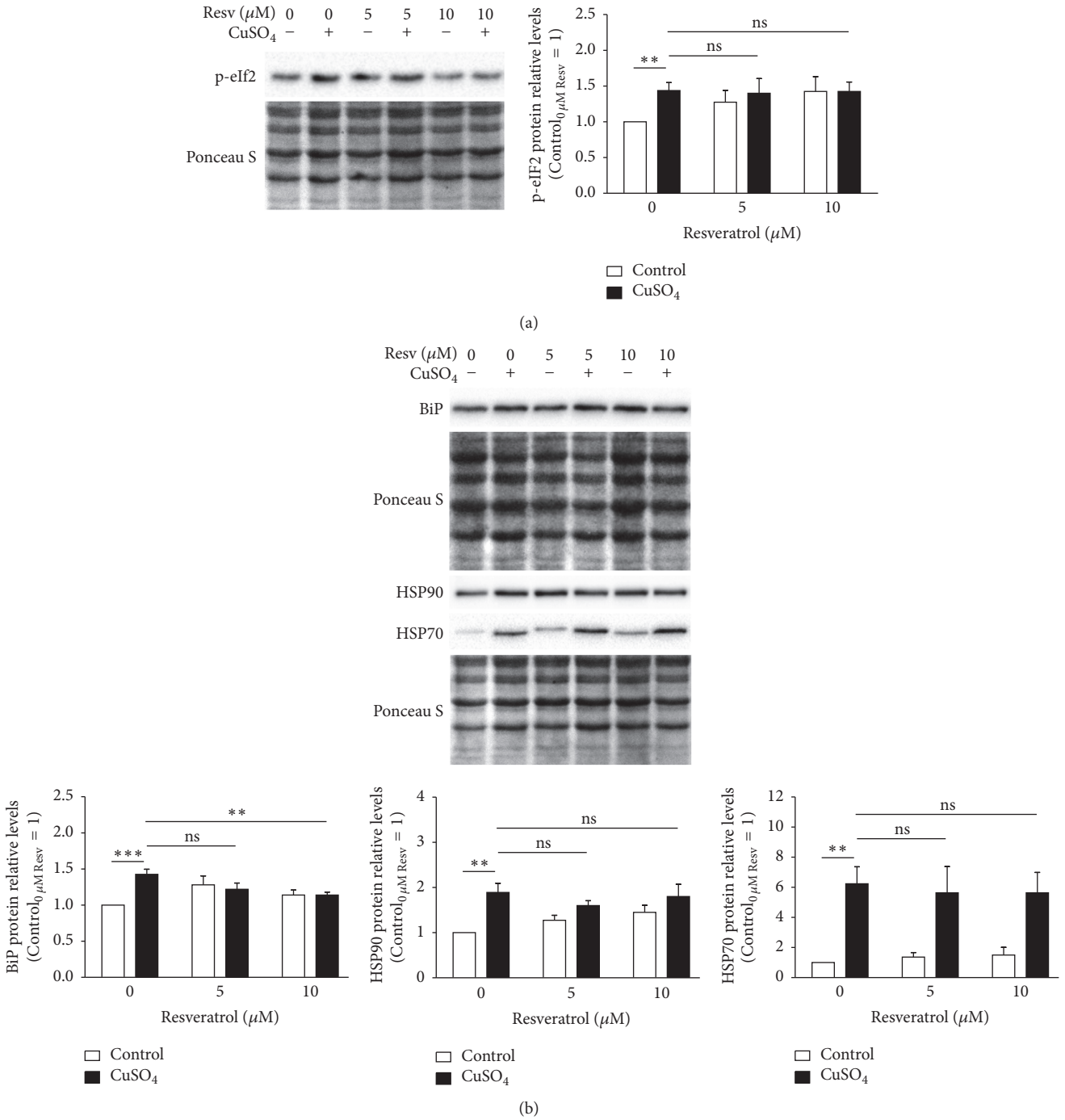

Figure 5: Resveratrol attenuates copper-induced BiP upregulation but has no effect on eIF2 phosphorylation or HSP90 and HSP70 expression. (a) Phosphorylated eukaryotic translation initiation factor 2 (p-eIF2) and (b) immunoglobulin binding protein (BiP), heat shock protein (HSP) 90, and HSP70 protein relative levels were determined by western blot at 72 h after the removal of 350 μM $CuSO_4$ (or Na_2SO_4, for controls) in fibroblasts that were allowed to recover in the presence or absence of resveratrol (5 or 10 μM). Representative blots are depicted and densitometric quantification is plotted assuming that protein levels of each analysed protein in control cells without resveratrol equal 1. Ponceau S staining was used to normalize protein loading. Data represent mean ± SEM of at least three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$; and ^{ns}non-significant, for the comparisons between the indicated groups.

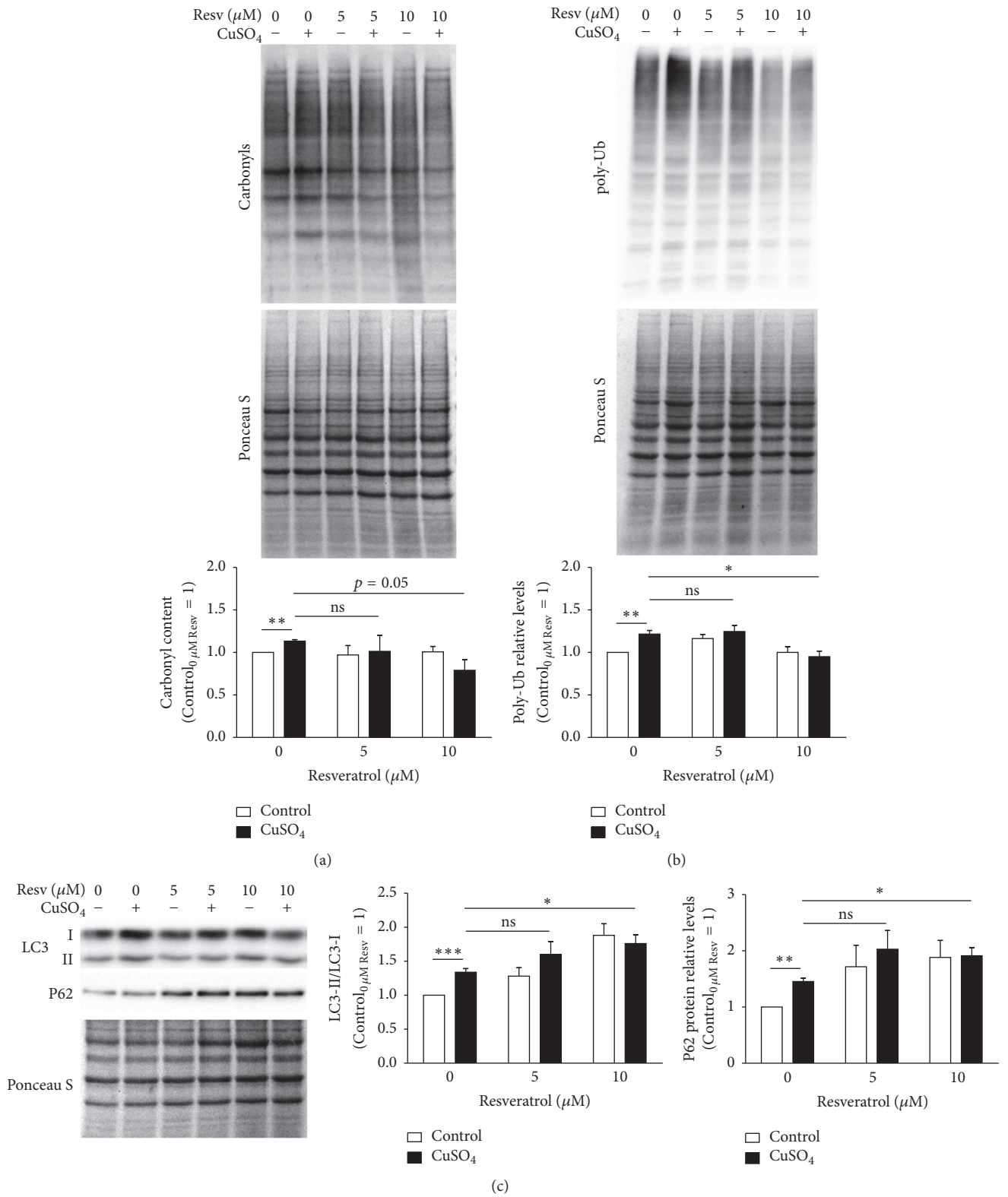

FIGURE 6: $CuSO_4$-induced accumulation of carbonylated and polyubiquitinated proteins is reduced by resveratrol, through lysosomal autophagy induction. (a) Protein carbonyl content and (b) polyubiquitinated (poly-Ub) proteins were evaluated in fibroblasts submitted to the indicated conditions by western blot. Representative blots are depicted and densitometric quantification was normalized by attributing the value 1 for control cells in the absence of resveratrol. Ponceau S staining was used as protein loading control. (c) Lysosomal autophagy was studied by the conversion of LC3-I to LC3-II, a critical step for autophagosome formation, and quantification of P62, an ubiquitin-binding protein that target Ub-substrates to autophagosomes. LC3-II/LC3-I ratio and P62 relative levels were evaluated upon densitometric quantification and plotted assuming that control cells without resveratrol present a value of 1. Data represent mean ± SEM of at least three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and ^{ns}non-significant, for the comparisons between the indicated groups.

restoring poly-Ub protein levels to the control cells ones, in a statistically significant manner ($p = 0.026$).

Depending on the conformation of the polyubiquitin chain that they possess, poly-Ub proteins may be degraded either in the proteasome or by lysosomal macroautophagy [36], mentioned as autophagy from here on in order to simplify. Autophagy plays a crucial role in the recycling of dysfunctional organelles and damaged protein aggregates and it was shown to be induced by resveratrol in order to prevent oxidative stress cellular damage [28, 29]. In the present study, the induction of autophagy was evaluated by the conversion of LC3-I to LC3-II, an essential step for autophagosome formation, by calculating the ratio of the LC3-II/LC3-I protein levels using western blot. Furthermore, the level of P62 protein, an ubiquitin-binding protein that serves as a link between LC3 and Ub substrates during autophagosome formation was also evaluated by western blot technique (Figure 6(c)). $CuSO_4$-SIPS cells presented a statistically significant 1.4-fold increase in LC3-II/LC3-I ratio, when compared to young control fibroblasts. Furthermore, cell treatment with 10 μM resveratrol after copper removal further increased this ratio (to 1.8-fold, $p = 0.017$), when compared to copper-treated cells that were allowed to recover in the absence of resveratrol. Accordingly, P62 protein levels were increased by 1.5-fold in $CuSO_4$-SIPS, when compared to control. Moreover, exposure to 10 μM resveratrol after copper removal resulted in additional increase in P62 protein expression (to 1.9-fold, $p = 0.039$).

4. Discussion

The $CuSO_4$-SIPS cellular model has proven to have major value for studying molecular events that are responsible for the aging process [5, 6, 37]. Furthermore, it brought additional evidence supporting copper contribution to the age-related functional deterioration and to the progression of age-related disorders. The present study shows that $CuSO_4$-induced cell senescence results in reduced Sirt1 expression. As Sirt1 is activated by the polyphenolic compound resveratrol, the mechanism and possibility of attenuating this senescent effect upon Sirt1 were addressed. In fact, it was demonstrated that resveratrol supplementation attenuated copper-induced appearance of some typical senescence features. In addition, the mechanism behind such antisenescence effect of resveratrol was shown to involve the modulation of cellular proteostasis either by the protection of proteins from oxidative damage or by the induction of protein degradation processes.

The effect of resveratrol on cellular senescence has been investigated, but the results are contradictory: while some authors reported resveratrol ability to attenuate cellular aging [12–14], others showed that it induced the appearance of senescence [38–41]. In either case, the molecular mechanisms involved in such effects were not fully clear. We believe that this discrepancy is explained by the different experimental conditions utilized in these studies: resveratrol ability to induce cell senescence was often reported using tumor cell lines [38–40] treated with high concentrations of the compound (above 25 μM) that in some cases resulted in pro-apoptotic effects [41]; in turn, antiaging effects were described in nontumor cell lines, incubated with lower doses of resveratrol [12]. In line with these evidences, herein, the administration of 5 or 10 μM resveratrol immediately after copper sulfate removal was able to attenuate the induction of WI-38 fibroblast cellular senescence, as the percentage of SA beta-gal positive cells was decreased, the typical morphological alterations were less evident, and the blockage of cell cycle was alleviated. However, in this study, resveratrol was not able to attenuate copper-induced upregulation of senescence-associated molecules, such as p21, ApoJ, and TGFβ1. This indicates that the mechanism behind the positive antisenescence effects of resveratrol does not involve the inhibition of copper-induced expression of such senescence-associated genes.

It was recently reported that both RS and $CuSO_4$-SIPS models exhibit altered expression of several ER molecular chaperones and enzymes and activated ER UPR pathways [6]. Here, $CuSO_4$-SIPS fibroblasts exhibited greater total protein content, as measured by augmented PLI, increased expression of BiP, HSP70, and HSP90 molecular chaperones, a rise on the levels of carbonylated proteins, and higher amount of polyubiquitinated proteins, adding further evidence to the occurrence of proteostasis disruption during senescence. Nevertheless, our hypothesis that increased PLI reflects impaired proteostasis could be further supported by experimental evidence obtained for instance after inhibiting protein degradation mechanisms such as autophagy or the UPS. At present, the actual underlying molecular conditions triggering PLI increase are still unknown, but involvement of the typical cell enlargement associated with the senescence phenotype, or other mechanisms apart from proteostasis disruption, cannot be excluded. $CuSO_4$-SIPS fibroblasts that were allowed to recover in the presence of resveratrol presented improved cellular proteostasis, as their total protein levels were similar to controls, BiP chaperone expression was attenuated, and poly-ubiquitinated proteins level was reduced. Altogether, these data demonstrated that, in the presence of resveratrol, cells were able to circumvent copper-induced disruption of cellular proteostasis, intimately related to the appearance of the typical senescent phenotype.

The well documented antioxidant properties of resveratrol are the likely contributors to this cell proteostasis maintenance effect, as it can protect proteins from being oxidized in a concentration and time-dependent manner. In fact, using in vitro oxidative stressed erythrocytes, resveratrol ability to prevent protein oxidation reaches a maximum protective effect between 30 and 60 minutes after polyphenolic compound addition and is then slightly reduced with time [42]. In the current study, resveratrol supplementation for 72 h attenuated the amount of carbonylated proteins on copper-treated cells in a variation that was close to reach statistical significance. A time-course evaluation of protein carbonylation along these 72 h should add further information on the existence of time-dependent variations on resveratrol efficiency to protect proteins from oxidation.

Another important resveratrol contribution for the modulation of cellular proteostasis is its ability to regulate protein degradation mechanisms, such as the UPS [26, 27] or lysosomal autophagy [28, 29]. Both mechanisms were shown to be intimately related as autophagy is activated to compensate UPS inhibition [43]. In brief, autophagy is crucial to degrade dysfunctional organelles and damaged protein aggregates and involves the formation of autophagosomes that are targeted to lysosomes for the degradation of their inner content. Autophagosome formation occurs in successive stages that depend on the concerted action of several proteins [44]. The cytosolic soluble protein LC3-I is particularly important in this process because it is lipidated to originate LC3-II, which integrates the autophagosome membrane. As such conversion is essential for elongation and maturation of the autophagosomes, LC3-II/LC3-I ratio is usually used to detect autophagy activation. In addition, as P62 protein is crucial to target poly-Ub-substrates into autophagosomes through LC3 binding [44], its detection further indicates such activation. Here, $CuSO_4$-SIPS cells exhibited an increase both in LC3-I to LC3-II conversion and in P62 protein levels; when allowed to recover in the presence of resveratrol, LC3-II/LC3-I ratio and P62 protein levels were even higher, indicating an enhanced induction of autophagy and targeting of poly-Ub-substrates to autophagy. These results are in agreement with previous in vitro [45] and in vivo [28] studies demonstrating that oxidative stress conditions promote LC3-II/LC3-I ratio increase, further enhanced in the presence of resveratrol. Moreover, resveratrol has recently been described as able to promote the flux of proteins through the autophagosomal-lysosomal pathway, thus attenuating the dysfunctional effect of intracellular accumulation of damaged or defective proteins [27]. This promotion is in agreement with the results of the current study that favor resveratrol antisenescence effect as a consequence of its ability to improve cellular proteostasis through autophagy induction. However, the present study has some limitations regarding the actual induction of autophagy by resveratrol; further functional studies monitoring autophagosome number and the autophagic flux [46] in the presence of resveratrol, would clarify its effect on such processes. Moreover, given the proven crosstalk between autophagy and proteasomal degradation [47], we cannot exclude, in addition, the beneficial effects resulting from resveratrol ability to modulate the ubiquitin-proteasome system.

5. Conclusions

This study demonstrates that resveratrol is able to attenuate the induction of cell senescence resulting from $CuSO_4$ exposure. Such effects result from resveratrol ability to promote cellular adaptive mechanisms, as autophagy upregulation, which sustain cellular proteostasis and confer cellular resistance to stress. Cellular proteostasis maintenance was found to be crucial to prevent the development of the senescent phenotype. These data also uncover molecular targets whose modulation is likely to prevent age-associated cell and tissue function deterioration and improve human healthspan.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

Liliana Matos was supported by Ph.D. fellowship SFRH/BD/61820/2009 from Programa Operacional Ciência e Inovação 2010, Fundo Social Europeu, and Fundação para a Ciência e Tecnologia.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 6487825, 12 pages
https://doi.org/10.1155/2017/6487825

Research Article

Long-Term Treatment of Native LDL Induces Senescence of Cultured Human Endothelial Cells

Sung-Tack Oh,[1] Hoon Park,[1] Hyun Joong Yoon,[2] and Sung Yeul Yang[2]

[1]*Department of Obstetrics and Gynecology, Chonnam National University Medical School, 264 Seoyang-ro, Hwasun-eup, Jeollanam-do 58128, Republic of Korea*
[2]*Department of Biochemistry, Chonnam National University Medical School, 264 Seoyang-ro, Hwasun-eup, Jeollanam-do 58128, Republic of Korea*

Correspondence should be addressed to Sung Yeul Yang; syyang@jnu.ac.kr

Received 17 August 2016; Revised 23 November 2016; Accepted 28 December 2016; Published 19 January 2017

Academic Editor: Maria João Martins

The study aimed to evaluate whether the treatment of primary cultured human endothelial cells with native low-density lipoprotein (nLDL) could induce their senescence and to uncover some of the putative mechanisms involved. For this purpose, human umbilical vein endothelial cells (HUVECs) were subcultured and/or continuously cultured with nLDL (0, 2, 5, and 10 μg protein/mL), for up to 9 days. The results indicated that nLDL inhibited the proliferation of HUVECs by arresting the cell cycle at G1 phase. The G1-arrested cells showed increase in cytosolic senescence-associated-β-galactosidase (SA-β-Gal) activity, a biomarker of cellular senescence. The causative factor of the cellular senescence was nLDL itself and not oxidized LDL (oxLDL), since blocking LDL receptor (LDLR) with the anti-LDLR antibody opposed the nLDL-induced increase of SA-β-Gal activity and decrease of cellular proliferation. In addition, nLDL-induced cellular senescence by inhibiting the phosphorylation of pRb (G1 arrest) via p53 as well as p16 signal transduction pathways. G1 phase arrest of the senescent cells was not overcome by nLDL removal from the culture medium. Moreover, the nLDL-treated cells produced reactive oxygen species (ROS) dose- and time-dependently. These results suggested, for the first time, that long-term treatment of nLDL could induce the premature senescence of endothelial cells.

1. Introduction

Aging is one of the main risk factors for cardiovascular diseases. Aging of blood vessels is associated with the development of endothelial dysfunction [1, 2] and atherosclerosis [3–5]. According to recent studies, vascular endothelial cells' senescence, that is, vascular aging, might be a fundamental cause for the development of cardiovascular diseases [6–10]. However, the molecular mechanisms of vascular aging remain unclear.

Cellular senescence is a stress response phenomenon that results in (a) a permanent secession from the cell cycle and (b) the appearance of distinct morphological and functional changes associated with impaired cellular homeostasis [6]. Cellular senescence can be classified into two types according to the presence or the absence of telomere shortening [11, 12]. Cellular senescence resulting from repeated cellular replication is called "replicative senescence" [13, 14]. In this type of senescence, the progressive shortening of telomeres, by end-replication problems, and their eventual dysfunction are considered as fundamental mechanisms of senescence induction. Another type of cellular senescence results from acute senescence response to various stressful conditions, such as intracellular oxidative stress or persistent mitogenic stimulation. This second type of cellular senescence is known as "stress-induced premature senescence" and is not accompanied by telomere shortening [15–17]. Both types of cellular senescence can occur in living organisms and are associated with diseases and aging of individuals [18–20].

Oxidized low-density lipoprotein (oxLDL) can exert various unfavorable effects on vascular endothelial cells, such as the impairment of endothelial nitric oxide formation [21], induction of endothelial expression of adhesion molecules [22], induction of superoxide anion formation from the vascular tissue [23], and induction of apoptosis [24] and senescence [25] of endothelial cells. Although oxLDL plays

an important role in cellular senescence, the oxLDL levels in the blood of healthy adults, measured by an ELISA assay with a specific antibody, are about 0.12~0.13 ng/μg LDL apolipoprotein B, which is <0.02% of total LDL [26, 27].

Actually, native LDL (nLDL), a LDL particle free of any modification induced by any agent within the blood, is hardly oxidized due to active circulating antioxidants; in addition, Kupffer cells and sinusoidal endothelial cells in the liver would rapidly remove the oxLDL already produced [28, 29]. Circulating nLDL is most probably oxidized within the vascular wall and diffuses into the blood [30, 31]. These data are suggestive of a negligible contribution of oxLDL to the in vivo development of atherosclerosis and cellular senescence in the absence of pathological conditions, such as hyperlipidemia or diabetes mellitus.

Native LDL could potentially promote the recruitment of monocytes [32] and expression of cell adhesion molecules [33, 34] by disturbing the lipid dynamics of the endothelial cell membrane. These effects of nLDL on vascular endothelial cells could lead to the development of atherosclerosis [35]. However, it is unclear whether nLDL could contribute to the development of cellular senescence.

Native LDL-induced endothelial dysfunction and expression of cell adhesion molecules in endothelial cells match the phenotypes of vascular aging, that is, endothelial cell senescence [18, 36]. Hence, most likely, nLDL could induce the senescence of endothelial cells. Thus, we determined whether nLDL could induce the senescence of primary cultured human endothelial cells (HUVECs).

2. Materials and Methods

2.1. Materials. HUVECs (catalog number MC1133) was purchased from Modern Cell & Tissue Technology (Seoul, Korea) and microvascular endothelial cell medium-2 (EGM-2) was purchased from Cambrex (East Rutherford, NJ, USA). RIPA lysis buffer and protease inhibitor cocktail were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). West-ZOL® (plus) was purchased from iNtRON Biotechnology (Seongnam, Korea). EZ-Cytox® cell viability assay kit was purchased from Daeil Lab Service (Seoul, Korea). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was purchased from Amersham (Buckinghamshire, UK). Propidium iodide (PI) was purchased from Invitrogen (Carlsbad, CA, USA). β-Mercaptoethanol was purchased from Merck (Darmstadt, Germany). RNase A was purchased from Qiagen (Hilden, Germany). LDL, trypsin/EDTA solution, 4-methylumbelliferyl-β-D-galactopyranoside (MUG), 3-{(3-cholamidopropyl)dimethylammonio}-1-propanesulfonate hydrate (CHAPS), phenylmethanesulfonyl fluoride (PMSF), PVDF membrane, and all other reagents were purchased from Sigma (St. Louis, MO, USA).

Various antibodies were used in this study. Anti-human pRb and anti-human phospho-pRb (pS807/pS811) monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA). Polyclonal antibodies against p16-INK4A, cyclin E2, and p53 and monoclonal antibodies against cyclin D1 and p21-CIP1 were purchased from Cell Signaling Technology (Danvers, MA, USA). Monoclonal antibodies against LDL receptor (LDLR), CDK2 and, CDK4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin polyclonal antibody and HRP-conjugated anti-rabbit or anti-mouse secondary antibodies were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell Culture. HUVECs were cultured in EGM-2 medium at 37°C under a humidified atmosphere and 5% CO_2. The cells were either subcultured or cultured continuously on a Petri dish for up to 9 days. Young HUVECs [population doubling level (PDL), 12~15] were inoculated on a Petri dish, stabilized by overnight incubation, and then treated with various concentrations of nLDL (0, 2, 5, or 10 μg protein/mL). Media exchange and nLDL treatment were performed concomitantly at intervals of 3 days. For each subculture, the same number of cells were reinoculated on the same-sized Petri dish. After 3 days of nLDL treatment, the cells were washed thrice with PBS and treated with 0.25% trypsin/EDTA solution for 3 min. The detached cells were harvested, washed thrice with PBS, and used for various assays.

We cultured the cells by the subculture system by default. Both culture systems used here have disadvantages. Although the subculture system is commonly used, it is stressful to the cells to be harvested and inoculated every 3 days. Continuous culture system causes another type of stress to the cells as a consequence of the inoculation with a lesser number of cells. To eliminate the variables that could occur from each culture system, we used both a subculture system and a continuous culture system for some critical assays.

2.3. Determination of Cellular Proliferation. The cellular proliferation of HUVECs was analyzed by using WST-1 (water-soluble tetrazolium salt-1) based cell viability assay kit (EZ-Cytox) [37]. The nLDL-treated cells (1.0 mL) were harvested with trypsin/EDTA, mixed with 100 μL/mL of EZ-Cytox solution, and incubated at 37°C for 2 hr. The mixture was shaken for 1 min and 200 μL of the mixture was transferred to a 96-well microplate. Absorbance of these samples was read at 450 nm with a microplate reader (Epoch, Winooski, VT, USA).

This assay was carried out in the cells either cultured continuously or subcultured. For subculture, the cells on a Petri dish (6×10^4 cells; ϕ, 35 mm dish) were subcultured twice concomitantly with nLDL treatment. Cellular proliferation was assayed at the last day of each subculture and expressed as the relative changes in absorbance of the nLDL-treated cells against that of the nLDL-untreated cells of the same culture duration. For continuous culture, the cells on a Petri dish (5×10^3 cells; ϕ, 35 mm dish) were cultured on the same dish for up to 9 days with nLDL treatment and media exchange. Cellular proliferation was assayed every day and expressed as the relative changes in absorbance of each group of cells to that of the first day inoculated cells without nLDL treatment.

2.4. Determination of Cellular Senescence

2.4.1. SA-β-Gal Activity Assay. The senescence of HUVECs was evaluated by the quantitative SA-β-gal assay using cell

extracts [38]. SA-β-gal activity was measured by quantifying the generation rate of 4-methylumbelliferone (4-MU), the fluorescent hydrolysis product of MUG. Young HUVECs (PDL, 12~15) on a Petri dish (1.0×10^5 cells; ϕ, 10 cm dish) were subcultured and treated with various concentrations of nLDL (0, 2, 5, or 10 μg protein/mL) for up to 9 days. On the last day of each subculture, the cells were washed 6 times with PBS to remove residual growth medium protein that could interfere with subsequent cellular protein determination. The cells were then lysed in 450 μL of 1 × lysis buffer (5 mM CHAPS, 40 mM citric acid, 40 mM sodium phosphate, 0.5 mM benzamidine, and 0.25 mM PMSF, pH 6.0). The lysate was scraped, transferred to a microtube, vortexed vigorously, and centrifuged at 12,000 ×g for 5 min at 4°C. The supernatant was kept on ice until further use. Reaction buffer at 2 × strength [40 mM citric acid, 40 mM sodium phosphate, 300 mM NaCl, 10 mM β-mercaptoethanol, and 4 mM $MgCl_2$ (pH 6.0)] was mixed with 1.7 mM of MUG (SA-β-gal substrate) immediately prior to its use. The reaction buffer containing MUG (45 μL) was mixed with clarified lysate (30 μL) and 1 × lysis buffer (15 μL). Incubation lasted 1 hr, at 37°C in a water bath. After the incubation period, the reaction mixture was added to 400 mM sodium carbonate stop solution (900 μL) and stored at 4°C until measurement of fluorescence. An aliquot of the carbonate-stopped reaction mixture was transferred to a 96-well plate (150 μL/well) and fluorescence was read with a fluorescence microplate reader (VersaMax, Molecular Devices Corp., Philadelphia, PA, USA) with excitation at 360 nm and emission at 465 nm. The SA-β-Gal activity was calculated based on the fluorescence intensity of 4-MU/μg protein and expressed as the relative activity of the nLDL-treated group to the untreated group of the same subculture duration.

2.4.2. SA-β-Gal Staining. The cellular senescence was also evaluated by the SA-β-Gal activity-staining assay [39]. Using the same subculture conditions described in Section 2.4.1, and when appropriate, the cells were fixed with 3% (v/v) formaldehyde in PBS for 3~5 min and washed thrice with PBS. The washed cells were stained with SA-β-Gal staining solution [1 mg/mL X-Gal, 40 mM citric acid in 40 mM sodium phosphate buffer (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$] at 37°C for 16 hr under light protection. The stained cells were washed twice with PBS and coated with 70% glycerol in PBS. The cells were then observed under a phase-contrast microscope (TS100F, Nikon, Tokyo, Japan).

2.5. Cell Cycle Analysis. Cell cycle of the nLDL-treated HUVECs was analyzed according to the flow cytofluorometric assay using PI staining [40]. Briefly, the cells on a Petri dish (1.0×10^5 cells; ϕ, 10 cm dish) were subcultured and treated with nLDL (0, 2, 5, or 10 μg protein/mL) for up to 9 days. When appropriate, the cells were harvested with trypsin/EDTA, washed thrice with PBS, and suspended in 300 μL of PBS. The washed cells were fixed in 70% ice-cold ethanol for 1~2 hr and washed thrice by centrifuging at 700 ×g for 5 min in cold PBS. The fixed cells were treated with 50~100 μL of RNase A (100 μg/mL) for 15 min and then by 100 μL of PI (50 μg/mL) for 15 min under light protection. Cell cycle was analyzed with a FACSCalibur® flow cytometer (BD Biosciences, San Jose, CA, USA) with excitation at 535 nm and emission at 617 nm.

2.6. Western Blot Analysis

2.6.1. Blocking of LDLR with Antibody. To block LDLR, the cells on a Petri dish (2×10^4 cells; ϕ, 35 mm dish) were pretreated with anti-LDLR antibody (20 μg protein/mL) for 1 hr at 4°C. Subsequently, the cells were treated with nLDL (10 μg protein/mL) and cultured for up to 6 days. Senescence induction was analyzed by SA-β-Gal activity assay in subcultured cells and cellular proliferation by tetrazolium salt staining in continuously cultured cells every 3 days.

2.6.2. Analysis of Cell Cycle-Regulating Proteins. Cell cycle-regulating proteins in HUVECs were analyzed by Western blot. As described in Section 2.6.1., the cells were subcultured for up to 6 days and lysed in the RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.004% sodium azide) containing 1 mM PMSF, 0.5 mM sodium orthovanadate, and a protease inhibitor cocktail (10~20 μL per 1 mL lysis buffer). The mixtures were incubated at 4°C for 30 min and centrifuged at 12,000 ×g for 15 min at 4°C. The supernatants were stored at −80°C until further use.

Cell lysates (40 μg protein) were separated by 8~15% gradient polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk in TBS-T buffer (Tris buffered saline-Tween-20 buffer; 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween-20 in 25 mM Tris-base buffer, pH 7.4) at room temperature for 1 hr and incubated overnight with primary antibody at 4°C. After washing thrice in TBS-T buffer, the blot was incubated with the secondary antibody [HRP-conjugated anti-rabbit (1 : 2,000) or anti-mouse (1 : 5,000)] for 2 hr at room temperature. The antibody-specific proteins were detected using the West-ZOL (plus) Western blot detection system (iNtRON Biotechnology, Seongnam, Korea). The primary antibodies were used in the following dilutions: anti-p53 (1 : 1,000), anti-p21-CIP1 (1 : 1,000), anti-p16-INK4A (1 : 1,000), anti-CDK2 (1 : 1,000), anti-CDK4 (1 : 1,000), anti-cyclin E2 (1 : 1,000), anti-cyclin D1 (1 : 1,000), anti-pRb (1 : 250), anti-human pS807/pS811-pRb (1 : 250), and anti-actin (1 : 2,500).

2.7. Measurement of Intracellular ROS. ROS generation in the nLDL-treated HUVECs was analyzed by the method of Royall and Ischiropoulos [41] to evaluate whether intracellular ROS were implicated in the premature senescence of the cells. Young HUVECs on a culture dish (1.0×10^5 cells; ϕ, 10 cm dish) were subcultured and treated with various concentrations of nLDL (0, 2, 5, or 10 μg protein/mL) for up to 9 days. When appropriate, the cells were washed twice with HBSS and incubated at 37°C for 1 hr in a reaction mixture containing 11 μM DCF-DA and 10 mM Hepes, pH 7.4, in phenol-red free EGM-2 medium. After incubation, the cells

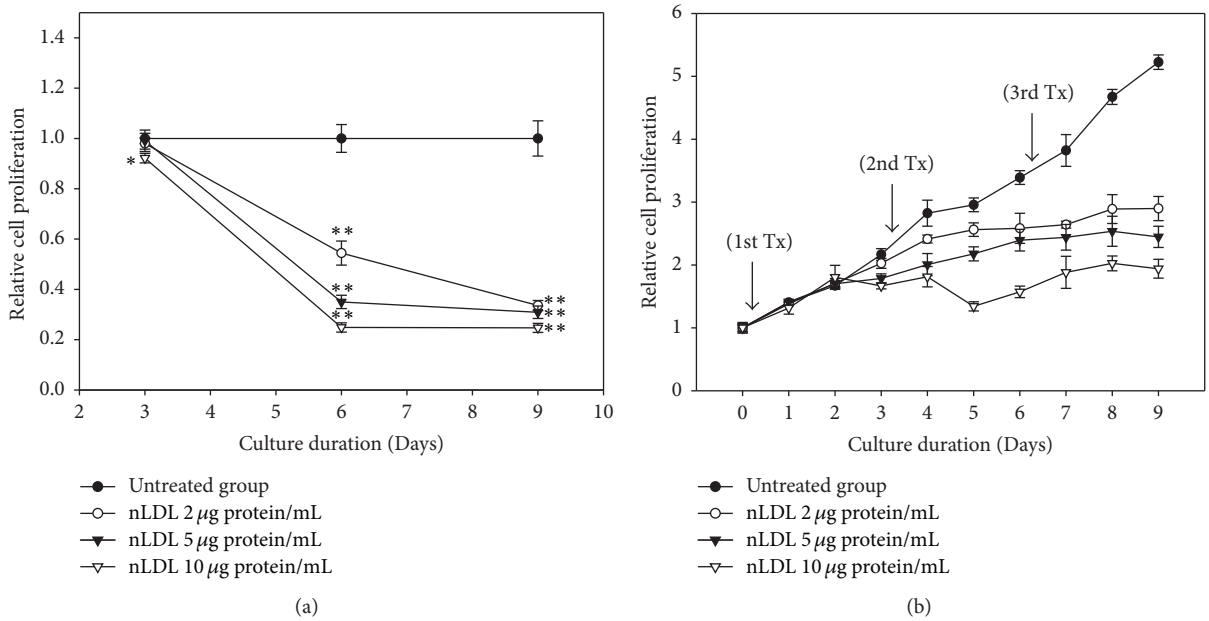

Figure 1: Effect of long-term treatment of nLDL on the proliferation of HUVECs. Young HUVECs (PDL, 12~15) were subcultured at every third day of each subculture with media exchange (a) and cultured continuously in the same culture dish with media exchange (b), for up to 9 days. The cells were treated with various concentrations of nLDL (0, 2, 5, and 10 μg protein/mL) concomitantly with media exchange every 3 days at both culture systems (1st, 2nd, and 3rd Tx). Cellular proliferation of the cells was analyzed by tetrazolium salt method. The degree of cellular proliferation was expressed as the relative ratio of cell number. The dose- and time-dependent differences in cellular proliferation between groups were analyzed statistically by repeated measures ANOVA assay ($p < 0.01$). Each nLDL-treated group was also compared with the respective nLDL-untreated group by independent t-test. $^{*}p < 0.05$; $^{**}p < 0.01$. Each result represents the mean ± SD ($n = 6$).

were washed twice with HBSS, supplemented with 1.5 mL of sonication buffer (50 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, and 0.1% CHAPS), and harvested by scraping. The cells were lysed by sonication (frequency, 24 Hz; duration, 10 sec/cycle; repetition, 6 cycles; time interval, 10 sec). An aliquot of supernatants (200 μL/well) was transferred to a 96-well plate and fluorescence was measured with a fluorescence microplate reader at excitation 502 nm and emission 523 nm. Intracellular content of ROS was expressed as the relative fluorescence intensity/μg protein of the nLDL-treated group to untreated group of the same culture duration.

Intracellular generation of ROS was also observed under fluorescent microscopy. DCF-DA-treated HUVECs were washed with HBSS twice and observed with an inverted fluorescence microscope (TE2000-U, Nikon, Japan) at 100x magnification.

2.8. Protein Quantification and Statistical Analysis. Protein concentration of each sample was measured by BCA® protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Bovine serum albumin was used as a protein standard.

Statistical analysis was performed by using SPSS Statistics 19 (IBM Corp., Armonk, NY, USA). The dose- and time-dependent differences between the groups were analyzed by repeated measures ANOVA assay and simple difference between the data was analyzed by independent t-test. $p < 0.05$ was considered statistically significant. Data was expressed as mean ± standard deviation (SD).

3. Results

3.1. Native LDL Inhibited Proliferation of HUVECs. The cellular proliferation assay was carried out to evaluate the effect of low concentrations of nLDL (0, 2, 5, or 10 μg protein/mL) on the proliferation of cultured HUVECs (Figure 1). The effect of nLDL treatment on cellular proliferation was analyzed in both a subculture system (Figure 1(a)) and a continuous culture system (Figure 1(b)). Native LDL treatment significantly inhibited cell proliferation dose- and time-dependently in both culture systems ($p < 0.01$). Cells treated with nLDL for 3 days in a subculture system showed no significant inhibition of cellular proliferation, except at 10 μg protein/mL ($p < 0.05$). Nevertheless, cells treated with nLDL for 6 or 9 days showed a significant inhibition of cellular proliferation ($p < 0.01$) for all concentrations tested. The inhibition of cell proliferation occurred after 2 days of nLDL treatment in a continuous culture system. The trypan blue assay of the nLDL-treated cells showed that cell death in this study was negligible (data not shown). These results showed that treatment with low concentrations of nLDL could inhibit the proliferation of cultured HUVECs in a dose- and time-dependent way.

3.2. Native LDL-Induced Senescence of HUVECs. Next, we evaluated the role of senescence of HUVECs in the nLDL-induced inhibition of cellular proliferation. The cells were treated with low concentrations of nLDL (0, 2, 5, and 10 μg protein/mL) and cellular senescence was analyzed by

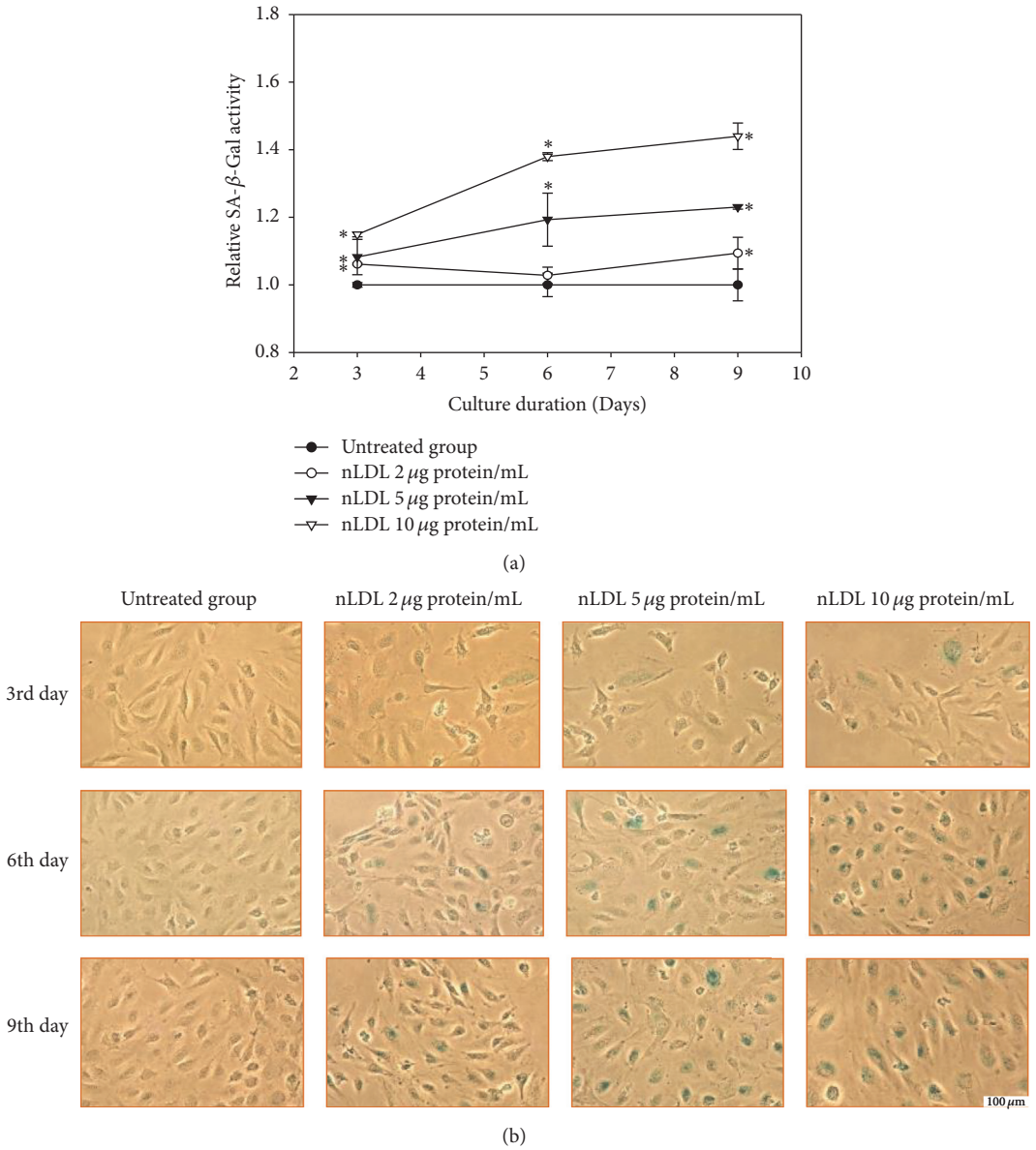

FIGURE 2: Senescence induction in HUVECs by long-term treatment of nLDL (0, 2, 5, and 10 μg protein/mL), for up to 9 days. (a) Quantitative assay of SA-β-Gal activity after nLDL treatment; (b), SA-β-Gal activity staining after nLDL treatment. HUVECs were treated with nLDL at the start of each subculture and assayed for SA-β-Gal activity at the end of each subculture. The SA-β-Gal activity was expressed as the generation rate of 4-methylumbelliferone (MU)/μg protein against that of the nLDL-untreated group. The dose- and time-dependent differences in SA-β-Gal activity between groups (a) were analyzed statistically by repeated measures ANOVA assay ($p < 0.01$). Each nLDL-treated group was also compared with the respective nLDL-untreated group by independent t-test ($^* p < 0.01$). Each result represents the mean ± SD ($n = 3$).

quantitative assay (Figure 2(a)) as well as staining (Figure 2(b)) of SA-β-Gal activity in a subculture system for up to 9 days. Native LDL significantly increased the enzyme activity dose- and time-dependently in the quantitative assay ($p < 0.01$). Native LDL also increased staining of the enzyme activity. The SA-β-Gal activity of each nLDL-treated group was also compared with the respective nLDL-untreated group by independent t-test. The SA-β-Gal activity was significantly increased at 3, 6, and 9 days of all concentrations of the nLDL treatment ($p < 0.01$). The increased SA-β-Gal activity preceded the decreased cellular proliferation for the lower nLDL concentrations. These results suggested that the nLDL-induced inhibition of cellular proliferation could at least partly result from cellular senescence.

3.3. Native LDL-Induced Senescent Cells Were Arrested at G1 Phase of Cell Cycle. In the next experiment, we analyzed the change in the distribution of cell cycle phase of the nLDL-induced senescent HUVECs, in a subculture system (0, 2, 5, and 10 μg protein/mL) for up to 9 days (Figure 3). Cell cycle analysis with flow cytometry after PI staining indicated that the distribution of G1 phase cells was significantly increased

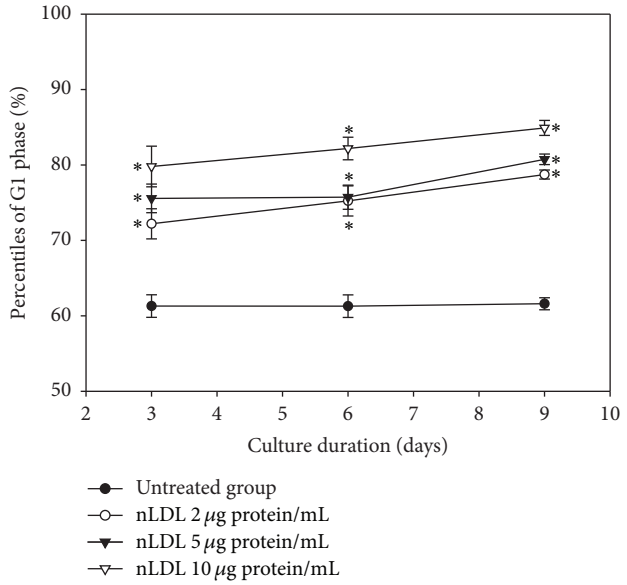

Figure 3: G1 arrest induction in HUVECs by long-term treatment of nLDL. Cell cycle was assayed by flow cytometry in the nLDL-treated cells, for up to 9 days, at the end of each subculture. The distribution percentiles of G1 phase cells after nLDL treatment (0, 2, 5, and 10 μg protein/mL) were shown as a line graph. The dose- and time-dependent differences in G1 phase distribution between groups were analyzed statistically by repeated measures ANOVA assay ($p < 0.01$). Each nLDL-treated group was also compared with the respective nLDL-untreated group by independent t-test ($^*p < 0.01$). Each result represents the mean ± SD ($n = 6$).

($p < 0.01$) and the distribution of S and G2/M phase cells was significantly decreased (data not shown, $p < 0.01$) in a dose- and time-dependent way. The distribution of G1 phase cells at each nLDL-treated group was also compared with the respective nLDL-untreated group by independent t-test. The distribution of G1 phase cells was significantly increased at all concentrations of nLDL tested ($p < 0.01$). These results indicated that the nLDL-induced senescent HUVECs were arrested at G1 phase of cell cycle.

3.4. Native LDL-Induced Cellular Senescence Resulted from NLDL Itself. To confirm that the nLDL-induced cellular senescence in HUVECs did not result from oxLDL generated from nLDL during in vitro incubation, we pretreated the cells with the monoclonal antibody against LDLR (anti-LDLR antibody) to block cellular LDLR before nLDL treatment (10 μg protein/mL). The SA-β-Gal activity assay (Figure 4(a)) was carried out in subcultured cells and the cellular proliferation assay (Figure 4(b)) was carried out in continuously cultured cells. Both assays were performed at the cells cultured for up to 6 days, since 10 μg protein/mL nLDL showed nearly maximum effect at 6 days (Figures 1(a) and 2(a)). The effect of nLDL on both cellular senescence parameters was significantly and negatively modulated by anti-LDLR antibody pretreatment at independent t-test ($p < 0.01$) as well as repeated measures ANOVA assay ($p < 0.01$). These results suggested that the nLDL-induced cellular senescence of HUVECs resulted from nLDL itself, and not oxLDL.

3.5. Cellular Senescence by NLDL Was Induced via Both p53 and p16-pRb Signal Pathways. To evaluate the signal transduction pathway involved in senescence induction with nLDL (10 μg protein/mL), we conducted Western blot analysis for some cell cycle-regulating proteins at the last day of each subculture (Figure 5) for up to 6 days. As expected, the content of p53, p21, and p16 proteins was significantly increased in the nLDL-induced senescent cells (B-1). Two cyclin/CDK complexes such as CDK4/6-cyclin D and CDK2-cyclin E are known to phosphorylate pRb to overcome G1 arrest. The protein content of these complexes, that is, CDK2, cyclin E2, CDK4, and cyclin D1, was significantly decreased in the senescent cells (B-2 and B-3); and, as a result, the phosphorylation of pRB was also significantly inhibited (B-4). These results suggested that the nLDL-induced cellular senescence in HUVECs could result from the inhibition of pRb phosphorylation (i.e., G1 arrest of cell cycle) by the inhibition of the two cyclin/CDK complexes (CDK4/6-cyclin D and CDK2-cyclin E) via both the p53 and p16 signal transduction pathways. Pretreatment with anti-LDLR antibody restored in varying degrees the changes in the levels of the cell cycle-regulating proteins induced by nLDL treatment (Figure 5). These results corroborated the results of Figure 4 that pretreatment of anti-LDLR antibody prevented senescence induction in HUVECs.

3.6. Native LDL-Induced Senescent Cells Were Arrested Permanently at G1 Phase. Although G1-arrested young cells can be reactivated to proliferating cells by treating with serum or other mitogenic agents, senescent cells theoretically cannot overcome the G1 checkpoint. In line with this, we tried to determine whether the nLDL-induced G1 phase arrest of HUVECs was a temporary or a permanent phenomenon. As a matter of convenience, we wanted to show the irreversibility of cellular senescence (i.e., G1 arrest) induced by the lowest concentration of nLDL. If cellular senescence induced by the lowest concentration of nLDL (2 μg/mL) was not reversed, cellular senescence by higher concentrations of nLDL would be irreversible. After inducing cellular senescence by nLDL treatment (2 μg protein/mL) with subculture for up to 9 days (first cycle of subculture), the cells were washed with EGM-2 medium twice and subcultured again in the same medium without nLDL for up to 6 days (second cycle of subculture). We thought that subculture for 6 days would be enough to show the cell proliferating trend at in vitro culture condition. Flow cytometric cell cycle analysis with PI was carried out subsequently.

The first cycle of subculture with nLDL treatment increased the distribution of G1 phase cells, as compared to the nLDL-untreated cells (Figure 3). After washing out nLDL, the second cycle of subculture for up to 6 days failed to return the cells to their original proliferative state ($p > 0.05$; Table 1). The G1-arrest of HUVECs induced by the lowest concentration of nLDL (2 μg protein/mL) was not reversed by washing out nLDL from the cells. This result indicated that

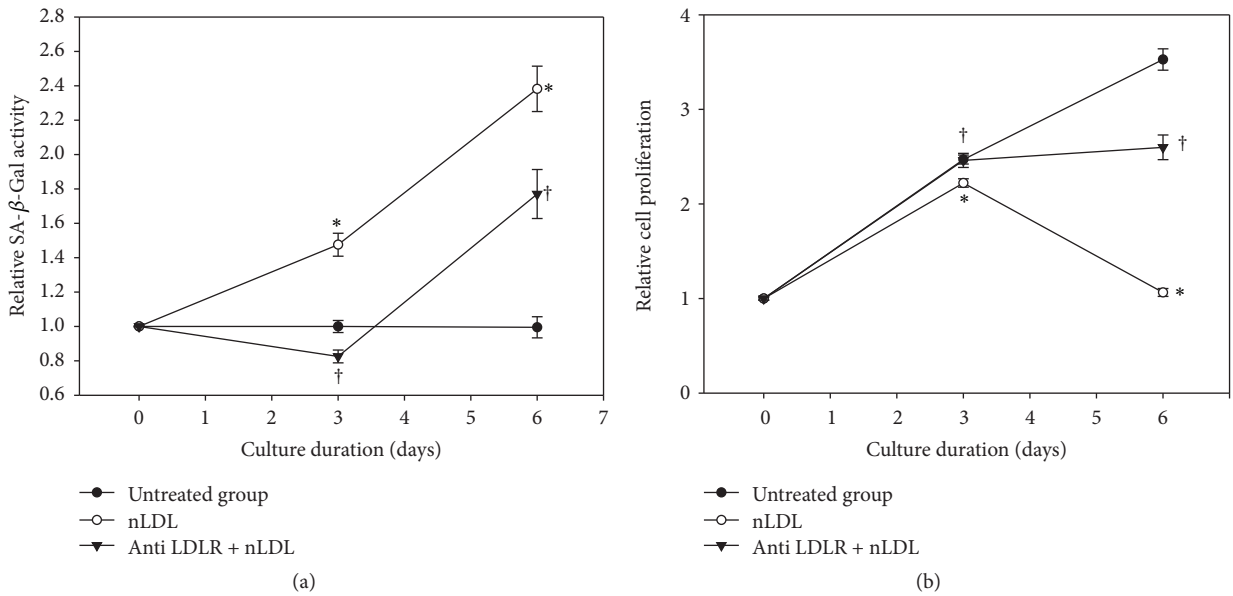

Figure 4: Effect of LDL receptor (LDLR) blocking with antibody on the nLDL-induction of senescence in HUVECs. The cells were pretreated with anti-LDLR antibody (20 μg protein/mL) before nLDL (10 μg protein/mL) treatment. The cells were cultured for up to 6 days. Senescence induction was carried out by SA-β-Gal activity assay in subcultured cells (a) and cellular proliferation by tetrazolium salt staining in continuously cultured cells (b), every 3 days. The time-dependent difference in SA-β-Gal activity or cellular proliferation between treatment groups was analyzed statistically by repeated measures ANOVA assay ($p < 0.01$). And also, each nLDL-treated group was compared with the respective nLDL-untreated group ($^{*}p < 0.01$) and each anti-LDLR antibody plus nLDL-treated group was compared with the respective nLDL-treated group ($^{\dagger}p < 0.01$) by independent t-test. Each result represents the mean ± SD ($n = 6$).

nLDL-induced senescence of cultured endothelial cells was an irreversible change.

3.7. Intracellular ROS Generation Was Implicated in NLDL-Induced Cellular Senescence. In this experiment, we determined the role of ROS in the nLDL-induced senescence of HUVECs. The cells were treated with nLDL (0, 2, 5, and 10 μg protein/mL) concomitantly with media exchange in a subculture system for up to 9 days. ROS generation in the nLDL-treated cells was measured by spectrofluorometry (Figure 6(a)) and fluorescence microscopy (Figure 6(b)) with DCF-DA as a chemical probe. The intracellular ROS generation was significantly increased in the nLDL-treated cells dose- and time-dependently ($p < 0.01$). Cells treated with nLDL for 3 days in a subculture system showed no significant ROS generation by independent t-test. Nevertheless, cells treated with nLDL for 6 or 9 days showed a significant increase in ROS generation ($p < 0.05$ or 0.01) for all concentrations tested.

4. Discussion

In this study, we used both a subculture system and a continuous culture system to eliminate the variables that could occur from each culture system for up to 9 days of culture. Long-term treatment with low concentrations of nLDL (2~10 μg protein/mL) inhibited the proliferation of HUVECs in both culture systems. The inhibition of cell proliferation was shown after 2 days of nLDL treatment, suggesting that as the concentrations of nLDL were very low, the effect required amplification within the cells. The result that the endothelial cell's senescence induced by nLDL treatment preceded their decreased proliferation suggested that the cellular senescence might be responsible for the decreased cellular proliferation. We first showed that nLDL could induce premature senescence of cultured cells.

The cellular distribution to G1 phase was increased by nLDL treatment. The nLDL-induced senescent cells were arrested at G1 phase of cell cycle. Moreover, the senescent cells did not escape from G1-arrest even on consecutive subculture for up to 6 days after removal of nLDL. These results suggest that the nLDL-induced G1-arrest of HUVECs is permanent and irreversible.

The oxidative status of nLDL itself is very important in this study, since oxLDL might induce premature senescence of cultured cells. The oxidative status of nLDL isolated from the serum of healthy men has been found variable. Colas et al. [42] reported that the degree of lipid peroxidation of LDL was about 245 fmol malondialdehyde (MDA)/μg LDL protein (49.5 pmol MDA/mg cholesterol). However, Han and Pak [43] reported a much lower degree of lipid peroxidation (0.8 fmol MDA/μg LDL protein). The lipid peroxidation level of nLDL used in this study was 8.4 fmol MDA/μg LDL protein. The oxidative status of the nLDL used in this study was about 30 times lower than the value by Colas et al. [42] but about 10 times higher than the value by Han and Pak [43]. Thus, the nLDL used in this study is within the normal range of oxidative status from healthy men.

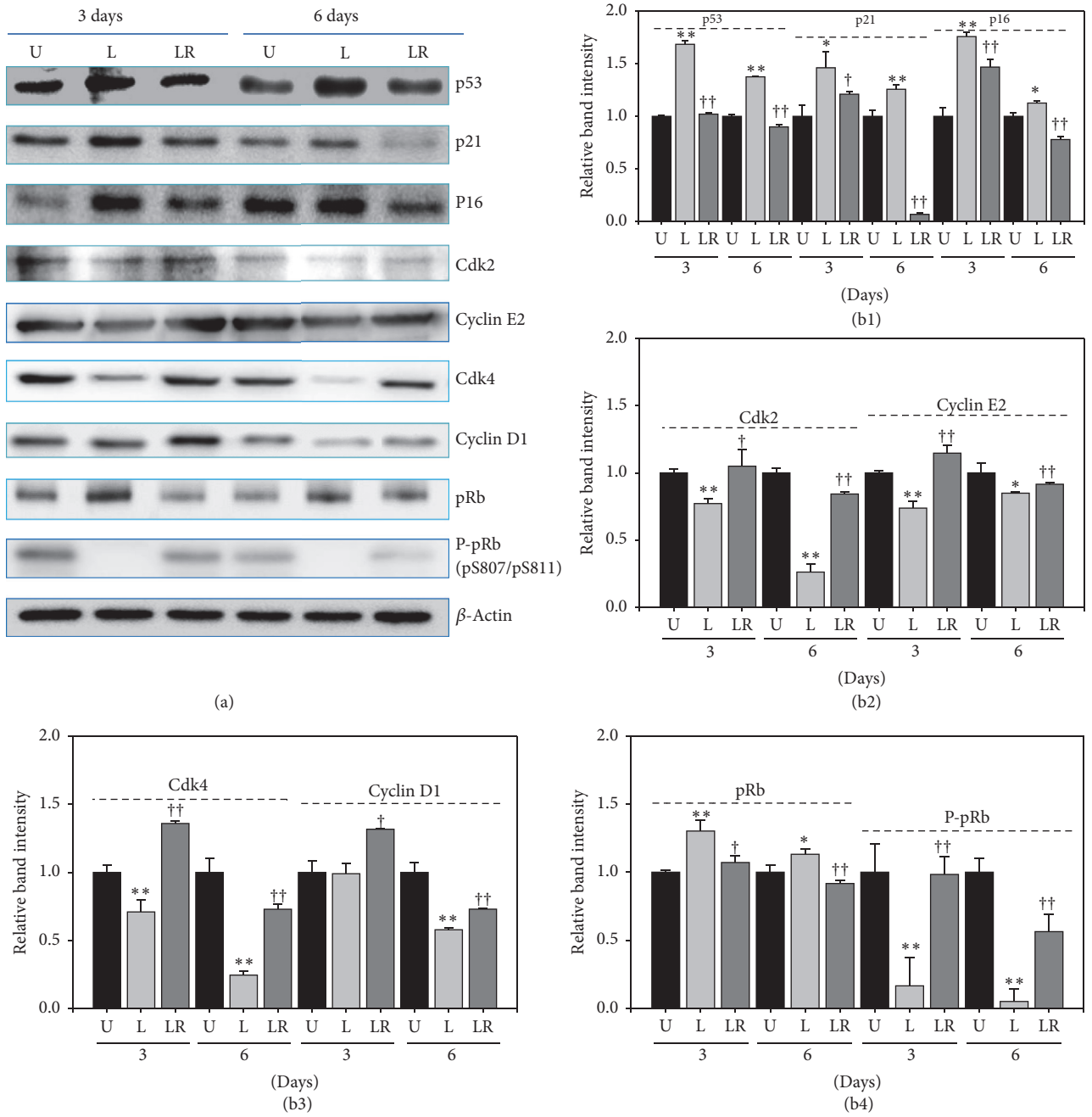

FIGURE 5: Changes in the content of some cell cycle-regulating proteins by long-term treatment of nLDL (10 μg protein/mL) in HUVECs and their restoration by pretreatment with anti-LDLR (20 μg protein/mL). The cells were untreated with nLDL (U), treated with nLDL (L), or treated with both anti-LDLR and nLDL (LR) at each subculture, for up to 6 days. At the end of each subculture, the content of some cell cycle-regulating proteins was assayed by Western blot analysis. (a) Western blot images; (b) quantification of Western blot bands. (b1) The contents of p53, p21, and p16 proteins were increased by nLDL treatment, with these effects compromised by the anti-LDLR pretreatment. (b2) The nLDL-induced decrease in Cdk2 and Cyclin E2 was compromised by the anti-LDLR pretreatment. (b3) The nLDL-induced decrease in Cdk4 and Cyclin D1 was compromised by anti-LDLR pretreatment. (b4) The content of pRb was increased and that of P-pRb (phosphorylated pRb) was decreased, with these effects compromised by the anti-LDLR pretreatment. Group L was compared with the nLDL-untreated group (U) by independent t-test ($^{*}p < 0.05$ and $^{**}p < 0.01$). Group LR was compared with group L by independent t-test ($^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$).

Table 1: Native LDL-induced G1 arrest of HUVECs was not reversed by nLDL removal. The cells were treated with nLDL (2 μg protein/mL) for 3, 6, and 9 days (first cycle of subculture) and the G1-arrested cells were subcultured again for 3 and 6 days without nLDL (second cycle of subculture). Cell cycle was analyzed by flow cytometry at the end of second subculture. The 3 and 6 days groups of the second cycle of subculture were compared with the 0 day group of the second cycle of subculture by independent t-test ($p > 0.05$). Each result represents the mean ± SD (n = 3-4).

First cycle of subculture of nLDL pretreatment (2 μg protein/mL)	Second cycle of subculture after nLDL removal	G1 phase	p value
3 days	0 days	76.2 ± 0.7	$p > 0.05$
	3 days	76.3 ± 1.7	
	6 days	77.8 ± 4.5	
6 days	0 days	77.1 ± 0.7	$p > 0.05$
	3 days	77.7 ± 0.9	
	6 days	79.3 ± 1.9	
9 days	0 days	80.7 ± 0.7	$p > 0.05$
	3 days	82.5 ± 0.9	
	6 days	84.8 ± 2.6	

Moreover, because nLDL could be oxidized to oxLDL during in vitro culture, we needed to ensure that the senescence was induced not by oxLDL but by nLDL itself. For this purpose, we blocked the receptor for nLDL (LDLR) with the anti-LDLR antibody before nLDL treatment as described by Allen et al. [44], on the basis that oxLDL does not bind to LDLR [45]. Anti-LDLR antibody pretreatment suppressed the nLDL-induced HUVECs senescence. This result suggested that nLDL itself may be endocytosed into the cultured endothelial cells to induce premature senescence of the cells. There is another subfraction of LDL, minimally modified LDL (mmLDL), that is sufficiently modified to be chemically distinguished from nLDL. Despite its modification, mmLDL retains the ability to bind to LDLR [46]. So far, this unusual character of mmLDL makes it hard to differentiate the effect of nLDL from that of mmLDL. Further studies are required to solve this problem.

Next, we tried to identify the signal transduction pathway involved in the nLDL-induced endothelial senescence. Growth arrest of senescent cells is maintained by the p53 and/or p16-pRb signal transduction pathways [47–49]. Hence, we conducted Western blot analysis of some of the cell cycle-regulating proteins that are related to these signal transduction pathways and G1 arrest of senescent cells. The level of p53 was increased. In addition, the level of dephosphorylated form of pRb was increased, but that of phosphorylated pRb at S807 and S811 [P-pRb (pS807/pS811)] was decreased, indicating that suppression of the phosphorylation of the pRb protein is the cause of senescence induction in the cells. The level of CDK inhibitors such as p21 and p16 was also increased, but the level of cyclin/CDK complex proteins such as Cdk2, Cdk4, Cyclin E2, and Cyclin D1 was decreased. These changes in the level of the cell cycle-regulating proteins in the nLDL-treated cells suggested that nLDL-induced G1 arrest of HUVECs could be due to the inhibition of pRb phosphorylation through both the p53 and p16-pRb signal transduction pathways.

Pretreatment of anti-LDLR antibody restored in varying degrees the changes in the level of the cell cycle-regulating proteins induced by nLDL treatment. This result corroborated the results of Figure 4 that pretreatment of anti-LDLR antibody prevented nLDL-induced senescence induction in HUVECs.

We also showed that long-term treatment with low concentrations of nLDL could stimulate the generation of ROS in HUVECs. This result suggested that ROS might be implicated in the premature senescence of cultured endothelial cells by nLDL treatment.

The senescence-inducing pathways could be initiated by diverse stressful conditions such as telomere shortening, DNA damage, oncogene activation, lack of nutrients, or growth factors, and oxidative stress [47, 49]. Native LDL reportedly can generate superoxide radical ($O_2^{-\bullet}$) instead of NO in endothelial cells or tissues, including HUVECs, by uncoupling of eNOS [50–53]. Relatively high concentrations of nLDL (2.4 mg cholesterol/mL) were used in these studies, to show the stimulating effect of nLDL on the generation of ROS in HUVECs. Here, we demonstrated that the premature senescence of the endothelial cells could be induced by long-term treatment, at very low concentrations of nLDL (4.4~22.2 μg cholesterol/mL).

Collectively, our results suggested that long-term treatment with low concentrations of nLDL could induce premature senescence of cultured endothelial cells. Native LDL is endocytosed into the cells through the LDLR and probably generates ROS to induce cellular senescence via both p53 and p16-pRb signal transduction pathways.

The findings from this study are not applicable to in vivo human pathophysiology, since in terms of antioxidant capacity in vitro culture condition is quite different from in vivo cellular environment. Nevertheless, the nLDL-induced senescence of cultured HUVECs could be used as a model system for the study of premature cellular senescence in in vitro aging conditions. Human body, including the circulatory system, has well-developed homeostatic defense systems that can protect from oxidative stress. Nevertheless, when the homeostatic balance between prooxidant and antioxidant

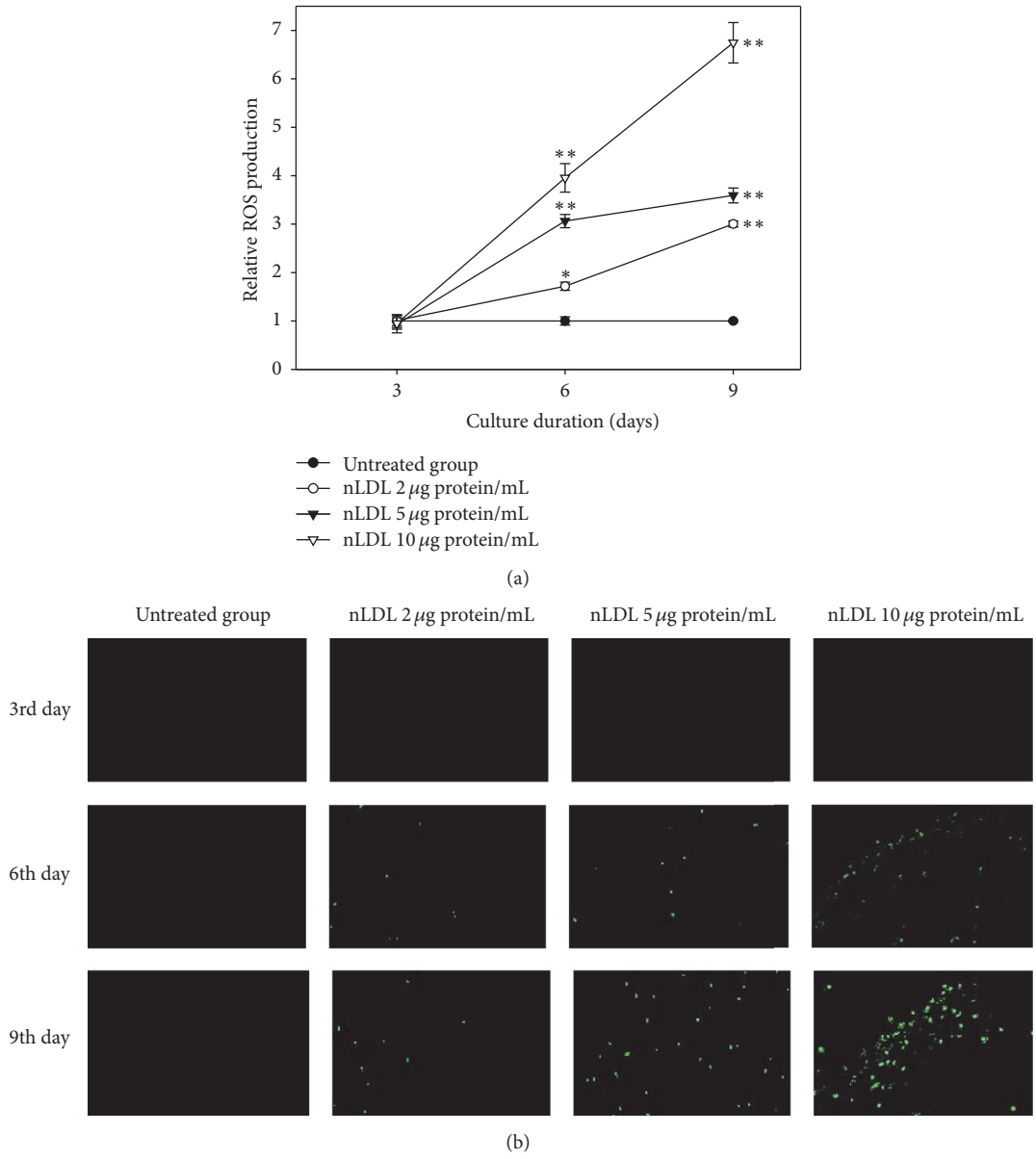

Figure 6: The generation of reactive oxygen species (ROS) with nLDL in HUVECs. The cells were subcultured and treated with nLDL (0, 2, 5, and 10 μg protein/mL) every 3 days of each subculture, for up to 9 days. ROS generation in nLDL-treated cells were analyzed by spectrofluorometry (a) and fluorescence microscopy (b) assays with DCF-DA at the end of each subculture. ROS generation was expressed as the relative fluorescence intensity/μg of protein of the nLDL-treated group, as compared to the untreated group. The dose- and time-dependent differences in ROS generation (a) between groups were analyzed statistically by repeated measures ANOVA assay ($p < 0.01$). Each nLDL-treated group was also compared with the respective nLDL-untreated group by independent t-test ($^{*}p < 0.05$ and $^{**}p < 0.01$). Each result represents the mean ± SD (n = 3-4).

system in the plasma and endothelial cells is disturbed for a relatively long period, nLDL as well as modified LDL, such as oxLDL, could induce the premature senescence of vascular endothelial cells.

In summary, this is the first report describing nLDL-induced senescence of vascular endothelial cells, at least, partly via oxidative stress under in vitro culture condition. The nLDL-induced senescence of vascular endothelial cells could be used as a model system for in vitro aging study. Further studies are required to apply this finding to in vivo human pathophysiology.

Disclosure

This article contains a part of the degree thesis "Induction of Premature Senescence with Native Low-Density Lipoprotein in Human Vascular Endothelial Cells" by Hoon Park (Doctor of Medical Sciences) at Chonnam National University, 2012.

Competing Interests

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

Authors' Contributions

Sung-Tack Oh and Hoon Park contributed equally to this work.

Acknowledgments

This study was supported by grants from Chonnam National University Hospital Research Institute (CRI 11024-1) and from Chonnam National University (2013-2591).

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 9270549, 23 pages
https://doi.org/10.1155/2017/9270549

Review Article

Is p53 Involved in Tissue-Specific Insulin Resistance Formation?

Justyna Strycharz,[1] Jozef Drzewoski,[2] Janusz Szemraj,[3] and Agnieszka Sliwinska[4]

[1]*Diabetes Student Scientific Society at the Department of Internal Diseases, Diabetology and Clinical Pharmacology, Medical University of Lodz, Lodz, Poland*
[2]*Department of Internal Diseases, Diabetology and Clinical Pharmacology, Medical University of Lodz, Lodz, Poland*
[3]*Department of Medical Biochemistry, Medical University of Lodz, Lodz, Poland*
[4]*Department of Nucleic Acid Biochemistry, Medical University of Lodz, Lodz, Poland*

Correspondence should be addressed to Agnieszka Sliwinska; agnieszka.sliwinska@umed.lodz.pl

Received 16 September 2016; Accepted 19 December 2016; Published 17 January 2017

Academic Editor: Miguel Constância

p53 constitutes an extremely versatile molecule, primarily involved in sensing the variety of cellular stresses. Functional p53 utilizes a plethora of mechanisms to protect cell from deleterious repercussions of genotoxic insults, where senescence deserves special attention. While the impressive amount of p53 roles has been perceived solely by the prism of antioncogenic effect, its presence seems to be vastly connected with metabolic abnormalities underlain by cellular aging, obesity, and inflammation. p53 has been found to regulate multiple biochemical processes such as glycolysis, oxidative phosphorylation, lipolysis, lipogenesis, β-oxidation, gluconeogenesis, and glycogen synthesis. Notably, p53-mediated metabolic effects are totally up to results of insulin action. Accumulating amount of data identifies p53 to be a factor activated upon hyperglycemia or excessive calorie intake, thus contributing to low-grade chronic inflammation and systemic insulin resistance. Prominent signs of its actions have been observed in muscles, liver, pancreas, and adipose tissue being associated with attenuation of insulin signalling. p53 is of crucial importance for the regulation of white and brown adipogenesis simultaneously being a repressor for preadipocyte differentiation. This review provides a profound insight into p53-dependent metabolic actions directed towards promotion of insulin resistance as well as presenting experimental data regarding obesity-induced p53-mediated metabolic abnormalities.

1. Introduction

Obesity is now referred to as a global pandemic, and its prevalence is expected to grow in enormously rapid way [1, 2]. Environmental factors such as high calorie intake accompanied by reduced energy expenditure are still perceived as its major drivers, what explains profound epigenetic research [3]. Nevertheless, the genetic aspect of obesity has never been ignored [4–6]. Obesity constitutes an excellent background for a plethora of life-threatening disorders such as type 2 diabetes (T2D), cardiovascular diseases, NAFLD (nonalcoholic fatty liver disease), and many cancers [7–10]. Inevitably, adiposity is tightly linked to metabolic syndrome (Mets). The latter one encompasses a group of metabolically related cardiovascular risk factors, also known to augment the progression of diabetes [11]. The escalating global incidence of T2D is alarming, being enriched by the expectance of diabetes to become 7th leading cause of death in 2030 [12]. Due to such dramatic data, there is a great need to investigate the pathomechanism underlying diabetes, which is always accompanied by numerous vascular complications [13]. p53 is an incredibly versatile molecule mainly involved in sensing cellular stresses, thereby acting as a potent tumour suppressor [14]. An accumulating amount of data suggests that p53 is involved in the pathomechanism of metabolic abnormalities triggered by obesity and hyperglycemia. This review summarizes findings regarding p53 role in the phenomenon of insulin resistance.

2. Insulin Signalling Pathway and Insulin Resistance

2.1. Insulin Signalling. Insulin is an anabolic hormone involved in the regulation of glucose and lipid homeostasis

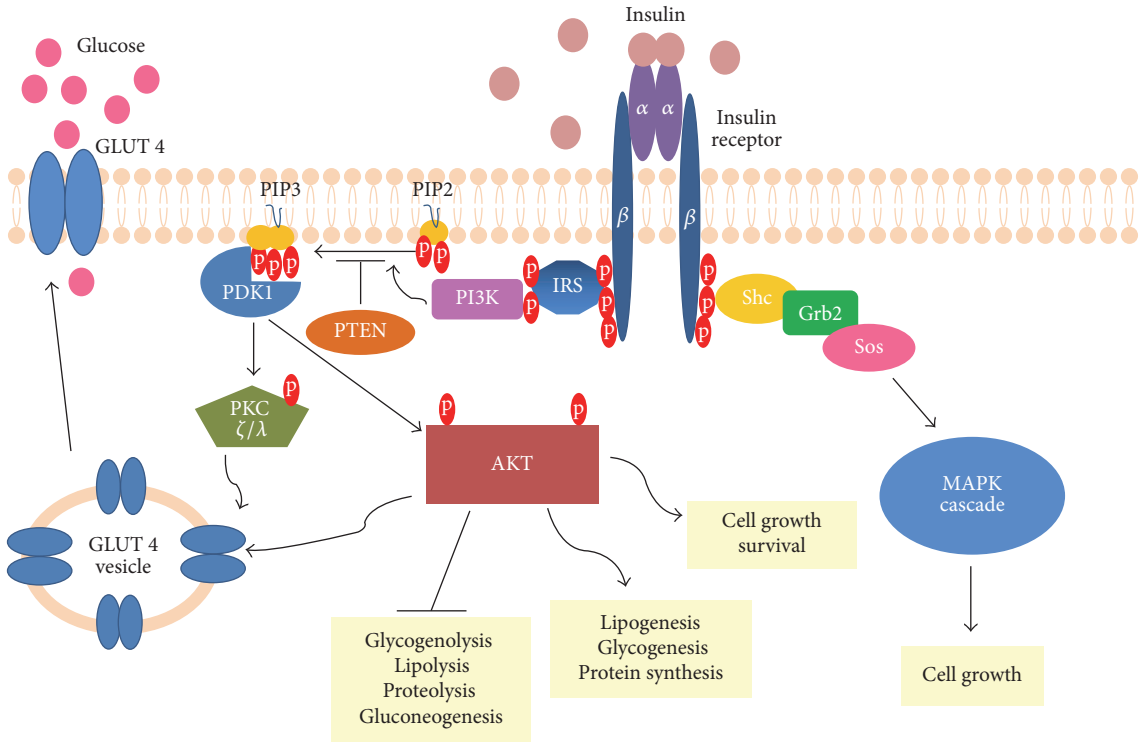

Figure 1: Insulin signalling pathway. Upon insulin stimulation, activation of insulin receptor and thus formation of many phosphotyrosine residues do occur. Binding of IRS proteins and their phosphorylation precedes recruitment of PI3K as well as subsequent PIP2 phosphorylation. Note that PIP3 can be converted to PIP2 through PTEN activity. PDK1 gets activated upon binding to PIP3, followed by the phosphorylation of PKC ζ/λ and AKT, which stimulate GLUT4 translocation towards cell membrane and therefore glucose influx. Phosphorylated AKT mediates the repression of glycogenolysis, lipolysis, proteolysis, and gluconeogenesis as well as the activation of lipogenesis, glycogenesis, protein synthesis, and cell survival. The stimulation of insulin receptor is also accompanied by binding of adaptor proteins, known as Shc. The subsequent binding of Grb2 and Sos proteins initiates the MAPK cascade pathway. The activation of AKT and MAPK cascade results in the promotion of cell growth.

[15]. Insulin receptor (IR) is a tyrosine kinase receptor, which requires dimerization and binding of insulin molecules for activation. This heterotetrameric receptor is made up of 2 extracellular α subunits and 2 transmembrane β subunits. Autophosphorylation of IR results in creation of many phosphotyrosine residues, which serve as docking sites for subsequent components of this signalling pathway. PTP-1B (protein-tyrosine phosphatase 1B) utilizes its phosphatase activity to reverse the process of receptor activation, thus being one of several negative regulators of insulin signalling [16]. Multiple phosphotyrosines allow for recruitment and phosphorylation of various substrate proteins, where IRS (insulin-receptor substrate) proteins deserve special attention [17]. Phosphorylated IRSs activate and direct PI3K (phosphatidylinositol-3-kinase) to plasma membrane. PI3K phosphorylates PIP2 (phosphatidylinositol 4,5-bisphosphate), thereby generating PIP3 (phosphatidylinositol-3,4,5-trisphosphate). PIP3 is the key lipid signalling intermediate undergoing dephosphorylation by two lipid phosphatases, SHIP2 (SH2-containing inositol 5′-phosphatase-2) and PTEN (phosphatase and tensin homolog) [18]. Increased level of PIP3 activates serine threonine kinase PDK1 (phosphoinositide-dependent protein kinase-1), thus allowing phosphorylation and subsequent activation of PKB/AKT (protein kinase B also known as AKT) and atypical PKC ζ/λ (protein kinase C) [19, 20]. Both of them increase insulin-induced glucose uptake by triggering translocation of GLUT4 (glucose transporter 4) from intracellular vesicles to cell membrane. Not only is AKT an antiapoptotic molecule [21], but also its activity results in all metabolic effects mediated by insulin [22]. AKT phosphorylates numerous downstream targets, thereby repressing glycogenolysis, lipolysis, proteolysis, and gluconeogenesis. On the other hand, AKT stimulates lipogenesis, glycogenesis, and protein synthesis. Undoubtedly, insulin is a potent growth factor triggering cell growth and differentiation [15]. Its mitogenic activity is exerted mainly through stimulation of mitogen-activated protein kinase (MAPK) cascade. Insulin signalling pathway is depicted in Figure 1.

2.2. Insulin Resistance. Insulin action is indispensable for the maintenance of energy balance. It affects a large number of kinases and enzymes during fasting and feeding periods to enable proper functioning of the entire organism. Insulin resistance is the condition characterized by lowered response of cells to circulating insulin. While the precise molecular mechanism remains to be elucidated, insulin resistance is always manifested by disturbances in insulin signalling, thus

leading to its attenuation. There are several molecular pathways which have been suggested to play a great role in this complex metabolic disorder [23]. As obesity is still the major risk factor, the role of free fatty acids is of crucial importance. Physiologically, insulin is secreted from β cells upon raised glucose level and acts on tissues enabling quick storage of energy surplus. Therefore, insulin signalling ultimately leads to translocation of tissue-specific glucose transporters to cell membrane, causing glucose influx. When calorie intake exceeds the demanded one, energy is primarily deposited in white adipocytes, which are the only cells for safe fat storage [24]. If this state is prolonged, hypertrophy and hyperplasia of adipocytes set in. Hence, the induction of adipose tissue hypoxia leads to low grade chronic inflammation, which triggers insulin resistance [25]. As insulin cannot further store energy in adipocytes, its raised amount is needed for compensation. Consequently, β cells undergo adaptive changes in order to produce and secrete an incredibly large amount of insulin. While insulin sensitivity is decreased, its inhibitory impact on lipolysis is relieved. This results in increased level of circulating free fatty acids subsequently taken up by muscles, liver, and pancreas. Thus, all these tissues become affected by lipotoxicity leading directly to the induction of nonadipose tissue insulin resistance. Then, gluconeogenesis and glycogenolysis are no longer suppressed, triggering glucose output from the liver. Meanwhile, inflammation in adipocytes exacerbates in parallel with their necrosis due to intensified macrophages infiltration. Hyperinsulinemia leads to β-cells exhaustion which makes their function decline. Therefore, the glucose intolerance along with hyperglycemia is established [26]. β cell mass reduction up to 60% precedes the first clinical manifestations of type 2 diabetes [27]. Clearly, insulin resistance is closely associated with obesity and is underlain by oxidative stress along with inflammation [26].

3. p53 Overview

TP53 gene has been declared the major tumor suppressor gene [28], as the prevailing number of its actions are perceived by the prism of conferring protection to the genome. Consequently, loss of wild type p53 is commonly linked to the phenomenon of tumorigenesis [29], making p53 reactivation a miraculous and promising target in anticancer therapy [30]. This transcription factor is broadly known to be activated in the presence of the plethora of genotoxic insults, like hypoxia, oxidative stress, DNA damage, and oncogene activation, to mention only the most important ones [14]. Depending on the stress severity and the extent of DNA damage, p53 decides whether to save the affected cell or perform the suicidal death to protect it from changing its phenotype to a malignant one. Activated p53 is forced to implement immediate antiproliferative program, yet this is accomplished due to a range of cellular events such as apoptosis, senescence (irreversible cell growth arrest), or cell cycle arrest [14, 31–33]. Induction of cell cycle arrest gives p53 a possibility to perform DNA repair [34], underscoring the relevance of p53 as a mediator of prosurvival and cytoprotective effect. On the other hand, apoptosis and senescence are claimed to be leading ways of p53 tumor suppressive program, as they are realized when the stress causes irreparable and highly hazardous DNA damage. Regardless of the type of implemented actions, p53 always aims to stop the propagation of deleterious mutations. Nevertheless, the exact molecular and mechanistic implications enabling p53 to choose between living or dying still arouse some controversy [35].

TP53 gene product is a broadly known transcriptional activator without being a direct transcriptional repressor [36]. Alternatively, p53 can mediate transrepression by preventing the binding of other transcriptional activators to their target sites [37]. Its nontranscriptional activities involve direct protein-protein interactions with Bcl2 family of apoptosis-regulatory proteins [38, 39]. Thereby, p53 induces mitochondrial outer membrane permeabilization and cytochrome c release, finally triggering apoptosis.

The major cellular gatekeeper [40] is entangled into the complex gene regulatory network composed of its mediators, effectors, and coregulators [41, 42]. Mediators are sensitizers of stresses determining p53 actions by marking it with adequate PTMs (posttranslational modifications). In this review, ATM (ataxia teleangiectasia mutated) and AMPK (AMP-activated protein kinase) are of great importance. Effectors constitute a group of more than one hundred genes, which exhibit p53-mediated specific cellular effects. p21 is considered as a master p53 effector participating in the cell cycle arrest and senescence [43, 44]. Its expression level is undoubtedly an indicator of p53 tumour suppressor activity. Moreover, p53 is known to increase expression of proapototic genes such as PUMA (p53-upregulated modulator of apoptosis) [45], NOXA [46], and Bax [47]. While coregulators closely cooperate with p53, they trigger histone modifications as well as changes in chromatin structure [48]. Considering the abundance of p53 target genes and functions, the variety of posttranslational modifications applied to this single protein should not be surprising [49]. Numerous PTMs influence p53 stability and determine its subsequent actions in response to different stimuli, enabling cellular lifeguard to act quickly and with extreme precision [50].

3.1. Regulation of p53 Activity. The "guardian of the genome" is subjected to sophisticated regulation [51]. Namely, its stability and amount are precisely regulated by its closest negative regulator, MDM2 (mouse double minute 2 homolog) [52, 53]. This E3 ubiquitin ligase forms a complex with p53 and determines its fate due to the attachment of ubiquitin molecules. Highly expressed MDM2 conducts p53 polyubiquitination thereby triggering its nuclear degradation. MDM2-dependent p53 monoubiquitination mediates nuclear exclusion of p53 and precludes its transcriptional activity [54]. Noticeably, p53 ubiquitination level is highly dependent on the MDM2 activity [54]. When the genotoxic stressor occurs, MDM2 activity and p53 degradation are repressed. p53 undergoes the process of stabilization and accumulation, followed by tetramerization and binding to target DNA sequences [55]. As p53 induces expression of its closest negative regulator, together they form autoregulatory feedback loop being relevant to the maintenance of balanced cellular conditions. Physiologically, p53 shuttles between two

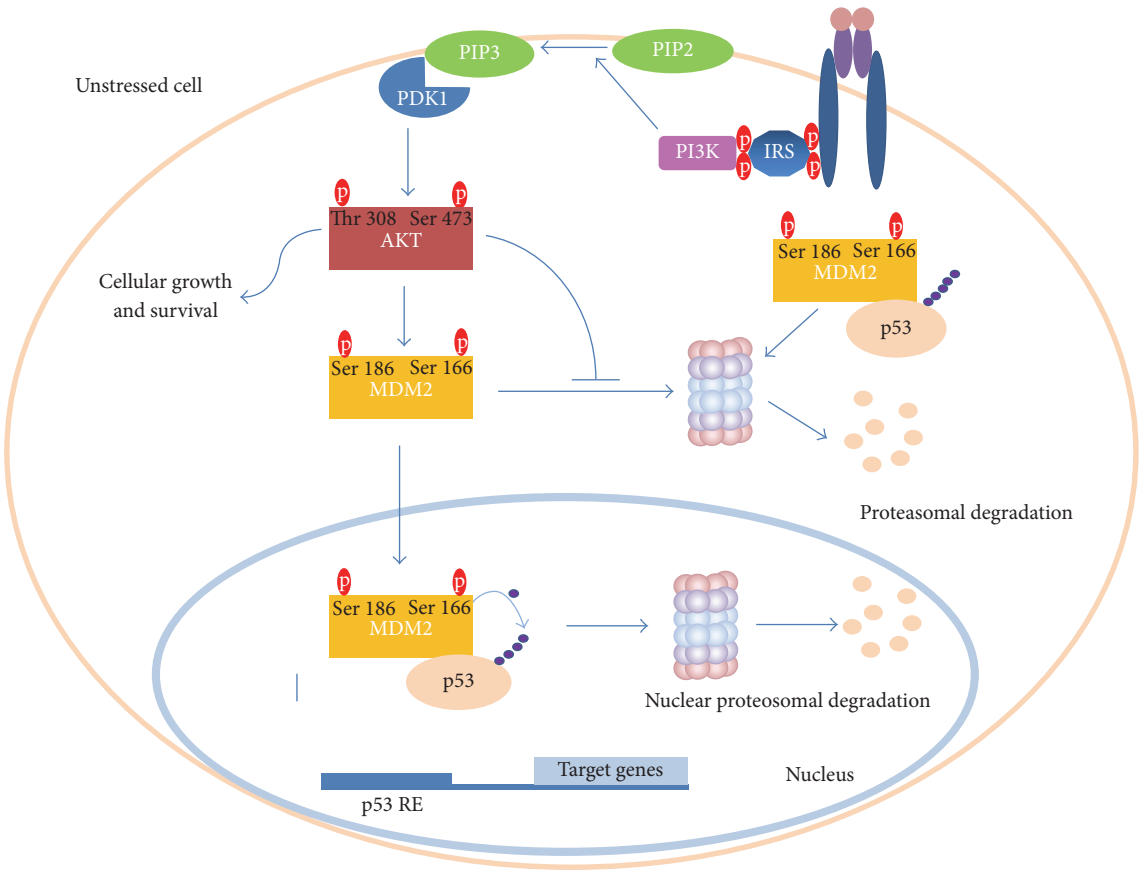

Figure 2: The crosstalk between MDM2/p53 and PI3K/AKT pathways under physiological conditions. Insulin signalling triggers the cellular growth and survival. Activated AKT performs MDM2 Ser166 and 186 phosphorylation thereby allowing for degradation of p53 in the cytoplasm and nucleus. Thus, p53 cannot transcriptionally regulate the plethora of target genes, where these enabling cell cycle arrest, senescence, and apoptosis are of crucial importance. Blue lines denote molecular mechanisms present under physiological conditions. RE stands for response element. See text for more details.

major cellular compartments, cytoplasm, and nucleus in a cell-cycle dependent manner [56].

As previously mentioned, AKT is subjected to phosphorylation upon insulin stimulation [57]. Ser473 and Thr308 phosphorylations are demanded for maximal activity of AKT [57]. This molecule has been reported to perform MDM2 Ser166 and Ser186 phosphorylations [58]. Hence, MDM2 entry into nucleus is promoted [59] and also its self-ubiquitination is limited [60]. Moreover, it decreases p53 transcriptional activity and stimulates MDM2 ubiquitin ligase activity, thereby leading to increased rate of p53 degradation [61] (Figure 2). When the stressor occurs, p53 aims to block the cell growth and survival. Thus, it triggers AKT degradation and transactivates PTEN, a prominent PIP3 phosphatase (Figure 3). Notably, PTEN was demonstrated to physically interact with p53 and promote its acetylation, tetramerization, DNA binding, and transcriptional activity by utilizing phosphate-dependent and phosphate-independent mechanisms [62, 63]. Moreover, PTEN-p53 interaction was potent enough to reverse MDM2-mediated inhibition of p53 [64]. Another research team showed that oroxylin-induced PTEN is capable of reducing the rate of MDM2 transcription and thus favours p53 stabilization [65].

In the case of stressor occurrence ATM kinase undergoes Ser1981 phosphorylation thereby being released from the inhibitory self-dimer complex [66, 67]. Concomitantly, it influences p53 stability by phosphorylating Ser15 (Ser18 in mice) [68, 69]. In order to preclude p53 degradation, ATM conducts MDM2 Ser395 phosphorylation [70]. Interestingly, ATM was also shown to inhibit MDM2 by affecting its RING domain oligomerization as well as its E3 ligase processivity [71]. In general, ATM activation releases MDM2-mediated inhibitory mechanism, thereby allowing p53 stability (Figure 3). ATM further potentiates p53 activity level by revolving around the suppression of an impressive amount of p53 inhibitors [72]. Prominently, p53 gets immediately deubiquitinylated thanks to USP10 (Ubiquitin Specific Peptidase 10) activity [73]. Moreover, ATM activity elicits cascades of molecular events going hand in hand with the promotion of efficient p53 translation level [72]. It is promoted through the binding of MDM2 to p53mRNA as well as by circumventing the MDM2-mediated RPL26 (ribosomal protein L26) degradation [73, 74]. Interestingly, MDM2-p53mRNA interaction is indispensable for p53 activation triggered upon DNA damage, therefore providing a deeper rationale for the p53-mediated MDM2 induction [75]. This regulatory mechanism

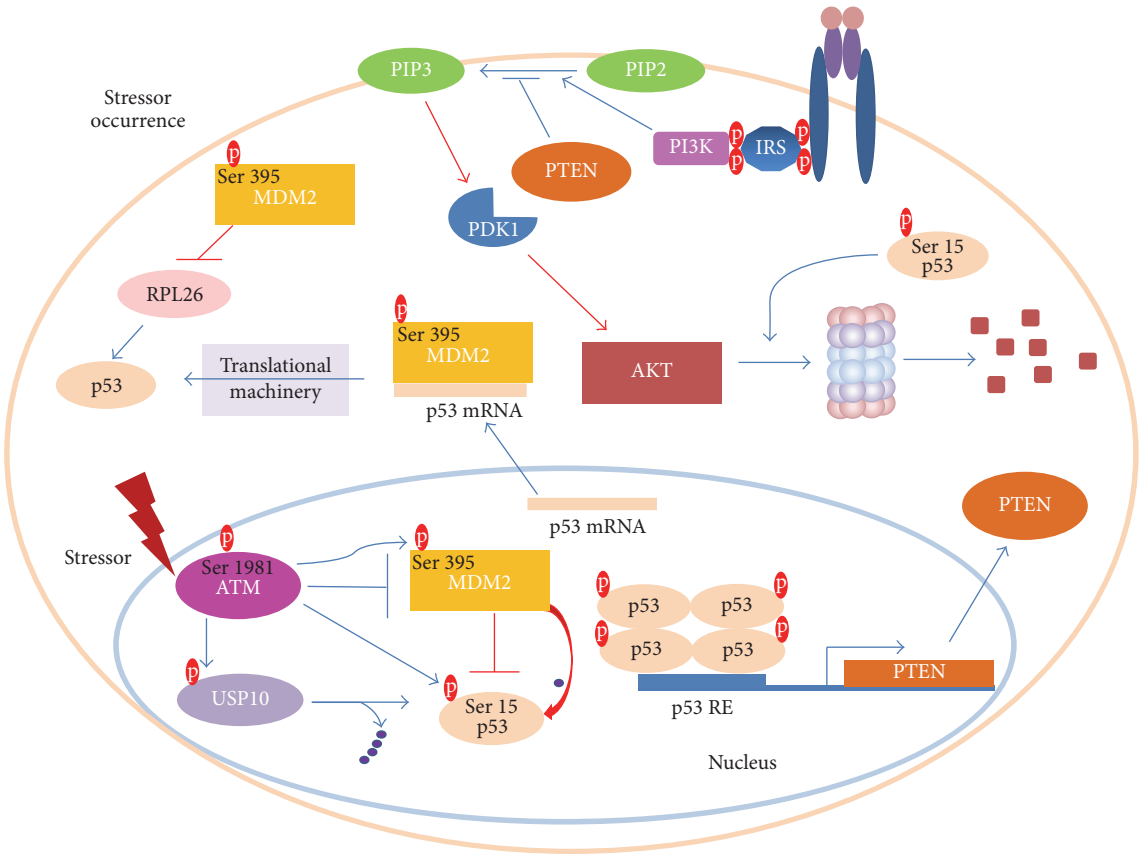

Figure 3: The crosstalk between ATM/MDM2/p53 and PTEN/PI3K/AKT pathways under the exposure to genotoxic and metabolic stressor. ATM kinase undergoes activation upon genotoxic stressor occurrence. It conducts MDM2 Ser395 phosphorylation as well as phosphorylating p53 at Ser15. MDM2 cannot negatively regulate p53, which immediately gets stabilized and accumulates. Positive regulation of p53 is performed due to its deubiquitination by activated USP10. Nuclear p53 tetramerization allows for transcriptional regulation of genes involved in provision of antioncogenic cellular protection. PTEN is one of the p53 target genes responsible for the inhibition of insulin signalling. In addition to the blockage of phospho-AKT formation, p53 triggers AKT proteosomal degradation. p53 translation is enhanced by the formation of p53mRNA-MDM2 complex and inhibited RPL26 degradation. While blue lines depict phenomena present upon stressor occurrence, red lines represent inhibited signalling events. RE stands for response element. Only limited number of regulatory events is shown.

adds a new dose of complexity to p53-MDM2 interactions and sheds a new light on the tumour suppressive role of MDM2.

3.2. p53 Functions. p53 roles are abundant and extremely diverse (Figure 4) [76]. Different cellular localization and concentration might be factors determining p53 function [77]. p53 is implicated, among others, in the antioxidant defense [78], suppression, and promotion of cellular differentiation [79, 80] as well as aging [81].

Interestingly, p53 has been declared a "guardian of differentiation" due to its prominent impact on several mesenchymal differentiation programs [79]. Interestingly, p53 plays a dual role in terms of adipogenesis [80]. While it is positively involved in brown adipogenesis, it suppresses white adipocyte differentiation [80].

p53 plays a dual role with respect to oxidative stress phenomenon [77]. Excessive ROS (reactive oxygen species) accumulation induces genomic instability, being a trigger for p53 activation. Consistently, p53 transactivates genes associated

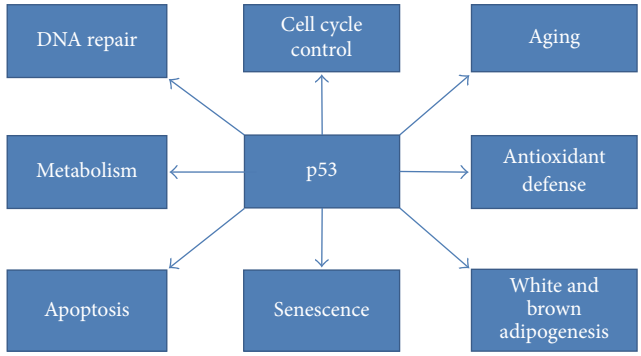

Figure 4: Cellular phenomena vastly influenced by p53.

with antioxidant defense, such as SESN1 and SESN2 (sestrin 1 and 2, resp.), which reduce the level of ROS and protect cells from the deleterious effects of oxidative stress. Antioxidant genes are subjected to permanent activation, as lack of functional p53 results in increased ROS level, followed by

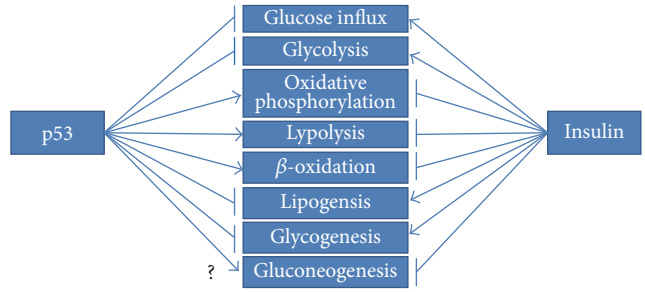

Figure 5: Opposed metabolic regulation executed by p53 and insulin.

DNA damage [78]. This sequence of events was confirmed in numerous normal and carcinoma cell lines, indicating the fundamental way in which p53 exerts its antioncogenic effect [78]. On the other hand, induction of prooxidant genes is a mechanism used by p53 to induce apoptosis in a ROS-dependent manner [77, 82]. As chronic inflammation underlies the process of tumorigenesis, it is another stimulus for p53 activation [83, 84]. p53 is entangled in a broad crosstalk with inflammatory elements, such as previously mentioned ROS, cytokines, or NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) [84]. Although NFκB-p53 relationship abounds in a variety of mechanisms enabling their mutual repression [85], p53 was suggested to elicit inflammation through the activation of NFκB pathway [86]. Surprisingly, NFκB might be an indispensable molecule for p53-dependent induction of apoptosis [87].

Moreover, mild oxidative stress condition intensifies the introduction of single-stranded damages into telomere structures [88]. Damage and shortening of telomeres form general telomere dysfunctionality, which provokes p53 activation and results in either senescence or apoptosis [89, 90]. Nevertheless, senescence is considered as a major mechanism protecting the cells from the effects mediated by this genotoxic stressor [91]. Shortened telomeres are a vivid sign of cellular aging, simultaneously creating a link with p53 signalling. However, the exact p53 role in aging is still inconclusive and highly debatable, as studies suggest that p53 can function as both its enhancer or repressor [81].

p53 is capable of responding to metabolic stresses. For instance, glucose deprivation state is sensed through AMPK (5′AMP-activated protein kinase), which activates p53 and elicits its transcriptional program [92]. Metabolic stressors which constitute a great threat towards replication fidelity force p53 to shut down mitogenic signalling expressed by the inhibition of IGF-1/AKT and mTOR (mammalian target of rapamycin kinase) pathways [93]. Depending on the stressor type p53 was proved to regulate the expression of TSC2 (tuberin), IGF-BP3 (insulin-like growth factor-binding protein 3), and PTEN as well as AMPK [94]. All these molecules negatively affect both the previously mentioned mitogenic pathways in a tissue or cell type specific way. Notably, the stimulation of PTEN and TSC2 expression was especially shown in all insulin-responsive tissues.

As new molecules associated with p53 still enter the arena, the intricate nature of the whole regulatory network advances in a very rapid way. Consistently, numerous profound studies still indicate new plausible roles of the guardian of the genome, surpassing expectations of even the most staunch p53 researchers [95].

4. p53-Orchestrated Regulation of Metabolism

The versatility of p53 has also dominated metabolism, which gives a new dimension to its antioncogenic actions. Utilizing transcriptional activation as well as other mechanisms, protein 53 has a great impact on glycolysis, oxidative phosphorylation, nucleotide biosynthesis, and autophagy as well as glutaminolysis or fatty acid oxidation [96]. All in all, p53 has emerged as an essential regulator of metabolic homeostasis [96]. This statement becomes especially prominent in the case of the Warburg effect, which is a pivotal mark of cancer cells. It is manifested by predominant dependence on glycolytic pathway for ATP synthesis, simultaneously neglecting oxidative phosphorylation. Wild type p53 has a vastly opposing role expressed by inhibition of glycolysis and promotion of oxidative phosphorylation. As vividly seen, metabolic-oriented actions of p53 are directed towards neutralization of the Warburg effect and the restoration of physiological way of ATP production. Nevertheless, the increased risk of carcinogenesis in patients suffering from obesity and diabetes gives some clues about a possible link to p53 function [97]. The effects of p53 actions are totally opposed to the effects mediated by insulin (Figure 5); therefore p53 can be perceived as the molecule engaged in creating insulin resistance state [98]. Below, examples of p53-orchestrated metabolic regulation are presented.

4.1. p53-Dependent Regulation of Glucose Influx, Glycolysis, and Glycogenesis. First, p53 leads to decreased glucose influx by directly repressing expression of two glucose transporters, GLUT1 and GLUT4 [99]. The latter one might also be repressed through indirect mechanism [100]. Namely, p53 was reported to downregulate PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1α), being engaged in the stimulation of GLUT4 expression in muscle tissue [100, 101]. Moreover, p53 indirectly represses GLUT3 expression by influencing other transcription factor, NFκB [102]. The detailed mechanism is focused on the suppression of IKKα and IKKβ kinase activities, being vital components of NFκB signalling cascade.

Additionally, p53 affects the first component of insulin signalling. Namely, insulin receptor (IR) promoter has been shown to undergo repression mediated by p53 and accompanied by two other factors, C/EBP (CCAAT-enhancer binding proteins) and Sp1 (specificity protein 1) [103].

p53-stimulated repression of glycolysis is executed in several ways. First, it performs inhibition of hexokinases HK1 and HK2 and phosphoglycerate mutase (PGAM), which catalyze the first and eighth glycolysis steps [104, 105]. The latter one is not subjected to p53 transcriptional repression, yet its protein stability is restricted [105]. Hexokinases' upregulation is one of the most significant signs of cancerous phenotype [106], simultaneously being the most crucial enzyme for glucose metabolism. p53 represses hexokinases and phosphoglycerate mutase by inducing the expression of miR-34a [104]. Nevertheless, these enzymes can be likewise subjected to opposite regulation, thus creating evidence for p53-stimulated positive regulation of glycolysis [107, 108]. The above aspect highlights the relevance and complexity of p53 actions. Importantly, p53-miR34 signalling pathway also contributes to the repression of GPI (glucose-6-phosphate isomerase), which catalyzes the second glycolytic reaction. Moreover, p53 overexpression itself has been reported to correlate with the decrease of another glycolytic enzyme, aldolase C (ALDC) [104].

TIGAR (TP53-induced glycolysis and apoptosis regulator) is a downstream p53 effector, which acts as a suppressor of phosphofructokinase 1 (PFK) enzyme [109]. PFK catalyzes the third step of glycolytic pathway, simultaneously being the rate limiting enzyme undergoing allosteric regulation by fructose-2,6-bisphosphatase. TIGAR acts as fructose-2,6-bisphosphatase, which causes the accumulation of fructose-6-phosphate, followed by its isomerization to glucose-6-phosphate. Not only is TIGAR claimed to be a glycolysis inhibitor, but also the pentose phosphate pathway (PPP) enhancer is. Additionally, it provides protection against ROS-dependent damage by the production of NADPH needed for the formation of reduced glutathione [109]. TIGAR has been shown to activate HK2 activity, which can be perceived as plausible mechanism enabling complex direction of glycolytic intermediates to the pentose phosphate pathway, thus causing even more profound repression of aerobic glucose oxidation [110].

RRAD (Ras-related associated with diabetes) was revealed to be overexpressed in some T2D diabetic patients [111]. Furthermore, it is likewise responsible for the reduction of glucose uptake in response to insulin stimulation in muscle and adipose tissue [112]. Recently, it was found to be a p53 target gene engaged in the repression of glycolysis under hypoxic conditions [113, 114]. The exact mechanism is centered on preventing the translocation of GLUT1 to the cellular plasma membrane. Although the study was conducted on lung cancer tissue, RRAD might be also subjected to p53 regulation in insulin-responsive tissues.

p53 was connected with restrained rate of glycogen synthesis in insulin-resistant subjects [115]. Study data provided by Fang et al. indicated that p63, which is a member of p53 family, is directly targeted by mir-20a-5p. Reduced amount of this miRNA led to increased expression of p63, followed by its physical interaction with p53, thus leading to increase of p53 and PTEN. Restriction of glycogenesis was proved to be conducted through PTEN-mediated AKT/GSK pathway inhibition.

4.2. p53-Mediated Enhancement of Oxidative Phosphorylation. Altogether, p53 is perceived as a negative glycolysis regulator due to direct as well indirect repression of essential glycolytic enzymes. P53 also performs glycolytic pathway limitation by profoundly promoting oxidative phosphorylation for ATP synthesis. The stimulation of glutaminolysis and fatty acid oxidation (FAO) as well as direct induction of genes influencing mitochondrial respiratory chain complexes vastly ameliorates this metabolic switch. Furthermore, such kind of metabolic control stays in agreement with p53 tumour suppressive responsibility and presents totally opposite effect in comparison to insulin signalling (Figure 5).

In physiological conditions, pyruvate, an end product of glycolysis, is converted to acetyl-CoA. The latter one serves as the entry molecule in tricarboxylic acid cycle (TCA). p53 increases the rate of acetyl-CoA formation due to direct regulation of genes affecting pyruvate dehydrogenase (PDH), the first enzymatic component of pyruvate dehydrogenase complex. P53 decreases the expression of PDK1 and PDK2 (pyruvate dehydrogenase kinase 1 and pyruvate dehydrogenase kinase 2, resp.), known to conduct repressive phosphorylation of PDH [104, 116]. In contrast, p53 was shown to increase the expression of PARK2 (parkin), which positively regulates PDH activity as well as strengthening cellular antioxidant defense [117]. Moreover, p53-mediated repression of lactate dehydrogenase A (LDHA) protects pyruvate from conversion to lactate, thus further promoting the formation of acetyl-CoA [104].

p53 affects tricarboxylic acid cycle by directly inducing the expression of mitochondrial GLS2 (glutaminase 2), which is commonly known to conduct the process of glutamine hydrolysis. This results in the formation of glutamate and consequent increase in the α-ketoglutarate level, a component of TCA. Consequently, p53 intensifies mitochondrial respiration and subsequent ATP production rate [118].

Direct p53 impact on oxidative phosphorylation is executed through the enhancement of SCO2 (synthesis of cytochrome c oxidase) expression level. The latter one regulates COX (cytochrome c oxidase) complex, a pivotal component of oxygen utilization [119]. AIF (apoptosis-inducing factor) is a mitochondrial flavoprotein presumed to positively affect the mitochondrial respiratory complex I. Crucially, p53 performs constitutive activation of AIF expression, thereby allowing efficient oxidative phosphorylation [120, 121].

4.3. Impact of p53 on the Regulation of Gluconeogenesis. As insulin exerts a repressive effect on gluconeogenesis, it is worth to establish the accurate p53 role in gluconeogenic regulation. Firstly, the liver p53 level seems to be dynamically regulated by nutrient availability, where activation of p53 and its target genes occurs in the case of food withdrawal through the AMPK-mediated mechanism [122]. Another point is that p53 was implicated to be a central node governing the

regulation of fasting in critical metabolic tissues, such as liver, muscles, and adipose tissue [123]. Interestingly, the absence of functional hepatic p53 results in defective gluconeogenesis thus promoting hypoglycemia [122]. However, study data implicates both p53-induced suppressive [124, 125] and stimulating [126–128] impact towards de novo glucose production. The most recent findings suggest that p53 conducts direct activation of sirtuin 6 (SIRT6), which in turn facilitates export of FoxO1 (forkhead box protein O1) to cytoplasm [124]. Consequently, FoxO1 is not capable of enhancing the expression of two vast gluconeogenic executors, PCK1 (phosphoenolpyruvate carboxykinase 1) and G6PC (glucose 6-phosphatase). Nevertheless, the accumulating amount of contradictory data reflects the need for further confirmation of the exact p53 role in the regulation of this particular metabolic pathway.

4.4. Implications of p53 in the General Glucose Homeostasis. As seen above, a large body of evidence indicates the vital influence of p53 on glucose metabolism. Besides biochemical implications, p53 signalling seems to underlie general physiological glucose homeostasis [129, 130]. Its impact has been observed in patients with A-T (ataxia teleangiectasia), which are highly susceptible to insulin resistance state, followed by the development of T2D [131]. ATM protein is the trigger for A-T disease, simultaneously being considered as a molecular target in metabolic associated disorders [132, 133]. It also acts as an upstream mediator of p53 function [134]. As ATM kinase performs p53 activation through Ser18 phosphorylation, the mice model with p53S18A was used to check the influence of ATM-p53 signalling pathway on the physiological regulation of insulin sensitivity [129]. This molecular defect turned out to be responsible for metabolic stress accompanied by glucose intolerance and subsequent insulin resistance. Insulin signalling was markedly reduced and associated with increased ROS level, as this metabolic imbalance was reversed by the antioxidant treatment [129]. Additionally, it was revealed that a higher dose of p53 transactivation domain contributes to intensified protection against any disturbances concerning glucose homeostasis [130]. In this study the specific mice model with p53Ser18 defect was utilized, enabling observation of effects associated with the loss of p53-dependent activation of hzf (hematopoietic zinc finger protein) in white adipose tissue [135]. Notably, hzf is a vast adipogenesis regulator. Mice exhibited adipose tissue specific insulin resistance as well as increased level of TNF-α along with notable decrease in adiponectin level. The above results confirm the significance of ATM-p53 signalling in maintenance of glucose homeostasis. Additionally, it exceptionally underscores the value of adipose tissue functionality, which if disturbed, leads directly to insulin resistance and inflammation.

On the other hand, p53 knockout mice did not exhibit any differences in insulin, glucose, and pyruvate tolerance tests in comparison to wild type mice [136]. Insulin-stimulated glucose uptake was not changed in adipocytes with siRNA-suppressed p53 expression. Totally different findings originated from studies performed on identically treated hepatocytes. Although glucose uptake turned out to be diminished, there were no changes in the expression of GLUT1 and GLUT2 in comparison to control hepatocytes. Similarly, the level of phosphorylated AKT was found to be equal in both types of cells, remaining exact p53-mediated mechanism to be elucidated. Nevertheless, p53 itself was not indispensable for general maintenance of glucose homeostasis [136]. Intriguingly, the lowered serum level of TP53 gene product was reported in diabetic subjects as well as patients with impaired glucose tolerance [137].

4.5. p53-Induced Regulation of Fatty Acid Metabolism. p53 has been observed to play a great role in both lipid catabolism and fatty acid β-oxidation [138]. It contributes to the first metabolic pathway by directly stimulating molecules engaged in the release of fatty acids from lipids. Namely, it activates proteins such as cytochromes P450-4F2 and 4F3, carnitine O-octanoyltransferase (CROT), and 2 types of carnitine palmityltransferase, CPT1A and CPT1C [138–141]. Carnitine acetyltransferases are responsible for conjugating fatty acids to carnitine, which enables their transport to the mitochondrial matrix [141]. Cytochromes P450-4F subject long fatty chain acids to hydroxylation, leading to its β-oxidation [139].

Starvation-induced lipolysis was reported to be governed by p53 through stimulation of its downstream target, Ddit4 (DNA Damage Inducible Transcript 4), in adipocytes [123]. The relationship between p53 and lipolysis was likewise associated with the chronic pressure overload and DNA damage response in obese adipocytes [142, 143].

Considering p53-mediated fatty acid oxidation regulation it must be stressed that all of them undergo activation in the glucose deprivation state. LPIN1 (lipin 1) and GAMT (guanidinoacetate N-methyltransferase) expression levels are subjected to p53-dependent increase [144, 145]. LPIN1 exhibits cooperation with two transcriptional regulators, PPARα (peroxisome proliferator-activated receptor alpha) as well as PGC-1α [146]. Their combined cellular effect is associated with the inhibition of the fatty acid synthesis and enhancement of fatty acid oxidation. As mentioned before, p53 has been revealed to directly repress and interact with PGC-1α, one of the master metabolic gene expression coactivators [101]. Additionally, PGC-1α constitutes a p53 regulator, forcing it to elicit transcriptional activation of genes involved in the metabolic regulation and cell cycle arrest [147]. GAMT expression induction results in a vast increase of FAO rate by the mechanism involving stimulation of creatine synthesis [145].

PANK1 (pantothenate kinase-1) influences the level of intracellular CoA (coenzyme A) by catalyzing the rate limiting step in CoA synthesis pathway [126]. It has been reported to be a direct positively regulated p53 transcriptional target gene. Mice lacking functional PANK1 gene and subjected to starvation demonstrated defective β-oxidation and gluconeogenesis. Therefore, p53 was implicated to be an enhancer of both of these processes.

Intriguingly, FAO induction is also mediated by RP-MDM2-p53 metabolic response pathway. RP (ribosomal protein) becomes activated upon suppression of ribosomal biogenesis occurring during metabolic stress. As RP binds

MDM2, it contributes to subsequent p53 induction [148]. Finally, MCD (malonyl-coenzyme A decarboxylase), enzyme critically involved in the process of FAO, undergoes p53-dependent upregulation. RP-MDM2-p53 signalling pathway has a promoting impact on the mitochondrial fatty acid uptake, as MCD enzyme regulates malonyl-CoA turnover and subsequent CPT1α activation.

Actions of the master tumour suppressor are also directed towards inhibition of lipid anabolism. Namely, G6PD (glucose-6-phosphate dehydrogenase) is markedly repressed upon p53 stimulation, what leads to lowered efficiency of pentose phosphate pathway (PPP) and consequently to reduced NADPH level. These events create a cellular signal, which decreases lipogenesis [149].

SREBP1c (sterol regulatory element-binding protein 1c), which belongs to the family of transcription factors regulating adipogenesis and lipogenesis [150, 151], is subjected to p53-dependent downregulation [152]. SREBP1c stimulates the expression of FASN (fatty acid synthase) as well as ACLY (ATP citrate lyase), which were likewise revealed to undergo p53-mediated repression [138, 152].

Besides direct regulation of metabolic enzymes, hypothalamic SIRT1/p53 pathway was implicated in the response towards ghrelin, the hormone regulating food intake [153]. However, mice lacking functional p53 were not characterized by the impaired ghrelin-dependent stimulation of growth hormone secretion. Moreover, p53-mediated regulation of adipogenic and lipogenic genes was found to be indispensable for ghrelin-induced storage of lipids in adipose tissue and liver [154].

5. Tissue-Specific p53-Mediated Changes Triggered by Obesity and Diabetic Conditions

Another piece of evidence linking p53 with glucose metabolism is associated with its response towards glucose oscillations as well as hyperglycemic stimuli. It has been established that hyperglycemia activates p53 and its downstream mediators in myocyte cells leading to its apoptosis [155]. Additionally, many studies have proved p53 activation upon high-fat and high-calorie diet treatment. Discovery of the new triggers for p53 induction encouraged the scientific community to investigate p53 role in multiple tissues.

5.1. p53 Role in the Pancreas. The relationship between p53-dependent apoptosis, hyperglycemia and mitochondrial dysfunction was widely investigated in pancreatic RINm5F cells [156–158]. First, it is clear that hyperglycemia causes oxidative stress contributing to pancreatic β-cells apoptosis [159]. The suggested mechanism is thought to be based on mitochondrial p53 mobilization, leading further to decreased mitochondrial membrane potential, followed by cytochrome c release and fragmentation of nuclear DNA [156]. Another study implied the plausible link between p53 Ser392 phosphorylation and p38 MAPK activation in hyperglycemia-stimulated β-cells mass decrease [157, 160]. Inhibition of p38 MAPK activity coincided with the blockage of p53 translocation and decreased rate of apoptosis. In other words, p53 ser392 phosphorylation was proved to be stimulated upon hyperglycemia and favor p53 mitochondrial translocation. In the last similar study, the MDM2-p53 regulatory status as well ATM and AKT regulation was examined [158]. AKT was found to undergo hyperglycemia-dependent phosphorylation and phosphorylated MDM2 at Ser166 [57, 59–61, 158]. Nevertheless, MDM2 mRNA expression and protein concentration were vastly decreased. High glucose seemed to support the formation of MDM2-p53 inhibitory complex in the cytosol, without any influence on its nuclear formation. ATM nuclear phosphorylation was reported to coincide with p53 Ser15 phosphorylation, indicating p53 activation [68, 69]. Despite the formation of MDM2-p53 complex, p53 was stabilized and its ubiquitination rate was limited. In a recent review the authors suggest many plausible mechanisms adversely affecting MDM2-p53 autoinhibitory feedback loop [161]. It seems that MDM2 undergoes activation upon high glucose stimulus, but its ubiquitin ligase activity is disturbed. This might happen due to hyperglycemia-induced oxidative stress and consequent multiple phosphorylation events. Special emphasis is placed on the role of ATM kinase, which could impair MDM2 ubiquitin ligase activity by the phosphorylation of some residues in RING-finger and acid central domains. Moreover, p53 polyubiquitination can be reduced due to ROS-dependent limitation of cellular ATP level. According to the authors, hyperglycemia supports the formation of p53 PTMs such as phosphorylation, poly ADP-ribosylation, and O-N-acetylglucosaminylation, which probably contribute to its stabilization and subsequent translocation to mitochondria [162]. All these findings are schematically depicted in Figure 6.

β cells apoptosis was found to be associated with AGE-RAGE pathway [163]. Not only can AGEs (advanced glycation end products) originate from food, but they can also be formed in the process of nonenzymatic glycation of proteins. RAGE (receptor for advanced glycation end products) is a receptor known to bind glycated proteins. An elevated level of AGEs is one of the most vivid signs of chronic hyperglycemia, supporting pathogenesis of diabetic complications [164]. p53 was also found critical for apoptosis of β cells in a glycation-serum-induced mechanism [165]. Studies performed on INS-1 cells and primary rat islets indicated that transcriptional activity of p53 becomes substantially increased in the presence of glycation serum (GS) and contributes to cell apoptosis. Intriguingly, β cells demise was avoided upon p53 inhibition, which underlined the relationship between major tumour suppressor and AGE-RAGE pathway.

In a study investigating mitochondrial functionality along with GSIS (glucose induced insulin secretion) MDM2 and p53 expression levels in β-cells originating from diabetic models were found vastly upregulated [166]. Lack of p53 inhibition provoked repression of pyruvate carboxylase (PC), being mitochondrial enzyme engaged in the production of oxaloacetate and NADPH. Downregulation of these TCA cycle intermediates set the scene for the impaired oxygen consumption ultimately leading to dysfunctional mitochondrial metabolism and defective GSIS. Given the obtained

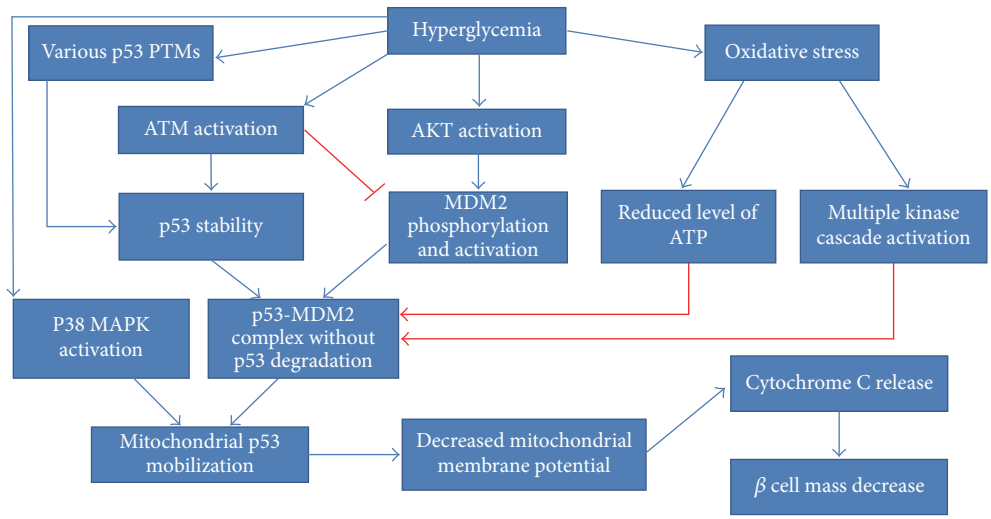

Figure 6: Hyperglycemia-induced changes leading to p53-mediated β-cell mass decrease. Red lines and arrows denote plausible mechanisms allowing p53 to avoid degradation in spite of p53-MDM2 complex formation.

results, the conjuncture of differential p53 role depending on the stage of type 2 diabetes was made. While slight increase in p53 level could repress GSIS, the progression of diabetes could further stimulate p53 upregulation and evoke β-cell apoptosis, thereby causing hyperglycaemia.

Moreover, glucolipotoxicity was shown to be a contributor to increased level of cytoplasmic p53 due to both oxidative and ER stress [167]. The suppressive interaction between p53 and Parkin was revealed to be a culprit for impaired mitophagy in concert with disturbance of electron system transport and consequent cellular ATP deficit. This sequence of events eventually resulted in glucose intolerance and impaired insulin secretion in animal models of both types of diabetes. While MDM2-p53 relationship still appears intriguing, another ubiquitin ligase, ARF-BP1, was reported to be critical for p53 activity in the context of age-related pancreatic β-cells viability and homeostasis. In support of this statement, activated p53 was proved to elicit acute diabetic symptoms concurrently with rendering life span *of arf-bp1* mutant mice shorter [168].

According to Tavana et al., NHEJ-p53R172P mutant mice, which demonstrated a combination of deficient p53 and non-homologous end-joining (NHEJ), exhibited severe diabetes and consequential death in early age [169]. Accumulation of DNA damage induced the increase of p53 and p21, which led to cellular senescence along with the reduction of β cell proliferation and overall depletion of pancreatic islet mass. Other studies suggested that independently of the stimulus causing β-cell metabolic stress, the formation of DNA double strand breaks along with consequent p53 induction constitutes the main mechanism leading to β-cells apoptosis [170]. Revolving around mechanistic details of p53 abundance, mir-200 dosage was delineated as a p53-amplifying factor promoting islets apoptosis in diabetic mice [171]. Another study provided an insight into the role of Δ40p53, the 44 kD p53 isoform with truncated transactivation domain in the context of β-cells biology [172]. Δ40p53 is engaged in setting the balance between all p53 isoforms. Its overexpression endows cells with highly stabilised p53, thereby instigating accelerated aging, attenuated cell proliferation as well as promoting some abnormalities in terms of insulin/IGF-1 signalling [172]. Although Δ40 isoform of p53 has no transcriptional activity it does impact full-length p53 activity due to its ability to form tetramers. Study results proved that Δ40p53 affected β-cell proliferation leading further to β-cells mass decrease. Glucose intolerance along with hypoinsulinemia was established in 3-month old mice, but the effect was progressing with age ultimately leading to overt diabetes and death. Increased level of p21 gene expression was also revealed. Intriguingly, in another study, stress-activated p21 was proved to evoke β-cell mass decrease through stimulation of intrinsic apoptotic pathway [173]. By contrast, p21 overexpression was found to be critical for β-cell recovery through the suppression of β-cell duplication rate after streptozotocin treatment [174]. Consistently, deficiency or genetic ablation of p21 deepened the effect of glucotoxicity on β-cells apoptosis, simultaneously promoting diet-induced diabetes [175]. The use of nutlin-3a, the inhibitor of the interaction between p53 and MDM2, stimulated p21 expression, thus preventing from the ER-stress-mediated effects of prodiabetic conditions and protecting β cells from apoptosis. Similarly, nutlin-3, as an activator of p53, was indicated to ameliorate streptozotocin-induced DM and proposed to provide antidiabetic effects [176], consequently raising some questions about the role of p53/MDM2 relationship.

Moreover, p53 was found to be entangled in the causal relationship between β cells demise and lipotoxicity through FFA-dependent reduced activation of AKT and simultaneous increase in p53 level [177]. Chronic FFAs exposure was shown to stimulate p53 and consequential transcriptional activation of mir-34a in β-cells, what elucidated alternative mechanism of β-cells apoptosis [178]. In another study, stearic acid contributed to the activation of PERK (of protein kinase-like endoplasmic reticulum kinase), which stimulated p53

for induction of mir-34a-5p in islets of diabetic mice and in vitro studies [179]. Finally, lipotoxicity was evoked through consequential reduction of BCL-2 (B cell CLL/lymphoma 2) and BCL-W (BCL-2-like 2), direct targets of mir-34a-5p. It resulted in not only the potentiation of the susceptibility of β cells to apoptosis, but also in the impairment of insulin secretion.

TCF7L2 (T-cell factor 7-like 2) is a transcription factor, which has attracted a great deal of interest recently due to its causative impact on β-cells pathophysiology as well as its association with type 2 diabetes [180]. Its contribution towards disturbed insulin secretion, incretin effect, and consequential lowered β-cells survival was enriched by the molecular link with major tumor suppressor gene. Namely, TCF7L2 was identified to employ p53-p53INP1 molecular pathway to negatively modulate the fate of islet β-cells [180].

Although the link between type 1 diabetes and p53 is beyond the scope of this review, potentiated activity of p53 was likewise unequivocally demonstrated in the context of this type of β-cell dysfunction [181]. The whole mechanism was underlain by the presence of ROS and inflammatory cytokines, which induced the activation of JNK and SAPK. GAD65 (glutamic acid decarboxylase 65 KDa isoform), which is a remarkably significant autoantigen underpinning pathomechanism of diabetes type 1, was reported to be subject to p53 regulation [182].

5.2. p53 Role in the Endothelium and Muscle Tissue. Taking into account the consequences of diabetes and obesity, the role of endothelial dysfunction should not remain undervalued. High-glucose incubation of endothelial cells evoked the reduction of SIRT1 in concert with higher acetylation and activation of p53, which provoked the senescence and dysfunction of endothelial cells [183]. The same results were obtained in vivo [184]. Endothelial "metabolic memory" formation in the presence of high glucose oscillations is also being connected with p53. Here, the crucial role for p53-MDM2 inhibitory feedback loop as well as prolonged activation of its target genes after stressor removal is specifically underlined [185]. Mitochondrial localization of p53 is supposed to underlie metabolic memory phenomenon, leading further to organelle dysfunction and oxidative stress accompanied by mtDNA damage [155]. Additionally, p53's remarkably negative impact has been noticed in endothelial progenitor cells (EPC) [186]. Both EPC cultured in diabetic milieu and EPC from diabetic donors exhibited accelerated senescence-like changes. This effect coexisted with the activation of AKT/p53/p21 signalling pathway, pointing out the major role of p53 in the impairment of diabetic neovascularization. On the other hand, diet-induced obesity mice model enabled demonstration of endothelial p53 as a vast inactivator of endothelial nitric oxide synthase (eNOS) [187]. Further studies showed an increase in p53-dependent PTEN transactivation, consequently affecting Akt-eNOS pathway. Subsequently, it decreased the expression of PGC-1α in skeletal muscle, followed by markedly reduced level of mitochondrial biogenesis and energy consumption. Additionally, deletion of endothelial p53 was manifested by increased muscle glucose uptake, due to lack of p53-GLUT1 inhibitory regulation. The upregulation of endothelial p53 was claimed to be a causative agent of all observed metabolic abnormalities, including intensification of insulin resistance as well as fat accumulation. Promisingly, exercise supported downregulation of p53 and TIGAR in skeletal muscle of Goto-Kakizaki (GK) rats without changes in SCO2 expression [188]. Therefore, exercise implementation constitutes a weapon against the hazardous activity of p53 leading to lowered level of oxidative stress along with enhanced level of mitochondrial DNA content.

5.3. p53 Role in the Formation of Insulin Resistance in the Liver. NAFLD (nonalcoholic fatty liver disease) is the general term defining liver abnormalities such as hepatic steatosis and nonalcoholic steatohepatitis (NASH), which can lead to fibrosis, cirrhosis, and hepatocellular carcinoma [189]. NAFLD is associated with insulin resistance due to hepatic triglyceride accumulation connected with raised level of circulating free fatty acids. Both of these factors further exacerbate the inflammation state ultimately forming a vicious cycle [190]. Upregulated p53 was confirmed to induce cytotoxicity exacerbating liver injury in various mice models of NAFLD [191]. Another study investigated the impact of p53 on apoptosis, which is a basic mechanism favouring hepatocytes elimination in NAFLD [192]. The amount of p53 was dependent on the severity of liver steatosis and thus linked to inflammation. While p53 upregulation was parallel with downregulation of antiapoptotic Bcl-2, proapoptotic Bax expression was not changed. Experimental murine NASH model exhibited p53 activation along with changes of its downstream effectors [193]. The induction of p21, suppression of antiapoptotic Bcl-XL, and formation of t-Bid confirmed the p53 role in the stimulation of intrinsic and extrinsic mitochondrial apoptosis pathways. The same nutritional NASH mice model revealed that the induction of p53 enhances p66Shc signalling, ROS accumulation, and hepatocyte apoptosis, thereby promoting the progression of steatohepatitis [194]. The expression changes obtained in this mice model were confirmed by evaluation of liver samples from patients characterised by different severity of NAFLD. Other findings connected NAFLD pathology with p53/miRNA34a pathway, known to repress SIRT1 (sirtuin1) expression [195]. Selective p53 transcriptional activity inhibitor called pifithrin-α (PFT) was administered to high-fat diet-induced NAFLD mice model. As the p53-mediated inhibitory impact was relieved, the activation of SIRT1/PGC1α/PPARα and SIRT1/LKB1/AMPK pathways was performed. Consequently, malonyl-CoA decarboxylase (MLYCD) underwent activation leading to the reduction of malonyl-CoA concentration. As mentioned above, this step is always followed by the enhancement of CPT1 activity, thereby inducing β-oxidation of fatty acids. Notably, the level of hepatic fat accumulation was diminished, which was manifested by the lessening of steatosis, oxidative stress and apoptosis. Intriguingly, PFT served as a factor lowering overall fat accumulation upon high-fat diet treatment [195]. Increased level of p53 in hepatic cells was reported in a

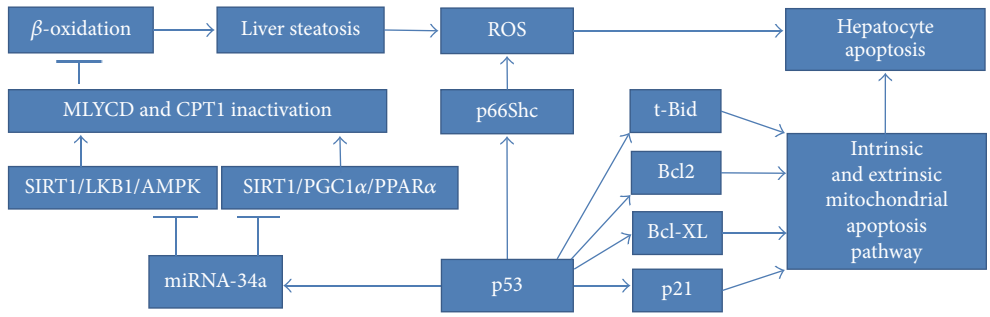

Figure 7: p53-induced cellular events promoting hepatocyte apoptosis.

streptozotocin-induced animal model of diabetes [196]. All of the above mentioned p53-based mechanisms leading to hepatocytes' apoptosis in the context of NAFLD are presented in Figure 7.

p53 level was upregulated by the treatment of hepatocytes with saturated fatty acids (SFA) along with its inhibition resulting in the prevention of SFA-induced cell death [197]. Once again, the antagonism between p53 and insulin activities was strongly highlighted, as insulin treatment antagonized the deleterious effects of p53. Moreover, as mentioned above, p53 overexpression in livers of diabetic or NAFLD patients could be caused by the downregulation of mir-20a-5p, consequent suppression of glycogen synthesis and the introduction of hepatic insulin resistance [115].

The previously observed connection between hepatic fibrosis and p53 accumulation in patients was investigated on, among others, hepatocyte-specific Mdm2-knockout mice [198]. Relief from MDM2 suppression prompted p53 to elicit spontaneous hepatic fibrosis. This liver dysfunction did not occur when p53 was subjected to concomitant deletion. p53 indirectly promoted the synthesis of CTGF (connective tissue growth factor), which is a molecule notorious for its relevant participation in fibrosis pathophysiology, via downregulation of miR-17-92 cluster gene [198].

Surprisingly, the effect of p53 activity measured in the context of glucose homeostasis in insulin-resistant diabetic mice turned out to be highly beneficial and described as insulin-like antidiabetic effect [199]. Overexpressed p53 improved plethora of biochemical indicators such as levels of glucose or serum triglycerides. Furthermore, glycogenesis genes were subjected to substantial upregulation concomitantly with downregulation of gluconeogenesis-associated ones. Positive changes in hepatic and pancreatic gene expression levels were reported for insulin receptor precursor, PPAR-γ, GLUT2, and GK (glycerol kinase). All in all, these results are in strong opposition to previous findings, thus demonstrating miraculous p53-stimulated effect on glucose homeostasis with special focus on the amelioration of hepatic insulin sensitivity.

5.4. p53 Role in the Formation of Insulin Resistance in Adipose Tissue. The very first implication for p53 elevation in classic insulin-responsive tissues was shown in adipocytes of genetically obese (*ob*/*ob*) mice [152]. The study aimed at evaluating expression of p53 and its target genes (p21, MDM2-2, Bax, and IGFBP-3) during fasting and refeeding periods. Surprisingly, the expression of all enumerated molecules was proved to be increased upon refeeding stimuli. In this study, SREBP-1c was revealed to be downregulated by p53, highlighting the role of p53 as an essential lipogenesis repressor. The substantially elevated level of p53 was vividly linked with obesity-induced insulin resistance, creating growing number of questions associated with the exact nature of its adipose tissue existence.

The groundbreaking study conducted by Minamino et al. established outstandingly important role of adipose tissue p53 upregulation in augmenting insulin resistance [127]. Most of all, white adipose tissue originating from high-fat-diet treated Ay mice model exhibited p53 and p21 expression increase. This observation suggested the induction of senescence-like phenotype. Moreover, the excessive levels of TNF-α (tumour necrosis factor) and CCL2 (chemokine C-C motif ligand 2) were found to occur due to senescence of both, macrophages, and adipocytes. The comparison of the results obtained from two mice strains, Ay $Trp53^{+/-}$ and Ay $Trp53^{+/+}$, provided ultimate evidence for p53 as a factor strongly exacerbating insulin resistance and glucose intolerance as well as inducing increased insulin plasma level. Additionally, it has established p53 role in enhancing expression of hepatic gluconeogenic enzymes. p53 was likewise indicated to participate in the formation of telomere-dependent insulin resistance. On the other hand, adipo-p53-deficient mice exhibited proper insulin signalling due to restoration of insulin-dependent AKT phosphorylation. Results demonstrated that presence of ROS leads to p53 activation, followed by NFκB-dependent induction of proinflammatory cytokines. Finally, studies conducted on human adipose tissue removed during abdominal surgery from diabetic and nondiabetic patients confirmed the increased level of p53 protein and CDKN1A mRNA expression. Significantly, inflammatory cytokines were elevated in adipose tissue originating from diabetic patients. All of the above results point to a complex p53 role in the pathophysiology of insulin resistance connecting it with inflammation, telomere shortening, and senescence of adipose tissue. In this study, the prominent role of cellular aging is highlighted, shedding a new light on age-related obesity and insulin resistance. Further studies conducted by this research group found the evidence suggesting that Sema3E (semaphorin 3E) and its receptor, plexinD1, may play a role in

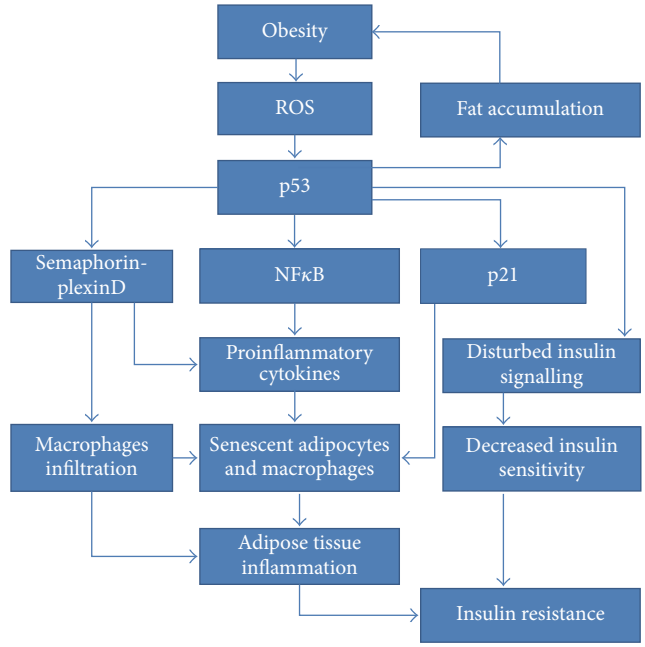

Figure 8: Obesity-induced p53-dependent molecular mechanisms occurring in adipose tissue and leading to insulin resistance.

creating adipose tissue inflammation upon p53 activation in DIO (diet induced obesity) mice model [200]. Sema3E is a secreted protein, simultaneously transcriptionally activated by p53. It was revealed to act as a chemoattractant, thereby directly triggering visceral fat inflammation along with all metabolic abnormalities exacerbating insulin resistance state. Thus, Sema3E is suggested to be another potential therapeutic target in the fight against insulin resistance. Findings originating from these highly relevant studies are visualized in Figure 8.

An intriguing relationship between p53 and hyperglycemia was observed in studies investigating the influence of AGEs on senescent preadipocytes [201]. RAGE-AGE axis was demonstrated to restore adipogenic function of senescent preadipocytes, whose adipogenic potential had been repressed by age-related p53 activity. Glycated proteins stimulated RAGE to suppress p53 expression and modify its functionality due to direct protein-protein interactions. Altogether, it seems that p53-stimulated preadipocyte senescence is a mechanism providing protection against excessive age-related adiposity, which might be reversed by AGE-RAGE axis.

Aside from hyperglycemia stimulus, hyperinsulinemia was also recognized as a factor strongly potentiating p53 activity [202]. The study conducted on 3T3-L1 adipocytes proved that increased p53 expression in concert with decreased level of phospho-MDM2 disturbed insulin signalling and glucose uptake, thus promoting hyperinsulinemia-induced insulin resistance. Prominently, p53 was subjected to time-dependent enhancement without signs of cytotoxicity.

Adipose tissue p53 elevation has been reported to associate with chronic pressure overload [142]. The exact pathomechanism was mediated by sympathetic nervous stimulation, subsequently resulting in lipolysis promotion. Notably increased level of free fatty acids triggered adipocytic p53 overexpression and promoted upregulation of proinflammatory cytokines in the NFκB-dependent manner. All of the above mentioned events elicited adipose tissue inflammation, followed by insulin resistance and successive hyperinsulinemia. Ultimately, p53 accumulation led to heart failure, simultaneously forming metabolic vicious cycle. The detailed mechanism of p53-induced inflammation upon pressure overload concerning endothelial and bone morrow cells has been elucidated, confirming its role in cardiac dysfunction [203]. Diabetic cardiomyopathy was also demonstrated to be underlain by diabetes-induced ROS-mediated increased levels of p53 and SCO2. Changes in gene expression extensively stimulated mitochondrial oxygen consumption for undue generation of ROS, thereby supporting lipid accumulation and cardiac dysfunction [204]. Cardiomyopathy of diabetic subjects was accompanied by raised levels of p53 and p21 and decreased levels of mir-181a and mir-30c, which were validated to directly target p53 [205].

The results provided by Vergoni et al. strongly suggested that ROS-induced DNA oxidation occurring in the early onset of obesity was a rationale for p53 activation in obese adipocytes [143]. Studies conducted on 3T3-L1 cells as well as mice models implied that raised levels of p53 and p21 were simply a response to DNA damage. These molecular shifts were followed by a classic cascade of events leading to insulin resistance such as adipose tissue inflammation, increased macrophages' chemotaxis, impaired insulin downstream signalling along with insulin-induced glucose uptake, and increased lipolysis in affected adipocytes. Conceivably, adipocytes were not found apoptotic due to the protective effect of raised p21.

Bogazzi and colleagues, who examined male C57BL/6J × CBA mice, proved that adipose p53 and growth hormone

(GH) pathways are converged, which evoked the insulin-resistant phenotype upon high-fat diet treatment [206]. Blockage of GH through p38 MAPK pathway was potent enough for normalization of p53 level in adipose depots, suggesting that GH action is indispensable for p53-triggered metabolic repercussions.

In contrast, a study conducted by Ortega et al. provided totally unexpected and contradictory results [207]. The study was focused on the evaluation of adipose tissue p53 expression depending on the inflammation and insulin resistance. Impressively, the results were obtained from subjects characterized by a distinct degree of obesity and severity of insulin resistance as well as for several in vivo and in vitro models. The authors postulated that the upregulation of p53 is solely associated with factors triggering inflammation, such as senescence-connected shortening of telomeres or progressive obesity. Surprisingly, the expression of adipose tissue p53 was inversely correlated with insulin resistance and hyperglycemia. The special attention was also focused on the level of p53 during adipogenesis, which was found increasingly repressed. Herein, the causal connection between inflammation and p53 expression was once again underlined, as mature adipocytes produce a significant amount of anti-inflammatory adipokines. High glucose level was another factor which intensified p53 downregulation in preadipocytes. Taken together, p53 was subjected to dual and opposed regulation. While inflammation stimulated its expression, insulin resistance and high glucose neutralized this effect.

p53 was found to be a repressor of preadipocyte differentiation [208]. Although its expression was shown to be elevated at the beginning of the process, it was then systematically down-regulated [207, 208]. Importantly, $p53^{-/-}$ MEF cells were shown to exhibit lowered insulin-stimulated level of phosphorylated AKT [208]. Another results indicated decreased level of adipogenesis marker genes due to overexpression of p53 [208]. Opposed p53-mediated regulation of white and brown adipogenesis was also revealed [80]. Moreover, p53 knockout mice treated with high-fat high-sucrose (HFHS) diet exhibited a higher level of fat accumulation in comparison to their wild type counterparts. These results imply that p53 provides protective effect against diet-induced obesity. On the other hand, p53 was shown to be an indispensable component for proper formation and function of brown adipose tissue (BAT) [80]. In a recent study p53 was revealed to influence brown adipose tissue *via* concurrent regulation of body weight and thermogenesis in the diet-induced obese mice [209]. It should be noted that p53 was subjected to different types of repression performed on different developmental stages and therefore its manipulation yielded different study results. For example, while mice lacking p53 gene did not develop diet-induced obesity due to previously mentioned mechanisms, acute BAT-specific repression of p53 in adult mice resulted in slight weight gain. Interestingly, pharmacologically activated p53 ameliorated body weight by positively influencing thermogenesis in obese adult p53-wild type mice model. Notably, brown fat is of great relevance to energy homeostasis, being involved in energy expenditure [210, 211]. While its reduced amount correlates with insulin resistance [212], it is also established as a highly determining factor for metabolic syndrome in mice [213]. In the light of emerging evidence enhancement of brown fat accumulation constitutes a novel and exciting approach in the battle against obesity [214]. Summing up, p53 can be perceived as a miraculous molecule allowing protection from obesity through confining white fat deposits as well as supporting brown fat formation and functionality. Data obtained by Nakatsuka et al. provide further confirmation for this statement by elucidation of the exact mechanism exerted by RXR (retinoid X receptor) antagonist, HX531 [215]. Its antiobesity effect was accomplished through stimulation of p53/p21 signalling pathway and related to suppressed differentiation of visceral adipocytes. Furthermore, G_0/G_1 cell cycle arrest was induced in mature adipocytes, which vastly reduced their hypertrophy. On the contrary, adipose tissue expansion has been attributed to p21 actions exhibited upon HFHS diet [216]. Not only was p21 presumed to have a stimulatory effect towards adipocyte differentiation, but it was also implicated in the promotion of adipose tissue hyperplasia by protecting it from apoptosis. Taken together, p21 was prominently entangled in the creation of obesity and insulin resistance upon high-fat diet treatment [216].

5.5. p53 Role in the Formation of Insulin Resistance in Insulin and Noninsulin Target Tissues. Homayounfar et al. evaluated the outcomes of p53 accumulation occurring in peripheral tissues of rats subjected to high fat diet (HFD) [217]. Surprisingly, p53 was found elevated in classic insulin target tissues (adipose, muscle, and liver) and in others (kidney and small intestine). Treatment with pifithrin-α (PFT) led to decreased amount of p53 in all examined tissues and proved the crucial role of crosstalk between ATM/MDM2/p53 and PTEN/Pi3K/AKT pathways. p53-mediated PTEN overexpression was implicated to have a causative impact on the impairment of insulin action. Finally, systemic application of PFT repressed p53 action simultaneously improving insulin sensitivity, reducing serum glucose level, and enhancing insulin cellular signalling. In the most recent study, obesity-induced p53-mediated deleterious metabolic effects were inhibited by two agonists of β-adrenergic receptor in all insulin target tissues [218]. Both of them were shown to stimulate elevation and activation of AKT along with a decrease of p53 and phospho-p53 levels. p53 inhibition was also revealed in noninsulin target tissues. In other words, agonists of β-adrenergic receptor cannot be considered as insulin sensitizers as the risk associated with the lessening of p53-dependent antioncogenic cellular protection is too high.

5.6. p53 and Age-Related Insulin Resistance. While diabetes is widely known to accelerate the process of aging, the reverse is likewise true. The connection between p53 and age-associated insulin resistance seems to associate with cellular senescence. Above issue was firstly raised by Minamino et al. [127], who indicated the causal relationship among obesity, ROS and DNA damage generation, shortage of telomeres, and p53 activation. This sequence of events consequentially led

to the introduction of senescent-like changes in adipose depots, thus stimulating an aging-associated insulin resistance. The same was implied in oxidative stress-exposed aging adipocytes [219]. An aging-replicating mice model, characterized by DNA repair impairment and DNA damage accumulation, indicated the formation of p53-associated cellular senescence in concert with a decline of pancreatic islet mass and the acquirement of severe diabetic phenotype [169]. Moreover, a deletion of ARF-BP1 phosphatase was concurrent with activation of p53 in β-cells, which underwent an age-dependent depletion [168]. Upregulation of p53 in the liver, muscle, and adipose tissue was shown for older and obese wild-type mice in comparison to control ones [220]. Interestingly, age-related p53 increase was prevented in PTP1B$^{-/-}$ mice, which were proved to be insensitive to diet-induced changes of insulin sensitivity and characterized by lean phenotype. According to Jeon et al., MCH$^{-/-}$ mice exhibited resistance towards aging-induced accumulation of body mass as well as insulin resistance [221]. In contrast to previous studies, p53 level decreased in response to aging in spleen and liver of MCH$^{-/-}$ and wild-type mice in comparison to their younger controls; however, the magnitude of p53 decrease was lower in MCH$^{-/-}$ ones.

6. p53 Polymorphisms in Diabetes and Complications

Besides expression-based findings, the connection between diabetes and p53 does have a genetic underlying. Namely, the SNP occurring at p53 codon 72 is constantly examined in the context of susceptibility to diabetes. While subjects with C nucleotide possess proline variant of p53 (R72), these carrying G allele have an arginine one (P72). Genetic studies revealed p53 codon 72 (Arg72Pro) polymorphism as a predisposing factor for T2D in Finish, Chinese Han, and European populations [222–224], which highlighted the independence of this variant in the race-specific context. The severity of insulin resistance observed in type 2 diabetic patients was demonstrated to associate with this polymorphism [225]. Arg72Pro was also connected with waist circumference and determined the association between T2D and BMI [226, 227]. In the most recent study, the above mentioned SNP was examined in the mice model and proved to increase the risk of obesity and general metabolic disturbances associated with insulin resistance and T2D [228]. Moreover, carriers of risk G allele demonstrated accelerated progression of diabetic nephropathy [229]. Correlation between Arg72Pro polymorphism and the early age of onset (<6 years) was also found in Italian female T1D patients as well as being associated with the development of polyneuropathy in Russian population [230, 231]. p53 polymorphism originating from 10 intron, A17708T, was reported to coincide with uremic complications in T2D subjects [232]. Interestingly, association between p53 and diabetes-related complications was shown for diabetic cardiomyopathy [203–205], retinopathy [233], neuropathy [234], nephropathy [235], vasculopathy [183], peripheral arterial disease (PAD) [236], defective wound healing [237, 238], testicular dysfunction [239, 240], thrombocytic complications [241], or augmented apoptosis-based response towards ischemia [242]. Worthily to note, polymorphisms of the previously mentioned p53 transcriptional targets, TCFL2 (rs7903146) and p53INP1 (rs896854), were indicated to underlie the risk for diabetes occurrence [243, 244].

7. Conclusions

All of the above findings provide a profound insight into p53 actions trigged by adiposity or expressed upon diabetic conditions. As can be seen, some experimental results are inconsistent, predominantly in adipose tissue. It is likewise associated with p53 role in glucose homeostasis, highlighting both its neutral and highly determining character. Its impressive impact has been shown in other classic insulin-responsive tissues. Clearly, p53 is responsible for the formation of tissue-specific insulin resistance as well as being entangled into phenomenon of endothelial "metabolic memory" and many diabetic complications. In the majority of studies, p53 was found to be elevated upon increased level of free fatty acids as well as high glucose stimulus. As both triggers lead to diet-induced obesity, activation of p53 affects numerous cellular phenomena, especially shown in the form of adipose tissue inflammation. Herein, it is impossible to undervalue the link between p53 and NFκB, whose reciprocal interactions were earlier claimed to be predominantly underlain by functional antagonism. The precise nature of their relationship seems to be highly context-dependent, as ROS-stimulated p53 elicits inflammation through NFκB-dependent mechanism. Herein, disturbed levels of both pro- and anti-inflammatory cytokines along with activated p53 contribute to the exacerbation of glucose intolerance, insulin resistance, and elevation of serum glucose level. In other words, p53 can affect the entire organism leading to systemic disturbances, which was brilliantly visualized by pifithrin-α administration. Consequently, the remarkable emphasis should be put on the existence of intricate and mutual regulatory events between p53 and insulin signalling molecules. Simultaneous regulation of AKT and p53 unambiguously forms a core for p53-induced abnormalities. Detailed mechanistic implications of this particular interaction depend on the phosphorylation status of specific sites located within structures of ATM, p53, MDM2, and AKT. The relationship between ATM and p53 seems to be especially intriguing, as obesity-associated DNA damage was found to promote p53 activity. PTEN contribution to insulin resistance has also been reported before [245], enriching the entire pathomechanism by strict relationship with p53. PTEN is suggested to be the key player of AKT-p53 crosstalk, taking responsibility for some of the effects induced primarily by p53.

Focusing further on adipose tissue, it seems that p53 influences its fate under physiological and pathophysiological conditions. While it is indispensable for the functionality of brown adipose tissue, it has been revealed to suppress hypertrophy and differentiation of white adipocytes. Prominently, p53 performs senescent-like changes of adipocytes which can be perceived as both deepening of inflammatory

state [210] and protection against the deleterious effects of hyperinsulinemia. This becomes especially important in the view of the mitogenic and anabolic nature of insulin activities, which are thoroughly different from the cellular effects exhibited by p53. Thus, it constitutes a great challenge for each researcher aiming to therapeutically inhibit p53, as its total deletion might result in the development of cancerous phenotype. Nevertheless, administration of pifithrin-α has been shown to protect from weight gain even upon high fat diet treatment.

Considering the abundance of p53 activities, its participation in pancreatic β-cell apoptosis should not be perceived as extremely unpredictable. However, p53 is assumed to strengthen β-cell mass decrease by participation in the mitochondrial-dependent apoptotic event with simultaneous avoidance of MDM2-mediated degradation. Apparently, p53 takes advantage of its all functional capabilities in the regulation of glucose metabolism and insulin sensitivity.

Elucidation of p53-mediated metabolic regulation is nowadays a highly developing area of research, exposing its impact on an increasing number of prominent biochemical processes. All metabolic activities promoted by p53 are directed towards suppression of oncogenesis as well as neutralization of effects exhibited by insulin. Therefore, insulin resistance seems to be an obvious consequence of the raised level of p53 in obesogenic conditions. However, an age-related activity of p53 adds a new dimension to the insulin resistance issue.

While the majority of observations emphasize that p53 is ubiquitously involved in the pathophysiology of insulin resistance, some study results suggest that p53 protects from obesity and diabetes. Apparently, its role in obesity-associated disorders has been undervalued for too long, now pointing to a strong need for further research. Hopefully, it might result in the creation of new and extremely promising therapeutic approaches. However, it is interesting, whether p53-mediated regulation of genes responsible for the metabolic regulation described in this review is similar in the diabetes-like conditions. Moreover, interactions between p53 and microRNA network merit further investigation in the diabetes-oriented research, as they may constitute a novel idea for the development of therapeutics. The abundance of p53 mediators and effectors do need a profound examination in order to elucidate further regulatory mechanisms controlling p53-associated actions in both tissue-specific and systemic insulin resistance. Certainly, the profound epigenetic research of p53 and the components of its network would be of crucial importance.

Competing Interests

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

Acknowledgments

This paper was partially supported by the Grant from the Medical University of Lodz (no. 503/0-077-09/503-01-002) and Grant no. 2015/17/B/NZ7/03019 from Polish National Science Centre.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 7928981, 9 pages
https://doi.org/10.1155/2017/7928981

Research Article

Impact of Yoga and Meditation on Cellular Aging in Apparently Healthy Individuals: A Prospective, Open-Label Single-Arm Exploratory Study

Madhuri Tolahunase,[1] Rajesh Sagar,[2] and Rima Dada[1]

[1]*Lab for Molecular Reproduction and Genetics, Department of Anatomy, All India Institute of Medical Sciences (AIIMS), New Delhi, India*
[2]*Department of Psychiatry, All India Institute of Medical Sciences (AIIMS), New Delhi, India*

Correspondence should be addressed to Rima Dada; rima_dada@rediffmail.com

Received 22 September 2016; Revised 17 December 2016; Accepted 22 December 2016; Published 16 January 2017

Academic Editor: Delminda Neves

This study was designed to explore the impact of Yoga and Meditation based lifestyle intervention (YMLI) on cellular aging in apparently healthy individuals. During this 12-week prospective, open-label, single arm exploratory study, 96 apparently healthy individuals were enrolled to receive YMLI. The primary endpoints were assessment of the change in levels of cardinal biomarkers of cellular aging in blood from baseline to week 12, which included DNA damage marker 8-hydroxy-2′-deoxyguanosine (8-OH2dG), oxidative stress markers reactive oxygen species (ROS), and total antioxidant capacity (TAC), and telomere attrition markers telomere length and telomerase activity. The secondary endpoints were assessment of metabotrophic blood biomarkers associated with cellular aging, which included cortisol, β-endorphin, IL-6, BDNF, and sirtuin-1. After 12 weeks of YMLI, there were significant improvements in both the cardinal biomarkers of cellular aging and the metabotrophic biomarkers influencing cellular aging compared to baseline values. The mean levels of 8-OH2dG, ROS, cortisol, and IL-6 were significantly lower and mean levels of TAC, telomerase activity, β-endorphin, BDNF, and sirtuin-1 were significantly increased (all values $p < 0.05$) post-YMLI. The mean level of telomere length was increased but the finding was not significant ($p = 0.069$). YMLI significantly reduced the rate of cellular aging in apparently healthy population.

1. Introduction

In the last decade there has been a significant increase in complex lifestyle diseases like depression, diabetes mellitus (DM), cardiovascular diseases (CVD), cancer, and infertility. These diseases are strongly associated with accelerated cellular aging [1, 2] and have become the bane of modern society [3–5]. Within a homogeneous sample of apparently healthy adult population, biomarkers have been defined recently [6] to characterize the complex processes of accelerated aging phenomenon. Although we do not have any gold standard biomarker to monitor healthy aging, based on the current knowledge of putative biomarkers, the cardinal biomarkers of cellular aging and metabotophic biomarkers which can influence them have become the focus of latest translational research to develop interventions to prevent chronic lifestyle diseases.

The cardinal biomarkers of cellular aging include DNA damage, telomere length attrition, and oxidative stress (OS) [7]. *DNA damage* causes genomic instability which is responsible for cellular dysfunctions in the pathogenesis of lifestyle diseases [8–10]. OS is the most important cause for DNA damage. Although many different oxidative DNA damage (ODD) products have been identified 8-OH2dG (8-hydroxy-2′-deoxyguanosine), a highly mutagenic oxidative DNA adduct has been the subject of intensive study and is a definitive biomarker of DNA damage [11]. *Telomere attrition* is due to altered telomere metabolism involving decrease in telomerase enzyme activity and OS. It contributes to genomic instability and is associated with aging and lifestyle diseases [12].

Oxidative stress, an imbalance between the prooxidants and the antioxidant defense mechanisms, becomes pathological at both extremes of the physiological range needed for normal cellular functions. It is involved in the pathogenesis of complex lifestyle and chronic diseases [13] including depression [14], obesity [15], and infertility [16, 17], the leading public health problems.

Several metabotrophic blood biomarkers influencing cellular aging include biomarkers of stress and inflammatory response, neuroplasticity, and longevity. *Sustained stress response* due to chronic stress stimuli causes constantly increased cortisol levels [18], which lead to systemic tissue abnormalities like increased adiposity and neurodegeneration. The level of stress responsiveness (cortisol levels) can be a biomarker for predicting susceptibility to lifestyle diseases [19]. Accelerated aging is characterized by a chronic, low-grade inflammation *("inflammaging")*. Inflammaging is a highly significant risk factor for most of chronic lifestyle diseases [20] and is a potential modifiable target [21]. IL-6 is the most prominent cytokine in inflammaging and is both a marker of inflammatory status and a hallmark of chronic morbidity [22]. Impaired *neuroplasticity* due to accelerated aging can have negative influence across the entire lifespan [23]. BDNF is a major regulator of neuroplasticity [24], which may be increased in specific regions of the brain by various interventions [25]. *Health span and longevity* are influenced by several factors. Sirtuin-1 (SIRT1), a histone deacetylase (HDAC), is prominent among them and recently has become a target for various interventions [26]. It systemically influences nutrition and energy metabolism and centrally has a role in circadian rhythm, survival against stress [27] and neuronal plasticity [28].

A variety of interventions have been studied [29, 30] to determine their influence on preventing lifestyle diseases and promoting health and longevity. They include drugs targeting specific hallmarks of aging, namely, physical exercise [31], nutrition, caloric restriction [32], and antioxidants [33]. However, no single intervention is shown to be an effective preventive and therapeutic strategy for modern complex lifestyle diseases and provide comprehensive benefits for delaying or reversing accelerated aging. Therefore, further research is needed to find optimum interventions for population at risk of lifestyle diseases. Yoga is an emerging integrative health discipline, which can positively modulate mind and body [34] and has been shown to improve clinical profile of patients with various pathologies [35] including depression, obesity, hypertension, asthma, type II diabetes, and cancer. However, recent reviews on Yoga suggest that potential underlying mechanisms need to be further explored [36]. Studies on biomarkers of disease and health in Yoga based interventions are limited and they have only highlighted diabetic and lipid profiles [37, 38], stress and inflammatory markers [39, 40] and neuroimaging correlates [41], in populations with specific medical conditions. Evidence is lacking regarding the efficacy of Yoga lasting short duration of 3 to 12 weeks in improving the biomarkers of cellular aging in apparently healthy people. Thus, the present study was designed to evaluate the impact of Yoga and Meditation based lifestyle intervention (YMLI) on cellular aging and longevity by analyzing cardinal and metabotrophic biomarkers in the peripheral blood of apparently healthy subjects.

2. Materials and Methods

2.1. Study Design and Participants. Ninety-six apparently healthy people were enrolled in this 12-week prospective, open-label, single arm exploratory study, from Aug 2015 to May 2016, designed to explore the impact of YMLI on cellular aging. The key inclusion criteria were male or female aged 30–65 years and leading unhealthy modern lifestyle. The key exclusion criteria were inability to perform the yogic exercises due to any physical challenges and those with recent changes in lifestyle during last 3 months. The study was initiated after ethical clearance (ESC/T-370/22-07-2015) and the registration of the trial with Clinical Trial Registry of India (CTRI REF/2014/09/007532).

2.2. Procedure

2.2.1. Yoga and Meditation Based Lifestyle Intervention (YMLI). Eligible subjects were enrolled in the study after screening and baseline characteristics were recorded. Participants underwent 12-week pretested YMLI program comprising theory and practice sessions [42, 43]. YMLI is designed to be an integrative health strategy incorporating the classic components of Yoga including Asanas (physical postures), Pranayama (breathing exercises), and Dhayna (Meditation) which are derived from a mix of Hatha Yoga and Raja Yoga. The YMLI for the current study was suitably modified for apparently healthy subjects. YMLI program included sessions 5 days per week for 12 wks. For the first two weeks the sessions were held at integrated health clinic (IHC), AIIMS, New Delhi, and taught by registered, specialized Yoga instructors (educational qualifications include Bachelor of Naturopathy and Yoga Sciences and P.G. Diploma in Yoga Therapy). Remaining 10 weeks were home based. Monitoring of compliance of the home based YMLI was through maintenance of a dairy and telephonic contact. The details of the activities in a day during YMLI program are given in Table 1. Each session in YMLI included a set of Asanas (physical postures), Pranayama (breathing exercises), and Dhayna (Meditation) for approximately 90 minutes. This was followed by an interactive lecture (only during the first two weeks of YMLI at IHC) on lifestyle, lifestyle diseases, and importance of their prevention for 30 minutes.

2.2.2. Laboratory Procedures. During this 12-week study the participants were evaluated for various biomarkers on day 0 and week 12. Fasting venous blood samples (5 mL) were collected and divided into two parts. One part was allowed to clot and the serum was separated within 30 min and the other part was transferred to heparinized/EDTA vials and was centrifuged at 2000g for 15 minutes at 4°C. Both serum and plasma were stored at −80°C until analyzed. ROS detection was done by chemiluminescence assay (Berthold detection luminometer, USA). Peripheral blood leukocyte telomere length was measured by qPCR method and telomerase activity was determined by using a telomerase assay kit (Roche,

Table 1: Details of activities in a day of Yoga and Meditation based Lifestyle Intervention (YMLI) program.

S. No.	Practice to be done			Duration
(1)	Session preparation instructions			5 min
(2)	Prayer			3 min
	Loosening practices (warm-up)			5 min
(3)	Asanas (Postures)	Supine	Shavasana	2 min
			Uttanpadasana	2 min
			Pawanmuktasana	2 min
		Prone	Makarasana	2 min
			Bhujangasana	2 min
			Salabhasana	2 min
		Sitting	Vakrasana	2 min
			Ardha-Matsyendrasana	2 min
			Vajrasana	2 min
		Standing	Tadasana	2 min
			Vrikshasana	2 min
			Ardhachakrasana	2 min
(4)	Relaxation		Shavasana	5 min
(5)	Pranayama (Breathing Exercises)		Nadishodhana Bhramri Shitkari Shitali Brahmamudra	20 min
(6)	Aumkar recitation			3 min
(7)	Dhyana (Meditation)			20 min
(8)	Shanti mantra			5 min
(9)	Interactive session (first 2 weeks only at Integrated Health Clinic, AIIMS, New Delhi)			30 min
Total				120 min

Switzerland), as per manufacturer's protocol. 8-OH2dG was estimated in white blood cell DNA (Cayman's EIA kit). ELISA kits were used for levels of TAC (Cayman Chemical, Ann Arbor, USA), cortisol (DRG Diagnostic, Germany), β-endorphin (Phoenix Pharmaceuticals, Inc.), IL-6 (Gen-Probe, Diaclone Diagnostic, France), BDNF (Raybiotech, Inc), and sirtuin-1 (Quayee Bio-Technology). Quality-control assays for biomarkers and validation were performed.

2.2.3. Endpoints. The primary endpoint was to assess the change in levels of cardinal biomarkers of cellular aging from baseline to week 12. The biomarkers included the following: 8-OH2dG, ROS, and TAC (markers of OS and ODD) and telomere attrition markers telomere length and telomerase activity. The secondary endpoints were assessment of metabotrophic blood biomarkers associated with cellular aging, which included cortisol, β-endorphin, IL-6, BDNF, and sirtuin-1 from baseline to week 12.

2.3. Statistical Analysis. Data were analyzed using SPSS 20 (IBM Corp, Armonk, NY). Descriptive statistics are reported as means and standard deviations. Changes in outcome variables were analyzed using paired-samples t-test. Exploratory analysis included comparisons for within gender subgroups using paired-sample t-test. Significance was accepted at $p < 0.05$.

3. Results

The flow diagram of participation details is provided in Figure 1. Of 96 subjects, 94 subjects were assessed for impact analysis. Two subjects were excluded from analysis due to poor compliance to the program. Baseline sociodemographic characteristics are shown in Table 2.

After 12 weeks of YMLI, there was significant improvement in both cardinal and metabotrophic biomarkers of cellular aging compared to baseline values (Table 3). The mean levels of 8-OH2dG and ROS were significantly lower and mean levels of TAC and telomerase activity were significantly increased (all values $p < 0.05$). The mean level of telomere length was increased but finding was not significant ($p = 0.069$). The mean levels of cortisol and IL-6 were significantly

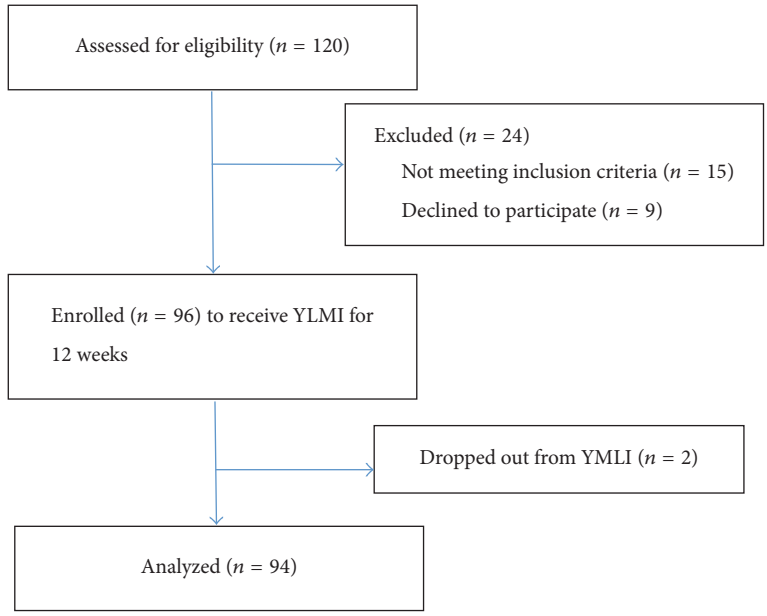

Figure 1: Flow diagram of study participation.

Table 2: Sociodemographic characteristics of participants.

Variable	Values
Age (years)	**40.26** (**10.13**)
Sex	
Female	**52** (**55.32**)
Male	**42** (**44.68**)
Socioeconomic status	
Kuppuswamy socioeconomic status scale	
Education	4.82 (1.24)
Occupation	5.26 (2.38)
Income	8.60 (2.74)
Total	**18.68** (**7.34**)
BMI (kg/m^2)	**26.30** (**3.40**)

Data were described as frequency (%) for sex and mean (SD) for others.

lower and mean levels of β-endorphin, BDNF, and sirtuin-1 were significantly increased (all values $p < 0.05$).

Few differences were noted in the gender subgroup analysis. Only male subgroup showed significant decrease in the levels of IL-6 and a more marked reduction in cortisol levels (males $p = 0.001$; females $p = 0.036$). After 12 weeks of YMLI, we also noted significantly reduced BMI in the study population ($p < 0.01$).

4. Discussion

The results of this study highlight the positive impact of YMLI on biomarkers of cellular aging and in promoting cellular longevity through changes in both cardinal and metabotrophic biomarkers. The findings suggest that the impact is mediated through improvement in genomic stability, telomere metabolism, and balance of cellular oxidative stress, well-regulated stress and inflammatory responses, and increase in neuroplasticity and nutrition sensing.

Genomic stability is central to cellular longevity and disease-free youthful healthy life and findings from our study suggest the reduction of genomic instability (decreased levels of 8-OH2dG) by YMLI. Unhealthy social habits (smoking, excess alcohol intake, etc.), sedentary lifestyle, exposure to environmental pollutants, and intake of processed and nutritionally depleted food have taken a toll on human health with onset of lifestyle diseases at a much younger age [3–5]. These environmental and lifestyle factors are responsible for genomic instability [10]. DNA damage to both mitochondrial and nuclear genome from endogenous as well as exogenous insults results in accumulation of genetic aberrations and genome hypermutability [8–10].

This is mainly due to aberrant DNA damage response (DDR) pathway, which is essential for DNA repair and for monitoring genomic integrity. Deficient DNA repair triggers systemic effects to promote pathological aging [10]. Reduction of DNA damage by YMLI suggests potential of yoga in activating DDR pathway to repair genomic damage and improve genomic stability and changes in metabotrophic factors seen in the study may be associated with these benefits.

Maintaining telomere length through regulation of telomere metabolism contributes to genomic stability and reduction in telomere attrition (increase in telomere length and telomerase activity levels) shown by our study after YMLI suggests the potential for yoga in telomere metabolism and cellular longevity. Telomeres, which serve as a biological clock, are highly conserved hexameric repeats and maintaining their length is vital for cellular longevity. Telomerase

Table 3: Change in outcomes in apparently healthy sedentary subjects participating in a Yoga and Meditation based lifestyle intervention ($n = 94$).

Characteristics	Baseline	12 wks	Change from baseline to 12 wks (diff. 95% C.I.)	Effect size*	p value
Primary endpoints: cardinal biomarkers of cellular aging					
Oxidative stress					
ROS (RLU/min/10^4 neutrophils)	1215.069 ± 88	1020.81 ± 79	194.3 (164, 224.5)	0.7	<0.0001
TAC (mmol Trolox equiv/L)	5.94 ± 1.52	7.4 ± 2.1	−1.16 (−1.9, −0.41)	0.4	<0.001
DNA damage					
8OH2dG (pg/mL)	1026.23 ± 630	790.98 ± 400	235.3 (72.73, 397.8)	0.22	<0.01
Telomere attrition					
Telomerase activity (IU/cell)	1.89 ± 1.42	2.94 ± 2.2	−1.05 (−1.68, −0.41)	0.3	<0.001
Telomere length (IU/cell)	2.36 ± 1.6	2.44 ± 1.4	−0.08 (−0.61, 0.45)	0.02	0.069
Secondary endpoints: biomarkers associated with cellular aging					
Cortisol (ng/mL)	118.83 ± 50.50	96.32 ± 38.6	22.51 (7.6, 37.42)	0.3	<0.01
Interleukin (IL6) (pg/mL)	3.16 ± 2.42	1.94 ± 2.3	1.22 (0.47, 1.97)	0.3	<0.001
β-Endorphins (ng/mL)	6.2 ± 3.5	8.2 ± 4.2	−2 (−3.22, −0.77)	0.3	<0.001
BDNF (ng/mL)	19.7 ± 6.75	37.1 ± 5.6	−17.4 (−19.48, −15.32)	0.7	<0.0001
Sirtuin (ng/mL)	26.69 ± 10.42	40.64 ± 11.6	−13.95 (−23.41, −4.49)	0.5	<0.01
BMI (kg/m^2)	26.30 ± 3.40	23.64 ± 3.55	2.66 (0.56, 3.12)	0.4	<0.01

*Effect size was calculated by dividing change by standard deviation at baseline of the specific outcome and interpreted using Cohen's d (small effect: 0.2 to 0.3, medium effect: 0.5, and large effect: 0.8).

is an important regulator of telomere length and accurate regulation of its activity, and a correct telomere-telomerase interaction is important to precisely safeguard telomere length and prevent telomere attrition [44]. ODD is prominent among the factors which can adversely affect telomere length [45]. Rapid telomere attrition due to ODD is associated with senescence and related disease conditions [46, 47]. Improved telomere metabolism after YMLI seen in the study may contribute to genomic stability. More research is needed to explore the mechanisms of how yoga and meditation intervention can positively modify telomere metabolism.

Our study suggests that improvement in maintenance of balance in cellular oxidative stress (decrease in ROS and increase in TAC) by YMLI. Supraphysiological ROS levels are due to endogenous and exogenous factors like smoking, excess alcohol consumption, exposure to electromagnetic radiation, infection, xenobiotic exposure, and psychological stress [48]. Even the levels of ROS below physiological limits are deleterious to normal cellular function and maintaining OS at physiological levels is important for cellular longevity. Increased OS causes damage to all molecules, including damage to DNA and telomeres. It also affects signal transduction and gene transcription by causing genome wide hypomethylation [49] and thus causes changes in the epigenome. Regulation of cellular oxidative stress within physiological limits after YMLI suggests the potential of this intervention in protecting cells from OS induced DNA damage and telomere attrition and in reversing epigenetic changes, which are accumulated due to unhealthy lifestyle and adverse environmental conditions. Other studies [50] support these findings and have shown reduced OS upregulation of telomerase activity and decreased ODD after YMLI. To combat OS people use antioxidants without monitoring ROS levels resulting in reductive stress [51] unlike in YMLI which regulates ROS levels so that no redox sensitive physiological functions are impaired.

Modern lifestyle and associated psychological stress have complex interactions with lifestyle habits, environmental conditions, and medical interventions to cause accelerated cellular aging, which adversely affect our mental, physical, and reproductive fitness [3–5]. Improved cellular longevity after YMLI suggests the potential role of Yoga in promoting this fitness. While psychological stress is a major manifestation on mind contributing to increased prevalence of neuropsychiatric disorders including depression, abnormal fat accumulation is a major somatic manifestation contributing to increased prevalence of metabolic syndrome and all the diseases that come under the umbrella of metabolic syndrome including, obesity, DM, and CVD [52]. Other peripheral manifestations of unhealthy modern lifestyle include aging of gonads leading to infertility [53] and recurrent pregnancy loss. Previous studies have demonstrated the clinical benefits of Yoga and Meditation in all these medical conditions [35]. Dada et al. have shown that YMLI can reduce testicular aging and result in significant upregulation in telomerase activity and decline in seminal OS and ODD [8]. The ongoing studies in our laboratory on the impact of Yoga and Meditation have provided significant

evidence for the reversal of cellular aging in subjects prone to accelerated aging due to depression. Microarray (Agilent 8 × 60k Microarray kit) and analysis of gene expression pre- and post-YMLI showed decreased IL6, IL10, and MAP10 and increased IL2 and IL4 [8, 54, 55]. Improved cellular longevity seen in our study after YMLI suggests that changes in both cardinal and metabotrophic biomarkers of cellular aging may be a mechanism for preventing chronic lifestyle diseases. Our study suggests that the changes in metabotrophic factors, which include increase in levels of β-endorphin, BDNF, and sirtuin-1 and decrease in levels of cortisol and IL-6, and the cellular processes involving them, may have important roles in reversal of cellular aging and improving cellular longevity after YMLI.

Improvement in stress and inflammatory response in our study after YMLI may be mediated by changes in cortisol, β-endorphin, IL-6, and other factors, with regulation by changes in brain through hypothalamic-pituitary-adrenal (HPA) axis. The response may involve regulation of adaptive pathways including integrated stress response (ISR) [18], which activate the eukaryotic translation initiation factor 2 alpha (eIF2α), that promote cellular recovery driving the signaling toward cell survival and longevity. The response may lead to decreased OS and reversal of senescent secretory phenotype of cells, including cells in brain, adipose tissue, endothelium, and gonads. Changes in secretory phenotype include decreased IL-6 [56], increased BDNF, and sirtuin-1 [57]. These regulated factors may lead to balance in OS and cellular longevity and contribute to tissue revival throughout the body from neuroplasticity in CNS to gonads, vessels, and muscles in the periphery. Secretory phenotype from somatic cells provides regulatory feedback to brain [58], which completes the vicious cycle of regulation between mind and body. Neurodegeneration is associated with pathogenesis of several neuropsychiatric conditions and neuroplasticity has a central role in their management and for vitality. Increased BDNF, sirtuin-1, and β-endorphin and decreased cortisol, which decrease cellular aging in brain, decrease neurodegeneration and increase neuroplasticity [23]. Increased cellular longevity and increased neuroplasticity may be a mechanism for alteration of gray matter volume in different regions of the cerebral cortex [59], increased mindfulness [60], and several other complex processes [55, 61] involved in reduction of stress and depression after Yoga and Meditation. Regulated mind-body communications may lead to minimization of subclinical inflammation and activation of nutrition and energy sensing pathways promoting longevity, where decrease in IL-6 and increase in sirtuin-1 play a prominent role, respectively. Previous studies have demonstrated increase in sirtuin-1 levels after interventions with caloric restriction [62]. Our study is the first to document increase in sirtuin-1 levels independent of caloric restriction after practicing Yoga. These improved processes may result in delaying onset and slowing down progression of diseases associated with accelerated cellular aging.

The impact of the intervention in both genders was assessed separately since men and women respond differently to day to day stress [63]. Interestingly, the gender subgroup analysis showed that reduction in cortisol and IL6 levels were more pronounced in male than in female subjects. No significant gender differences were seen in other biomarkers. Phase of the menstrual cycle should be taken into consideration since some biomarker levels are known to vary with different phases of the menstrual cycle [64, 65]. Our study showed significant decrease in BMI in apparently healthy subjects which came into normal range (23.64 ± 3.55) from baseline overweight range (26.30 ± 3.40). While latest research [66] suggests people with mean BMI of 27, who are overweight by current classification of obesity, are likely to survive longest in western population, similar data is not available for Indian population. Therefore, our findings need to be interpreted cautiously.

Stratification of cases was not done in this study to do subgroup analysis due to small sample size.

Lifestyle is an integrated entity, and an intervention, like YMLI, that has overall positive influence on our health appears most useful versus changing only one aspect at a time, as is seen by action of certain drugs. Yoga is holistic and a mind-body medicine and is more beneficial and advantageous than individual interventions like physical exercise, caloric restriction, and antioxidants. The practice of Yoga and physical exercise are different entities, the former results in energy conservation with economy of energy expenditure for mental and physical benefits, and the later results in energy expenditure more for physical exertions and metabolic needs, as is evident from a study which showed exercise causes erratic changes in biomarkers and results in OS [67] while Meditation brings about uniform biomarker and behavioral changes and improvement in cognition and decrease OS [68]. Therapeutic antioxidants can only decrease ROS rather than regulating it and may paradoxically shorten life span [69] due to imbalance in ROS mediated immune response [70]. YMLI regulates ROS rather than simply lowering them by balanced stress-related processes and appropriate gene expressions [71]. The only limitation of our study is that it is a single arm proof of concept study and did not include controls. It is important to adopt a lifestyle which slows the decline in health by reversing or delaying accelerated aging due to unhealthy lifestyle. The biomarkers of cellular aging can form the basis for determining the risk of chronic lifestyle diseases and the efficacy and usefulness of interventions to decrease disease risk. Hence, findings from this study are supportive of YMLI as a significant clinical utility especially in prevention of and management of complex multifactorial diseases and reducing the rate of functional decline with aging.

5. Conclusion

Though we cannot change our biology or chronological age we can definitely reverse/slow down the pace at which we age by adopting YMLI. This is the first study to demonstrate improvement in both cardinal and metabotrophic biomarkers of cellular aging and longevity in apparently healthy population after Yoga and Meditation based lifestyle intervention. So our health and the rate at which we age entirely depends on our choices. Making Yoga and Meditation an integral part of our lifestyle may hold the key to delay aging or aging

gracefully, prevent onset of multifactorial complex lifestyle diseases, promote mental, physical, and reproductive health, and prolong youthful healthy life.

Competing Interests

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

Acknowledgments

The authors are thankful to Amit Tomar and Sudhir Choudary for Yoga instructions and all the subjects enrolled in the study.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 6469138, 13 pages
https://doi.org/10.1155/2017/6469138

Review Article

Nutritional and Lifestyle Interventions for Age-Related Macular Degeneration: A Review

Ângela Carneiro[1,2] and José Paulo Andrade[3,4]

[1]*Department of Surgery and Physiology, Ophthalmology Unit, Faculty of Medicine of University of Porto, Porto, Portugal*
[2]*Service of Ophthalmology, Hospital S. João, Al. Professor Hernâni Monteiro, 4200-319 Porto, Portugal*
[3]*Department of Biomedicine, Anatomy Unit, Faculty of Medicine of University of Porto, Al. Professor Hernâni Monteiro, 4200-319 Porto, Portugal*
[4]*Center for Health Technology and Services Research (CINTESIS), Faculty of Medicine, University of Porto, Rua Dr. Plácido da Costa, 4200-450 Porto, Portugal*

Correspondence should be addressed to José Paulo Andrade; jandrade@med.up.pt

Received 27 September 2016; Revised 2 November 2016; Accepted 14 December 2016; Published 5 January 2017

Academic Editor: Andreas Simm

Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world. In this narrative review, we will summarize the nutritional interventions evaluated in numerous observational studies and a few randomized clinical trials. The AREDS and AREDS2 studies demonstrated that supplements including vitamins C and E, beta-carotene, and zinc may reduce the progression to advanced AMD, in some patients, by 25% in five years. This is one of the few nutritional supplements known to have beneficial effects in any eye disease. Lutein/zeaxanthin supplementation may have beneficial effects in some individuals whereas omega-3 fatty acids supplementation needs to be further investigated and supported by more evidence. Genetic factors may explain the different patterns of response and explain differences found among individuals. More importantly, a combination of lifestyle behaviors such as the avoidance of smoking, physical activity, and the adoption of a healthy dietary pattern like the Mediterranean diet was associated with a lower prevalence of AMD. The adoption of these lifestyles may reduce the prevalence of the early stages of AMD and decrease the number of individuals who develop advanced AMD and consequently the onerous and climbing costs associated with the treatment of this disease.

1. Introduction

Age-related macular degeneration (AMD) is the first cause of blindness in the Western countries according to World Health Organization [1] and this disease can significantly reduce the quality of life [2]. As the central vision is lost in advanced AMD, the aging patients suffer from limitations to function independently as they have a diminished ability to recognize the faces of other people and to read the small letters in newspapers, on food packages, and on medicament labels [3].

The early stages of AMD are characterized by the presence of pigmentary abnormalities and drusen near the fovea [1]. Late AMD has two forms: (a) geographic atrophy with major loss of the retinal pigment epithelium (RPE) and choriocapillaris; (b) neovascular AMD with newly formed blood vessels in the macular region that lead to leakage of blood and serum, causing an irreversible damage and progressive vision loss [4].

The prevalence of AMD increases sharply with age but despite major geographical and lifestyle differences it appears to be similar in Caucasian populations from the United States, Australia, and Europe [5, 6]. In a meta-analysis performed in 2004 by Friedman and collaborators the prevalence rates for the late forms of the disease increase from less than 0.5% in subjects aged 50–60 years to 12–16% in individuals with at least 80 years. Concerning the early AMD, an increase from about 1.5% in Caucasians aged 40–49 years to more than 25% in individuals aged 80 years or more was found [5].

In the recent past, AMD was an ignored and untreated disease of the elderly. In the present, hundreds of millions

of the healthcare budgets of the Western countries are spent on the new treatments available for the neovascular form. According to a 2013 United Nations report concerning world population aging, the percentage of people aged 60 years or older is growing faster than any other age group due to the fertility decline and increase of longevity. With these longer lifespans, the prevalence of AMD is also rising in the rest of the world. In Asia, the number of people with AMD increased in the last two decades, doubling the number of those reported for the whole world [6, 7]. For example, in Japan, the prevalence of neovascular AMD has changed drastically. AMD was not registered in 1994 and now is the fourth-leading cause of visual disability [8], although it remains relatively low compared with Western countries [8].

There are therapies for neovascular AMD, but no effective treatment exists for early AMD and geographic atrophy, a fact that is dramatic in terms of public health in the next two decades [9]. Therefore, there is a considerable interest to prevent and delay the onset of AMD by identifying and modifying the risk factors [10]. The progression of the disease is slow, and the randomized multicenter clinical trials are extended in time, expensive, and hard to perform and analyze. However, even a modest protective effect on the prevention and/or progression of AMD would have a significant impact on patient welfare and on the burden to society.

2. Risk Factors

AMD is a complex disease with numerous inherited risk factors including one major genetic risk locus on chromosome 1 in the complement factor H region and a second locus in the HTRA/ARMS2 region on chromosome 10, in parallel with minor genetic factors identified through genome-wide association studies [10–12].

Similar to genetic polymorphism and family history of AMD, increasing age is another very important, consistent, and nonmodifiable trait. Aging is clearly associated with the exponential rise in incidence and prevalence of AMD [13]. Other nonmodifiable traits associated in the literature with the increased risk of AMD are light skin color, light iris color [14, 15], and perhaps the female gender [6, 16]. These risk factors guide the development of new therapies and novel treatment strategies but the present situation demands efforts to identify and alter modifiable risk factors.

The modifiable risk factors were found using epidemiological studies. It is important to stress that single epidemiological studies cannot be interpreted when isolated from other evidence unless they can be replicated in independent cohorts. Biological plausibility based on the pathophysiology of the disease added to the associations found in several epidemiological studies add confidence to the solidity of the results, but they do not prove the existence of causality [10].

The epidemiological studies all around the world provided important information concerning the distribution of the different patterns of the disease in several countries and identified several environmental and modifiable risk factors. The most recognized environmental risk factor for AMD is smoking and a dose-dependent relationship was found and the risk of late AMD is multiplied by 2.5 to 4.5 and appears to be cumulative over time [17, 18]. On the other hand, smoking or other environmental factors were not clearly associated with early AMD [17, 19–21].

Curiously, as AMD shares several characteristics, with Alzheimer's disease, such as intra- and extracellular deposits, it was called the "Alzheimer of the eye" [22, 23]. Additionally, AMD closely mirrors AD in terms of lifestyle and many risk factors (aging, hypertension, smoking, obesity, hypercholesterolemia, and arteriosclerosis). However, the similitude is relative as the genetic background is completely different [22, 23].

As there are no other effective means of primary prevention other than smoking cessation, other possible changes in lifestyle including nutritional changes acquire enormous importance. Multiple studies revealed oxidative stress as one of the mechanisms implicated in the pathogenesis of AMD and diet is generally the main source of antioxidants [24]. Concerning eye tissues, their biological integrity is dependent on the balance between the production of free radicals and their catabolism [24, 25].

3. The Human Eye and the Oxidative Stress

The production of free radicals increases with age, but some of the endogenous defense mechanisms decrease creating an imbalance that leads to progressive damage of cellular structures [25]. The vast antioxidative network, includes vitamins (C, D, and E), enzymes (superoxide dismutase, catalase, and glutathione peroxidase), carotenoids (alpha- and beta-carotene, lycopene, lutein, and zeaxanthin), and many other compounds (flavonoids, lipoic acid, uric acid, selenium, and coenzyme Q10) [26]. They act as a protective chain, and the different antioxidants have a synergistic effect and protect each other from direct destruction in the processes of neutralizing free radicals [25]. The generation of singlet oxygen free radicals will extract hydrogen atoms from molecules with available six double bonds, such as the omega-3 fatty acid docosahexaenoic acid (DHA), leading to lipid peroxidation [24]. The lipid peroxides cross-link with proteins, nucleic acids, and other compounds adversely affecting tissues structure and function [27].

The eye presents an antioxidant system with multiple components intervening from the aqueous humor to the retina. The retina possesses an antioxidant system with several components, but vitamins C and E and the carotenoids lutein and zeaxanthin are the most important [28]. Both vitamins cooperate to decrease the retinal epoxide adducts and also participate in the protection from blue light-induced damage [29]. The carotenoids are concentrated in the plexiform area of the macula, but zeaxanthin preferentially predominates in the central fovea whereas lutein is mostly present in the periphery. Zeaxanthin is, therefore, a more effective antioxidant in the area where the risk of oxidative damage is higher [30]. In addition, both carotenoids are found in the outer segments of rods and probably of cones, particularly rich in DHA and, therefore, potentially vulnerable to lipid oxidation [28, 31].

DHA comprises 40% of the polyunsaturated fatty acids (PUFAs) in the brain and 60% of the lipid constituents of the membrane of the retinal photoreceptors; in humans, DHA is obtained from the diet [28, 32]. Also, DHA may be converted from eicosapentaenoic acid (EPA) but this synthesis involves several steps including elongation and peroxisomal beta-oxidation [33]. DHA and EPA have pleiotropic effects through signal transduction, gene regulation, and plasma membrane dynamics [34]. Health benefits arise from the ability of DHA and EPA to reduce the production of inflammatory eicosanoids, cytokines, and reactive oxygen species [35] and modulation of the expression of numerous genes involved in the inflammatory pathways [36]. DHA is present in small quantities in most tissues but is a major structural lipid of the retina presenting particularly high levels in this neural tissue [37, 38]. DHA may be involved in the permeability, thickness, fluidity, and other properties of the membrane of photoreceptors [38] and its insufficiency is linked to changes in the function of the retina [38, 39]. The anti-inflammatory actions may inhibit the formation of new choroidal vessels that appear in exudative AMD [39, 40]. EPA and other major dietary omega-3 fatty acids appear to have a similar action [38]. The renewal of retinal membranes demands a steady supply of these omega-3 fatty acids by RPE cells. If there is an imbalance in the retinal lipids, the photoreceptor degradation and accumulation may lead to the formation of drusen, formed mainly by the debris of lipid and lipoprotein in the RPE layer and sub-RPE space [34].

4. Pathogenesis of AMD and Oxidative Stress

Although the exact pathogenesis of AMD is not known, oxidative stress is considered to be involved as the human eye, and particularly the retina is exposed to the production of free radicals leading to a prooxidative environment [28]. This vulnerability is related to several factors: (a) the high vascularization, which results in high oxygen tension; (b) the light-induced stress in fovea due to the increased cellular density and metabolism; (c) the retinal tissues that present high quantity of unsaturated fatty acids and photosensitizing compounds, which are highly susceptible to oxidation; (d) the exposition of the retina to cumulative radiation [24, 28, 41].

The putative mechanisms are probably light-initiated oxidative damage and a reduction of the macular pigment [42, 43]. The long exposure to bright light and the oxidative damage and the presence of oxidized metabolites in the outer segment of the photoreceptors of the RPE may contribute to the formation of the drusen and the pigment disturbances in the macula [44, 45].

It is, therefore conceivable that dietary antioxidants and/or supplements may be beneficial to preventing and/or delaying the progression of AMD due to the decrease of oxidative stress and reduction of inflammatory events [28].

5. Diet and the Ocular Antioxidant System

The dietary intake of vitamins, carotenoids, essential fatty acids, and other oligoelements in Western countries, is generally enough to supply the needs of healthy individuals. The major dietary sources of the 18 carbon fatty acids alpha-linolenic acid (ALA) and linoleic acid (LA) are vegetable oils [26]. The ingestion of these fatty acids is essential as humans do not possess the enzyme systems necessary to insert double bonds at the n-3 or n-6 positions [26, 46]. Diets rich in fish, meat, and eggs will provide EPA, DHA, and arachidonic acid, but they can also be synthesized from ALA and LA [24, 47]. However, the conversion rate of ALA to omega-3 PUFAs is relatively low in humans [48]. Due to this fact, that involves several steps, oils rich in ALA do not significantly increase the DHA and EPA levels [33, 46, 48].

In contrast, lutein and zeaxanthin are not synthesized by humans. They are found in vegetables such as fruits and spices (lutein) and lettuce, broccoli, and spinach (zeaxanthin) [10].

The role of nutrition and nutritional supplements has been raised by a number of observational and randomized clinical trials. Mainly three types of nutritional factors have been investigated for their potential protection against eye age-related diseases: antioxidants (mainly vitamins C, E, and beta-carotene), zinc, the carotenoids lutein and zeaxanthin, and omega-3 PUFAs. The results of the most important randomized clinical trials concerning nutritional effects of these nutritional factors on AMD are presented and discussed below [28].

6. The Age-Related Eye Disease Study (AREDS)

AREDS was a complex randomized clinical trial designed to evaluate the effect of vitamins C (500 mg), E (400 IU), beta-carotene (15 mg), with or without zinc (80 mg), and copper (2 mg), on the progression of AMD [28, 49]. Most of the 4757 patients, aged 55 to 80 years, were taking nutritional supplements at the time of the study enrollment and, to standardize this occurrence, a daily dose of a multivitamin and minerals tablet was provided (Centrum®) and 66% of the participants opted to use this supplement. Note that AREDS supplements are provided in much higher concentrations than the recommended daily intake [50]. Therefore, the effects found were attributed to the AREDS supplements [50]. The patients were followed up for a mean period of 6.3 years [28].

The kind of vitamin E used in AREDS is alpha-tocopherol, the form that is mainly present in tissues and blood [51]. Vitamin E exists as eight fat-soluble compounds of tocopherols and tocotrienols, and each subgroup has several subtypes (alpha, beta, gamma, and delta). Nuts and seeds, whole grains, and dark leafy vegetables (spinach, collard greens) are rich sources of alpha-tocopherol that is absorbed and accumulated [50, 51]. Note that the doses used in the AREDS trials were 400 UI daily whereas the recommended daily intake is only 22.4 UI [50].

Beta-carotene is a carotenoid often referred to as provitamin A [51]. Due to its antioxidative properties beta-carotene was included in the multivitamin supplements, but a solid role in the prevention of AMD was not strongly supported [51]. Also, due to the effective antioxidant activity, vitamin C (ascorbate) was included in the AREDS formulation. However, there was no association between vitamin C intake and AMD in the Pathologies Oculaires Liées à l'Age (POLA)

study or in the Eye Disease Case-Control Study Group [51–53].

At the beginning of the study, 1117 patients had few if any drusen (Category 1) and 1063 patients had extensive small drusen, pigment abnormalities, or at least 1 intermediate size drusen and were classified as Category 2. Category 3 included 1621 participants possessing extensive intermediate drusen, geographic atrophy not involving the center of the macula, or at least 1 large drusen. Finally, 956 participants were included in Category 4 as they had advanced AMD or visual acuity less than 20/32 due to AMD in one eye [28]. The results showed that treatment with zinc alone or in combination with antioxidants reduced the risk of progression to advanced AMD in patients of Categories 3 and 4 [28]. This reduction of the risk at 5 years for those taking the supplements plus zinc was 25% when compared to placebo controls. The treatment effect persisted following 5 additional years of follow-up after the end of the clinical trial [54]. During the study, 407 participants without geographic atrophy at baseline developed, at least, moderate geographic atrophy, not necessarily involving the center of the macula [28]. No significant differences among treatments with antioxidants, with zinc, or antioxidants plus zinc on the progression of AMD were found [49].

After the publication of the AREDS report in 2001, the AREDS formulation became the standard of care. Due to serious concerns about the safety of beta-carotene that increased the risk of lung cancer in smokers, this compound was replaced in the randomized clinical trial known as AREDS2 [28]. In addition to this concern, it was found that beta-carotene was not detectable in the human retina contrary to lutein, zeaxanthin, and mesozeaxanthin [10]. Moreover, in the Beta-Carotene and Retinol Efficacy Trial (CARET) study, the combination of retinol (vitamin A) and beta-carotene increased the risk of lung cancer and cardiovascular events [55]. This evidence discourages the use of beta-carotene in the prevention of AMD due to not only the side effects but also the poor efficacy [51]. In addition, vitamin A alone was found to have no solid results concerning the prevalence of AMD [51] and it was not included in the original AREDS formulation.

7. The Age-Related Eye Disease Study 2 (AREDS2)

The AREDS2 was a phase 3 study and controlled clinical trial, involving 4203 patients, aged 50 to 85 years [56]. Conducted in 2006–2012, the selected participants were at risk of progression to advanced AMD with bilateral large drusen or large drusen in one eye and advanced AMD in the fellow eye [28, 56]. The main aim of AREDS2 was to improve the original AREDS formulation, turning it more effective and safer. The lutein/zeaxanthin daily dose chosen was in the ratio 5 : 1 (10 mg of lutein : 2 mg of zeaxanthin) based on a small-scale study [57, 58]. These daily doses are substantially higher than a typical Western diet (1-2 mg of lutein : 0.2 mg of zeaxanthin). The primary randomization considered four groups of participants: (a) lutein (10 mg) + zeaxanthin (2 mg); (b) fish oil (350 mg of DHA + 650 mg of EPA); (c) lutein + zeaxanthin + DHA + EPA; and (d) placebo [56].

The expected 25% incremental improvement over the original AREDS results was not obtained [56]. The addition of lutein + zeaxanthin, DHA + EPA, or both to the AREDS formulation did not further reduce the risk of progression to advanced AMD. In the analyses evaluating the patients assigned to lutein/zeaxanthin (2123) versus the patients not assigned to lutein/zeaxanthin (2080), an added 10% reduction was found in the risk of progression to advanced AMD in patients assigned to lutein/zeaxanthin versus those not assigned to lutein/zeaxanthin. However, the patients that received DHA + EPA did not show this effect. Lutein/zeaxanthin was not related to increase in lung cancer incidence, contrary to beta-carotene that was associated with a rise in lung cancer in former smokers [28, 56]. Mesozeaxanthin is now available in the market but there is no randomized clinical trial supporting its superiority to lutein/zeaxanthin [10].

In brief, the evidence on beneficial and adverse effects from AREDS2 and other studies suggests that lutein (2 mg) + zeaxanthin (15 mg) could be more adequate than beta-carotene (15 mg) in the AREDS-type supplements, particularly for former or current smokers. Other studies also found a correlation between plasmatic high levels of lutein/zeaxanthin and low risk of AMD, like the POLA Study and others [52, 59, 60].

Large-scale epidemiologic observational studies, such as the Eye Disease Case Control Study (USA), agree with the findings of beneficial effects of dietary intake of carotenoids [61] and several other studies demonstrated an increase in macular pigment associated with the intake of carotenoids [60, 62, 63]. There are other epidemiological studies with other conclusions. For instance, the Rotterdam study showed a reduced risk for AMD in subjects with high dietary intake of beta-carotene, vitamins C, and E and zinc [64], while the Physicians' Health Study did not reveal benefits for vitamins C or E [65].

Interestingly, data from the United States differs from European data, which could be justified by differences in nutritional patterns or supplement intake. For example, the majority of the participants in AREDS study used vitamin supplements (Centrum) in addition to the supplementation tested in the study [4]. The mean baseline plasmatic vitamin C was 62 micromole/L in AREDS participants, whereas it was 31.6 micromole/L in men and 40.5 micromole/L in women enrolled in the French POLA study [4, 66]. The plasmatic values of vitamin C of this French study were comparable to those of the EUREYE Study implemented in seven European countries [67].

The systematic review of prospective studies of dietary intake found no evidence that diets high in antioxidant vitamins prevent AMD [68, 69]. More specifically, there is no evidence from clinical randomized trials that healthy people should take vitamin and antioxidant supplements to delay or prevent the onset of AMD [68]. Recently, it was reported that the high dietary intake of folate was associated with a decreased risk of progression of geographic atrophy, using a large prospective cohort with a high number of

incident cases [70]. This association could be modified by genetic susceptibility, particularly associated with the C3 genotype [70]. In this same study, thiamin, riboflavin, niacin, and vitamins B6 and B12 were not significantly related to progression [70]. Folate is provided by green vegetables, fruits, nuts, beans, and peas sharing the same food sources as lutein and zeaxanthin [70].

8. Zinc Supplementation

Zinc levels are high in ocular tissues, but the distribution is not uniform [47]. It is the most abundant trace metal in the retina and is preferentially located in the inner nuclear and PR layers, that is, the regions that are affected by AMD [71]. Total zinc concentration in the RPE is high enough to release reactive zinc in the high micromolar range [71]. In photoreceptors, the loosely bound zinc ions may play a role in the regeneration of rhodopsin and in the phototransduction cascade [71]. The US Food and drug Administration (FDA) recommends 11 mg/day of zinc for men and 8 mg/day for women. A daily replenishment of approximately 1% of the total body zinc is obtained with the diet and prolonged periods of zinc depletion cannot be compensated [71]. Oysters and other seafood contain more zinc per serving than any other food [50]. Beef, poultry, and pork generally provide the necessary intake [50]. Although zinc is present in beans, cereals, and nuts, the plant-based phytates may inhibit their absorption [50]. Therefore, if meat and seafood are scarce in the diet the consumption of zinc may not be sufficient as the body does not have a storage of zinc [50, 71].

Zinc is a cofactor of many active ocular enzymes, including superoxide dismutase and catalase [8, 72]. Zinc also binds complement factor H, inducing large multimeric forms that loose complement 3b inhibitory activity [73]. This action can help to suppress the chronic inflammatory events in the retina that can lead to AMD [73]. In vitro studies suggested that zinc supplementation attenuates endothelial cell activation and may affect the progression of AMD [74]. In an animal model of light-induced retinal degeneration, the zinc supplementation induced changes in gene expression enhancing antioxidative retinal capacity and the reduction of oxidative damage [75].

In AMD, the content of zinc in human RPE/choroid in AMD is decreased by 24%. On the other hand, high levels of zinc are present in drusen, probably affecting the oligomeralisation of complement factor H [47, 71]. Also, the expression of some transporters, sensor, and trafficking/storage proteins were found to be downregulated in AMD maculas [47, 71]. These changes in retinal zinc homeostasis justify its presence in the AREDS formulation and in other trials. As high doses of zinc may interfere with copper absorption the AREDS formulation included a copper supplement (2 mg/day) [4].

A systematic review of these trials with zinc was published recently [76]. Ten studies were included: four randomized clinical trials, four prospective cohorts, and two retrospective cohort studies analyzing the effect of zinc intake, both from supplements and/or foods in the treatment and primary prevention. The review concluded that current evidence on zinc intake for the prevention of AMD is inconclusive. However, AREDS revealed that 80 mg of zinc oxide, alone or in combination with antioxidants, significantly reduced the risk of progression to advanced AMD in individuals with moderate AMD. The risk of visual acuity loss was of similar magnitude, but not statistically significant. Other two randomized clinical trials showed a statistically significant increase in visual acuity in early AMD patients and the other one revealed no effect of zinc treatment on visual acuity in advanced AMD patients [76]. Furthermore, results from the remaining six cohort studies on associations between zinc intake and incidence of AMD were inconsistent. The authors of the systematic review concluded, based on the strength of AREDS, that zinc treatment may be effective in preventing progression to advanced AMD but zinc supplementation alone may not be sufficient to produce clinically meaningful changes in visual acuity [76].

Due to possible secondary effects in the stomach of the high doses of zinc, AREDS2 evaluated the effects of a lower dose of zinc (25 mg versus 80 mg) but no significant difference in AMD progression was reported [56]. Note that, as stated previously, most of the participants in both AREDS trials also took simultaneously Centrum that included the recommended daily intake of zinc, increasing the daily dose of zinc [4]. As a trend favoring the higher dose of zinc was found [77], the present AREDS2 formulation includes 80 mg of zinc. It should be noted that the doses of daily zinc are much higher than the recommended by FDA but the secondary effects described were minimal.

More recently, it was reported that response to AREDS supplements is in accord to genetic factors and the effectiveness of antioxidant/zinc supplementation is influenced by genotype [78]. Particularly, in AREDS patients with the high-risk CFH/low-risk ARMS2 genotype, the zinc-containing treatments may worsen outcomes for patients who have moderate to severe AMD [77].

9. Lutein and Zeaxanthin Supplementation

As already mentioned the carotenoids, lutein/zeaxanthin, are concentrated at the central fovea composing the macular pigment [2, 42] and protect the macular region from photooxidative injury by scavenging reactive oxygen species and filtering the potential damaging blue light and can decrease a toxic byproduct of the visual cycle (A2E) stimulated by this blue light [10, 42]. Lutein/zeaxanthin supplements may offer protection to decrease the number of lipofuscin granules and increase the stability of lysosomes [42]. Using animal models, lutein/zeaxanthin preserved macular health and improved functional abnormalities [14]. The absorption of poor-focused short wavelengths by the macular pigment decreases chromatic aberration and improves visual resolution [15, 79].

Lutein/zeaxanthin supplementation from foods can increase pigment macular density, but this capacity varies among individuals [79]. Physical activity may contribute to a denser macular pigment directly due to the reduction of inflammation and oxidative stress, or indirectly by reducing obesity. In fact, obesity is related to a lower density of the macular pigment in this study and others and may increase

oxidative stress [13]. The status of the macular pigment may be improved by healthy diets and physical exercise [10, 79].

Despite the strong biological plausibility, the epidemiological studies have not found consistent results [80]. More importantly, as stated above AREDS2 trial failed to confidently demonstrate, in the primary analyses, the protective effects of lutein/zeaxanthin [56]. However, there was a 26% risk reduction when the analysis was restricted to a subgroup of participants at the bottom 20% of the dietary intake of lutein/zeaxanthin [56, 81]. This secondary analysis supports the hypothesis that supplements are more effective only when the background dietary intake is below a sufficient threshold [81].

This hypothesis is supported by a long-running (two decades of follow-up) very large prospective cohort from the Nurses' Health Study and Health Professionals Follow-up Study that found an association between a higher intake of lutein/zeaxanthin with a 40% lower risk of advanced AMD [80]. The intake of carotenoids was not associated with intermediate AMD. This finding was interpreted as an effect on the progression of AMD but not on the initiation of the disease [80]. Although there is no causal inference this is probably the best available evidence in the absence of a near future and well-designed large-scale randomized trial as AREDS2 [80].

Some of other studies found an association between carotenoids and intermediate AMD. However, only one of 3 prospective cohort studies reported a significant inverse association between the intake of lutein/zeaxanthin and intermediate AMD [42]. In late AMD the dysfunction or loss of macular photoreceptors may not be rehabilitated [82]. A meta-analysis of 8 high quality randomized clinical trials involving 1176 AMD patients in which a comparison of lutein/zeaxanthin intervention with placebo was performed [42]. The outcome measurements included visual acuity, contrast sensitivity, glare recovery time, and subjective perception of visual quality. They found that lutein/zeaxanthin improved visual acuity and contrast sensitivity of AMD patients and, more importantly, there was a dose-response relationship [42].

The intake of xanthophyll carotenoids is far below the recommended level and there is a tendency for a decrease in Western countries [83]. A recommendation for an increased intake of lutein/zeaxanthin from food sources or supplements should be advised, especially for AMD patients. The results of the Taurine, Omega-3 Fatty Acids, Zinc, Antioxidant, Lutein (TOZAL) study showed that AMD patients require at least 6-month supplementation to have a positive outcome such as changes in visual acuity and other visual parameters [84].

10. Omega-3 Supplementation

In AREDS2, the addition of DHA + EPA to the original AREDS formulation (vitamin C, vitamin E, beta-carotene, zinc, and copper) did not further reduce the risk of AMD. This finding was unexpected as epidemiological studies have for more than 10 years pointed to a beneficial effect of dietary omega-3 in the prophylaxis of AMD [85]. There is consistent evidence that the intake of high doses of DHA present in oily fish is associated with a decreased risk of neovascular AMD [32, 39]. Concerning the dietary consumption of n-3 fatty acids the Eye Disease Case Control Study in the United States demonstrated an association between the higher intake of n-3 fatty acids and a lower risk of AMD among individuals on a diet low in LA [8, 25]. The Blue Mountains Eye Study, in Australia, demonstrated a protective effect of n-3 fatty acids in late AMD in individuals in the highest quintile of intake [43]. Subjects in the AREDS trial who reported the highest consumption of n-3 fatty acids were also less likely to have neovascular AMD at baseline [44]. Other studies have reported an inverse association between the dietary consumption of n-3 fatty acids and neovascular AMD [44, 45, 57]. Some researchers criticize AREDS2 stating that the design, setting, and selection of patients of AREDS2 trial had not allowed ascertaining the potential of omega-3 supplements [85] due to an inadequate duration of treatment, inadequate dose, or both [56, 85]. Another possible explanation is that the effect may be modified by underlying genetic risk factors [86, 87]. It is also possible that there is an optimal level of supplementation with omega-3 fatty acids and if the level is not optimal it could be ineffective or detrimental. A more appropriate conclusion is that in a well-nourished population AREDS2 was not more effective than the original formulation [22].

A prospective randomized prospective study, the Nutritional AMD Treatment-2 (NAT-2) trial, had a true placebo group and demonstrated that patients who achieved red blood cell membrane EPA/DHA levels were significantly protected against AMD versus those patients with low levels of EPA/DHA [32, 39]. However, in the same NAT-2 study, with the supplementation with 840 mg/day DHA (87 individuals) or placebo (80 individuals) for 3 years, the dynamic drusen remodeling was not affected by DHA supplementation [34]. This drusen remodeling showed a tendency to be influenced by CFH genotype [34].

In an open-label experiment, it was reported that high doses of EPA (3.4 g) and EPA (1.6 g) on a daily basis for 6 months improved the visual acuity of patients with dry AMD [88]. Also regarding the intake of omega-3 PUFAs, a Cochrane meta-analysis published in 2012 and updated in 2015 concluded that there is currently no evidence to support that increasing levels of omega-3 PUFAs in the diet prevent or slow the progression of AMD, in accordance with AREDS2 trial [89, 90].

In short, the majority of evidence suggesting a positive effect of dietary omega-3 intake on the development and progression of AMD comes from observational studies and remains to be demonstrated in randomized clinical trials [39]. In a recent paper, the systemic confounders that may affect the serum measurements of omega-3 and omega-6 PUFAs in patients with retinal disease were discussed [91]. In that pilot study, they demonstrate that serum lipid profiles are significantly altered by variables like fasting and medication. The patient fasting status may affect the serum levels of unsaturated and saturated fatty acid levels influencing the results and conclusions of the studies [91].

Summing up the current evidence it is reasonable to advise AMD patients to consume dietary fatty fish (e.g.,

salmon, tuna, sardine, mackerel, and trout) or fish oil supplements. It is prudent to inform the patients of the doubts concerning their efficacy and the lack of support from large clinical randomized trials.

11. The Mediterranean Diet

Dietary patterns where nutrients of the food can interact synergistically is a recent paradigmatic major change in nutritional sciences. Numerous studies show that a healthy diet, the maintenance of an adequate body weight, and an active lifestyle are important to maintaining health and avoiding the physical and cognitive degeneration associated with aging [3, 26, 92].

The Mediterranean diet is one of the most studied healthy dietary patterns. This diet is rich in vegetables and fruits, thereby providing high amounts of bioactive antioxidant compounds. Furthermore, olive oil and fatty fish rich in omega-3 fatty acids are present in the Mediterranean dietary pattern [26, 93].

From the scientific point of view, there are major differences between the Mediterranean diet and the so-called Western diet. In fact, the INTERHEART and INTERSTROKE studies [94, 95] considered the existence of three main dietary patterns, evaluated by a dietary risk score: (a) Western diet with high intake of fried foods, salty snacks, eggs, and red meat; (b) oriental diet, high in tofu and sauces like soy and others; (c) prudent diet with a high intake of fruits and vegetables with some characteristics of the Mediterranean diet. It was found that the Western diet increased the population risk for acute myocardial infarction and stroke by approximately 30%. In contrast, the prudent diet decreased the same risk by 30%. The oriental diet was neutral probably because the high intake of fruits, fish, and vegetables was offset by the high salt intake and other factors [26, 94, 95].

Defining a Mediterranean diet is difficult considering that the geographical region includes more than 17 countries [26]. According to De Lorgeril, the Mediterranean dietary pattern is inspired by the traditional diet found in Greece and South Italy [26]. It presents the following characteristics: (a) it has high consumption of fruits, legumes, and other vegetables, bread and other cereals, potatoes, beans, nuts, and seeds; (b) olive oil is the main source of monounsaturated fat; (c) dairy products, fish, and poultry are consumed in low to moderate amounts; only low quantities of red and processed meat are present; (d) wine is consumed during meals in low to moderate amounts [26, 96].

Summarizing the prospective observational studies, a meta-analysis was published by Sofi and collaborators in 2008 and updated in 2010 [97, 98]. The studies analyzed prospectively the association between adherence to a Mediterranean diet, mortality, and incidence of diseases, with a total of more than 2 million subjects followed for a time ranging from 3 to 18 years. Greater adherence to a Mediterranean diet was associated with an improvement in health status, namely, a major reduction in overall mortality (9%), mortality from cardiovascular diseases (CVD) (9%), and incidence of mortality from cancer (6%). The reduced incidence of Parkinson's and Alzheimer's diseases (13%) demonstrated that the adherence to the Mediterranean diet prevented these major chronic neurodegenerative diseases associated with aging [26, 97, 98].

The *Prevención con Dieta Mediterránea* (PREDIMED) trial, a multicenter randomized clinical trial in Spain [99], showed that relatively small changes in diet have beneficial effects [99]. The clinical trial included 7447 subjects at risk of CVD. The conclusions demonstrated that the adoption of a Mediterranean-style diet reduced the risk of cardiovascular complications by 30%, including a reduction of the risk of stroke by 40% over a follow-up of approximately 5 years [99]. In 2013, the Cochrane Heart Group published a review analyzing the Mediterranean dietary pattern for the primary prevention of CVD [100]. The authors concluded that the Mediterranean diet may reduce some cardiovascular risk factors (total cholesterol levels, LDL cholesterol levels) [100]. Additional randomized clinical trials are necessary, but there is sufficient scientific evidence to recommend the Mediterranean dietary pattern to the individuals who want to age in good health while still appreciating food [26, 101].

12. Mediterranean Diet and AMD

Assessing the dietary pattern with respect to AMD is relatively recent. Association of a particular nutrient to AMD is difficult and impossible to dissociate from other aspects of the diet. More importantly, there are synergistic relationships of the food components [3, 26, 86].

In the Carotenoids Age-Related Eye Disease Study (CAREDS), the high adherence to a Mediterranean diet was associated with a lower prevalence of early AMD [86]. Previously, it was reported that advanced AMD was related to overall diet quality using the Health Eating Index (HEI) [102]. Data from the Melbourne Collaborative Melbourne Study showed that a diet rich in fruits, vegetables, nuts, and chicken was associated with a lower prevalence of AMD [103]. Conversely, the Western diet was associated with increased odds of AMD [104]. The oriental pattern of diet was linked to decreased odds of AMD [104].

Merle and collaborators used the data from 2525 individuals of the AREDS trial and dietary information collected from food-frequency questionnaires and the alternate Mediterranean diet score [86]. This score was constructed from individual intake of vegetables, fruit, legumes, whole grain, nuts, fish, red and processed meats, alcohol, and the ratio of monounsaturated to saturated fats. They reported that a high score was associated with a 26% reduced risk of progression to advanced AMD after adjustments for demographic, behavioral, ocular, and genetic factors. The consumption of fish and vegetables was associated with a lower risk of progression to advanced AMD [86]. The administration of AREDS supplements did not change the protective effect of the Mediterranean diet on the risk of progression [86]. In addition, they reported that the Mediterranean score was associated with a lower risk of advanced AMD among individuals carrying the CFH Y402H nonrisk (T) allele. CFH is one of the main genes implicated in AMD, and the risk allele leads to complement activation

increasing the AMD risk. The authors hypothesize that the effect of the Mediterranean diet is stronger among subjects with the nonrisk allele [86]. The biological plausibility of the benefits of the Mediterranean diet involves a decrease in oxidative stress and inflammation. Accordingly, individuals with higher adherence to the Mediterranean diet have higher plasma concentration of some important and beneficial biomarkers [105].

In a population of the central region of Portugal, it was reported that higher adherence to a Mediterranean diet score was associated with a lower AMD risk [6]. In this Coimbra study, the consumption of fruit, beta-carotene, and vitamin E was particularly beneficial [6]. In an update of the study presented in the American Academy of Ophthalmology meeting (Chicago, 2016), it was reported for the first time that coffee, also rich in antioxidants, was protective. Of the participants of this study who consumed caffeine (one cup of espresso, approximately 78 mg of caffeine), 54.4% did not have AMD (data not published).

As one of the key components of the Mediterranean diet, olive oil was studied in the ALIENOR (Antioxydants, LIpides Essentiels, Nutrition et maladies OculaiRes) study, a population-based study aimed at verifying the associations of nutritional factors such as antioxidants, macular pigment, and fatty acids [106]. After the adjustments for the multiple potential confounders, there was a decreased risk of late AMD among olive oil users in 654 subjects (1269 eyes) aged 72.7 years on average with complete data suggesting a protective role of olive oil consumption for late AMD in an elderly French population [106]. On the other hand, no association was found between olive oil consumption and early AMD and the use of another type of fats was not associated with any stages of AMD. Similar results were found in an Australian cohort (6734 individuals aged 63.7 years on average at baseline) where an inverse association between the intake of olive oil and the prevalence of late AMD was found [107].

Olive oil is a mixture of lipids and antioxidant compounds and it contains monounsaturated fatty acids (MUFAs) and polyphenols that have strong antioxidant and anti-inflammatory properties [86]. A laboratory study in ARPE-19 human retinal pigment epithelial cells reported that the major polyphenol of olive oil, hydroxytyrosol, prevented the degeneration of retinal pigment epithelial cells induced by oxidative stress [108]. Probably, the protection does not rely on oleic acid, the main MUFA component, as the associations between MUFA intake and AMD are inconsistent in literature. It is suggested that the protection derives from the phenolic compounds including oleocanthal, hydroxytyrosol, and oleuropein [106]. However, more studies are needed to find the mechanism by which olive oil has beneficial effects on late AMD [106].

No association was found between oils rich in omega-3 and any stage of AMD. The ALIENOR study also found no association between olive oil consumption and early AMD [106]. This lack of association suggests that the risk factors are dissimilar at different stages of the disease. Some risk factors may favor the accumulation of drusen and the pigmentary disturbances. Others may have an influence on neoangiogenesis and/or apoptosis. Another explanation, as suggested by some studies, is that early AMD may represent a very heterogeneous group of patients, and some of the patients present a low risk of developing late AMD [109–111].

13. The Genetic Risk, Lifestyle, and AMD

In epidemiological studies a healthy diet, avoiding food which is high in sugar, fat, alcohol, refined starch, and oils, and absence of smoking and physical activity (low-intensity exercise for one or two hours per day, outside when possible) were associated with reduced occurrence of early or advanced AMD, or both [3]. This risk reduction was greater when multiple lifestyles were considered together as shown by CAREDS [3]. In this study, women (50–74 years of age) who displayed a combination of healthy lifestyle factors such as healthy diet, physical exercise, and not smoking had a 3-fold lower odds for early AMD when compared to women who displayed unhealthy lifestyles. The several mechanisms of the healthy lifestyle leading to protection are difficult to disentangle from one another [3]. The energy expenditure of the physical exercise leads to an increase of daily nutrient intake. Both physical activity and diet may contribute to a better vitamin D status, also associated with lower risk of AMD [112].

Insufficient physical activity increases the occurrence of numerous metabolic and vascular diseases [113] and may facilitate the progression of some cases of AMD [113, 114]. The sedentarism may contribute to increasing inflammatory events and dysfunction of the endothelium [113]. This hypothesis is supported by the finding that elevated C-reactive protein in patients with neovascular AMD is partly explained by physical inactivity [115]. On the contrary, physical activity can upregulate the enzymatic systems related to antioxidant protection, reducing the oxidative events [113].

There is another important factor that can modify the benefits of the healthy lifestyles: the genetic risk [116]. In fact, in the Unites States, the Y402H (rs1061170) variant within the complement factor H (CFH) gene and the A69S (rs10490924) variant within the age-related maculopathy susceptibility 2 (ARMS2) locus increased the risk (1.5- to 3-fold) for both early and late AMD in individuals of European ancestry [116, 117]. Wang and collaborators reported that the ingestion of lutein only reduces the incidence of AMD among persons with 2 or more alleles from common CFH and ARMS2 variants [118]. Meyers and collaborators studied the joint associations of diet, lifestyle, and genes in AMD and concluded that physicians should recommend the adoption of healthy lifestyles at early ages in individuals that have a family history of AMD and also they should motivate AMD patients to follow the same advice [116].

Stressing the benefits and safety of the use of high-dose antioxidants for long periods in the prevention or delay of the progression of AMD in early stages has not been established [119]; the adoption of healthy lifestyles at early ages may ultimately benefit significantly the patients and family at genetic risk. Public health interventions to consume plant-rich, high lutein diets, physical activity, and cessation

of smoking are recommended strategies for AMD prevention. These recommendations are particularly important in individuals at genetic risk, with a family history of AMD or both. The combination of healthy lifestyles practices may be more important in the reduction of AMD than a focus on one. Collectively, these changes may reduce the oxidative stress, blood pressure, and blood lipids. In short, they may reduce the systemic inflammation that contributes to the pathogenesis of AMD and other chronic diseases including neurodegenerative diseases, although there is a lack of clinical trials to sustain with solid evidence these benefits [116]. These recommendations can lead to an improvement of outcomes through genotype-directed therapy [12]. The knowledge of the modifiable risk factors along with information concerning genetic risk variants for AMD has greatly improved the management of patients and the ability to predict which patients will develop or progress to advanced forms of AMD [87]. Individualized prevention and treatment strategies and personalized medicine are becoming a reality [87].

Changes in the lifestyle, such as eating a healthy diet like the Mediterranean diet, and if necessary complementing the dietary patterns of selected individuals with appropriate supplements, may play an important role and avoid the progression to advanced stages of AMD [86]. There are good rates of compliance with the dietary advice; that is, patients change their diet but patient education by health professionals can be improved [120].

14. Conclusion: Nutritional Advice to Patients with AMD

The nutritional advice of clinicians to their patients must be balanced and discuss what is supported by scientific evidence and the current doubts. This advice should be supported by high level of evidence, preferably from randomized clinical trials [119].

Current evidence shows that all AMD patients should be given indications to increase the consumption of green leafy vegetables and to eat fatty fish, at least twice a week. A Mediterranean type of diet may provide additional benefits in other age-related diseases beyond AMD [26].

Patients with moderate or advanced AMD should be advised to use AREDS-based supplements. Current smokers or ex-smokers are advised to avoid formulations with beta-carotene [120].

If the patients are unsure of their dietary lutein intake, they should be advised to use lutein/zeaxanthin AREDS-based supplements, given the lack of side effects found in most trials [120]. If they have a normal or high dietary lutein intake they can consume the modified beta-carotene free AREDS formulation [120].

These dietary modifications may not only delay the onset of AMD but it can also slow the progression of the disease. Supplementation with AREDS-based supplement slows the progression of AMD but does not prevent its development.

More importantly, the adoption of a Mediterranean diet and physical activity and avoiding smoking and sedentary behavior may reduce the prevalence of the early stages of AMD, decrease the number of individuals who develop advanced AMD, and consequently reduce the burden associated with the treatment of this disease.

Competing Interests

The authors declare that they have no competing interests.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 2069290, 14 pages
http://dx.doi.org/10.1155/2016/2069290

Research Article

Serum from Varicose Patients Induces Senescence-Related Dysfunction of Vascular Endothelium Generating Local and Systemic Proinflammatory Conditions

Justyna Mikuła-Pietrasik,[1] Paweł Uruski,[1] Krzysztof Aniukiewicz,[2] Patrycja Sosińska,[3] Zbigniew Krasiński,[2] Andrzej Tykarski,[1] and Krzysztof Książek[1]

[1]*Department of Hypertensiology, Angiology and Internal Medicine, Poznań University of Medical Sciences, Długa 1/2 Str., 61-848 Poznań, Poland*
[2]*Department of General and Vascular Surgery, Poznań University of Medical Sciences, Długa 1/2 Str., 61-848 Poznań, Poland*
[3]*Department of Pathophysiology, Poznań University of Medical Sciences, Rokietnicka 8 Str., 60-806 Poznań, Poland*

Correspondence should be addressed to Krzysztof Książek; kksiazek@ump.edu.pl

Received 16 August 2016; Revised 25 October 2016; Accepted 1 November 2016

Academic Editor: Delminda Neves

Although the role of endothelium in varicose vein development is indisputable, the effect of the pathology on biological properties of endothelial cells remains unclear. Here we examined if the presence of varicose veins affects senescence of endothelial cells (HUVECs) and, if so, what will be the local and systemic outcome of this effect. Experiments showed that HUVECs subjected to serum from varicose patients display improved proliferation, increased expression of senescence marker, SA-β-Gal, and increased generation of reactive oxygen species (ROS), as compared with serum from healthy donors. Both increased SA-β-Gal activity and ROS release were mediated by TGF-β1, the concentration of which in varicose serum was elevated and the activity of which *in vitro* was prevented using specific neutralizing antibody. Senescent HUVECs exposed to varicose serum generated increased amounts of ICAM-1, VCAM-1, P-selectin, uPA, PAI-1, and ET-1. Direct comparison of sera from varicose and healthy donors showed that pathological serum contained increased level of ICAM-1, VCAM-1, P-selectin, uPA, and ET-1. Calendar age of healthy subjects correlated positively with serum uPA and negatively with P-selectin. Age of varicose patients correlated positively with ICAM-1, VCAM-1, and ET-1. Collectively, our findings indicate that the presence of varicose veins causes a senescence-related dysfunction of vascular endothelium, which leads to the development of local and systemic proinflammatory environment.

1. Introduction

Varicosity refers to the presence of tortuous, lengthened, and/or twisted veins, typically in the lower limbs superficial or deep inside [1]. In Western nations, the prevalence of varicose veins reaches up to the half of the adult population [2, 3] and rises significantly with age [4, 5]. There is a consensus that varicose veins develop from faulty valves in the veins and weakened vessel walls. Mechanistically, the weakening and a subsequent venous dilatation are evoked by degenerative changes within all layers of the vein wall, in which imbalance between collagen and elastin plays the prominent role [6]. Apart from abnormalities that resulted from the rearrangements in the wall proteins, etiology of varicosity includes hyperproliferation of smooth muscle cells and fibroblasts and an injury-related activation of vascular endothelium [4, 7]. The symptoms and complications of the disease include skin discoloration, pain, itch, ulceration, superficial or deep vein thrombosis, and hemorrhage [8].

Although the role of dysfunctional endothelium in the development of varicose veins is well established [9], the effect of varicosity on endothelial cell biology and, indirectly, on the endothelium-related pathophysiology of varicose vein complications remains elusive. In order to address this issue in a comprehensive manner we compared in this paper serum from patients with varicose veins and from healthy age-matched volunteers in terms of its effect on such critical, functional features of endothelial cells, like proliferation,

Table 1: Characteristics of patients with varicose veins and the control individuals from whom serum samples were taken.

Parameter	Varicose patients	Healthy donors
n	40	40
Sex (male/female; *n*)	15/25	17/23
Age (mean ± SD/ range; y)	52 ± 15/21–81	51 ± 15/17–77
	Comorbidities* (number)	
No comorbidities	23	29
Hypertension	6	6
Hypothyroidism	2	0
Asthma	2	0
Obesity	2	2
Psoriasis	3	1
Allergy	1	2
Adrenal insufficiency	1	0

*Comorbidities have been treated as follows: hypertension: angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers; hypothyroidism: synthetic analogue of thyroxine; asthma: inhaled sympathomimetic drugs; allergy: antihistamine drugs; adrenal insufficiency: hormone of the adrenal cortex.

senescence, production of reactive oxygen species (ROS), and secretory properties. These *in vitro* investigations were followed by a comparative *ex vivo* analysis of both groups of sera with respect to the level of eight arbitrarily selected mediators of vascular inflammation (ICAM-1, VCAM-1, E-selectin, P-selectin, uPA, PAI-1, ET-1, and TFPI) and by studies in which a concentration of these molecules in the sera was correlated with the calendar age of the serum donors.

2. Material and Methods

2.1. Materials. Unless otherwise stated, all chemicals and culture plastics were purchased from Sigma (St. Louis, MO). Exogenous, recombinant form of human TGF-β1 and the specific TGF-β1 neutralizing antibody were obtained from R&D Systems (Abingdon, UK).

2.2. Varicose Vein Patients and Healthy Donors. The study was performed with serum samples obtained from patients with primary varicose veins of lower extremities and from volunteers (blood donors) in whom the presence of varicose veins was excluded and who were treated as the control group. Varicose patients had symptomatic disease ($C_2E_PA_{S2,3}P_R$) and the inclusion criteria for the study were based on clinical examination. Blood from varicose patients was taken 1 h before planned surgery of varicose vein removal. Blood samples were centrifuged immediately after collection and serum obtained was stored in aliquots at −80°C until required. The study was approved by the institutional ethics committee (consent number 441/13). Demographic data regarding varicose patients and the control individuals are presented in Table 1.

2.3. Endothelial Cell Culture and Senescence. Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in DMEM with 20% fetal bovine serum (FBS), L-glutamine (2 mM), HEPES (20 mM), EGF (10 μg/mL), heparin (5 U/mL), penicillin (100 U/mL), and streptomycin (100 μg/mL). Senescence of endothelial cells was induced by serial passaging at 7-day intervals until complete exhaustion of cell capacity to divide. Cells from passages 1-2 were treated as "young" cells, while cultures that ceased to divide and displayed hypertrophic appearance were considered as "senescent." During experiments, young and senescent endothelial cells were plated in culture dishes at high density (80–90% of confluency) and then were simultaneously exposed to 20% serum from varicose vein patients and healthy volunteers for 72 h. Then the cells were carefully washed with phosphate buffered saline (PBS) and exposed for 72 h to serum-free medium to generate autologous conditioned medium.

2.4. Immunoassays. Concentration of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, P-selectin, urokinase plasminogen activator (uPA), plasminogen activator inhibitor 1 (PAI-1), endothelin-1 (ET-1), tissue factor pathway inhibitor (TFPI), growth-related oncogene 1 (GRO-1), transforming growth factor β1 (TGF-β1), tumor necrosis factor α (TNFα), and interleukin 6 (IL-6) in serum and/or in conditioned medium generated by endothelial cells was measured using appropriate DuoSet® Immunoassay Development kits (R&D Systems), according to manufacturer's instructions.

2.5. Proliferation and Senescence Assays. Endothelial cells were seeded into culture dishes at low-density (5×10^4 cells per well), allowed to attach for 2 h, and then growth synchronized by serum deprivation for next 4 h. Afterwards, the cells were exposed to 20% serum obtained from patients with varicose veins and from healthy volunteers for 72 h. After the incubation, cells were detached enzymatically, counted using the Bürker chamber, and the number of population doublings (the measure of cell proliferation) was calculated using the following formula: population doublings = $\log_2(C_t/C_o)$, where C_o indicates the number of cells inoculated, and C_t is the number of cells harvested.

The activity of cellular senescence biomarker, senescence-associated β-galactosidase (SA-β-Gal), was quantified in the same cultures by measuring the rate of conversion of 4-methylumbelliferyl-β-D-galactopyranose to 4-methylumbelliferone at pH 6.0, essentially as described in [10].

In some experiments, both parameters were measured upon endothelial cell exposure to exogenous, recombinant form of human TGF-β1 used in the concentration corresponding to the cytokine's level in serum from patients with varicose veins. In another group of experiments, SA-β-Gal was reanalyzed in the endothelial cells subjected for 72 h to serum from varicose patients preincubated with specific TGF-β1 neutralizing antibody (400 ng/mL) [11] for 6 h.

2.6. Measurement of Reactive Oxygen Species (ROS). ROS production was assessed in endothelial cells exposed to 20% serum from patients with varicose veins and from healthy

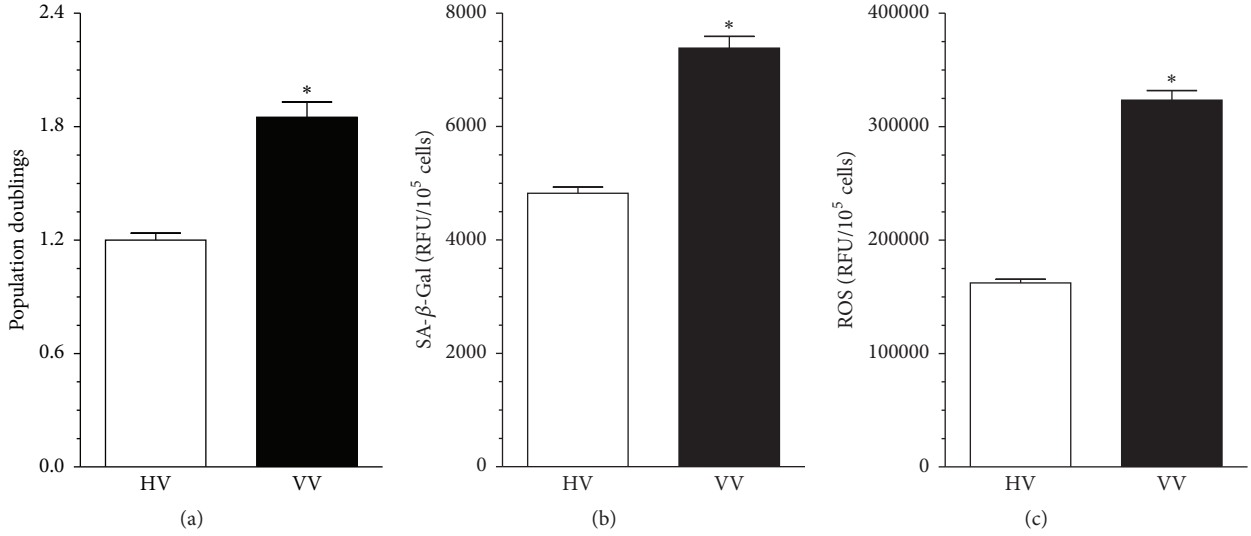


Figure 1: Effect of serum from healthy volunteers (HV) and patients with varicose veins (VV) on proliferation (a), senescence (b), and oxidative stress (c) in cultured endothelial cells. Subconfluent cultures of endothelial cells were exposed to the tested sera (20%) for 72 h and then the number of population doublings achieved (a measure of cell proliferative capacity), the activity of SA-β-Gal (a marker of cellular senescence), and the production of ROS (an indicator of oxidative stress) were examined. Results (expressed as means ± SEM) derive from experiments performed with sera from 12 (proliferation) or 16 (senescence, oxidative stress) individuals per group. Asterisks indicate significant differences as compared with HV. RFU: relative fluorescence units.

volunteers for 72 h. In brief, 1×10^5 cells were incubated in the presence of 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes, Eugene, USA) for 45 min at 37°C. The fluorescence intensity in cell lysates was monitored in a spectrofluorimeter Victor2 (Perkin-Elmer, Turku, Finland) with excitation at 485 nm and emission at 535 nm. In some experiments, ROS production was examined upon endothelial cell exposure to exogenous, recombinant TGF-β1 used in the concentration corresponding to the cytokine's level in serum from patients with varicose veins and in the presence of serum from the varicose patients upon its preincubation with the specific TGF-β1 neutralizing antibody (400 ng/mL) for 6 h.

2.7. Statistics. Statistical analysis was performed using GraphPad Prism™ v.5.00 software (GraphPad Software, San Diego, USA). The means were compared with Mann-Whitney test or Wilcoxon signed-rank test, when appropriate. Correlations were analyzed using the Spearman test. Results are expressed as means ± SEM. Differences with a P value <0.05 were considered to be statistically significant.

3. Results

3.1. Serum from Varicose Patients Causes Endothelial Cell Dysfunction in the TGF-β1-Dependent Mechanism. Low-density cultures of endothelial cells (HUVECs) were exposed to 20% serum from patients with varicose veins (VV) and from healthy, age-matched volunteers (HV) for 72 h and then three functional parameters, that is, cell proliferation, activity of SA-β-Gal (biochemical marker of senescence), and production of reactive oxygen species (an indicator of oxidative stress), were examined. Experiments showed that serum from VV patients stimulated proliferation of endothelial cells (the number of population doublings reached increased by 54 ± 8%, $P < 0.0001$; Figure 1(a)), increased the activity of SA-β-Gal (by 53 ± 6%, $P < 0.0001$; Figure 1(b)), and increased the level of ROS (by 99±8%, $P < 0.0001$; Figure 1(c)) as compared with the serum from the control donors.

Intensified proliferation of endothelial cells combined with accelerated senescence and augmented oxidative stress in response to serum from VV patients encouraged us to attempt to identify serum-derived factor(s) which could be responsible for these effects. To this end we selected, based on literature data, four agents (TGF-β1, GRO-1, TNFα, and IL-6) known to affect cell proliferation [12], senescence [11, 13], and oxidative stress [14, 15]. Comparative analysis of sera from VV patients and healthy volunteers showed that samples differed significantly only with respect to the concentration of TGF-β1. Namely, the level of this cytokine in serum from varicose patients was higher (by 87 ± 24%, $P < 0.003$; Figure 2(a)) than in the control group. Concentrations of GRO-1 (Figure 2(b)), TNFα (Figure 2(c)), and IL-6 (Figure 2(d)) in both groups of sera were almost identical ($P > 0.05$).

In order to clarify if the serum from the VV patients exerted its impact on endothelial cells via TGF-β1, exogenous recombinant form of this protein was applied to low-density HUVECs at the dose of 100 pg/mL, corresponding to TGF-β1 level in serum from varicose patients, and then proliferation, senescence, and oxidative stress were reexamined. This experiment revealed that proliferation of endothelial cells subjected to TGF-β1 was unchanged ($P > 0.05$; Figure 3(a)), whereas the activity of SA-β-Gal and the production of ROS were increased by 62 ± 9% ($P < 0.01$; Figure 3(b)) and by 39 ± 5% ($P < 0.04$; Figure 3(c)), respectively.

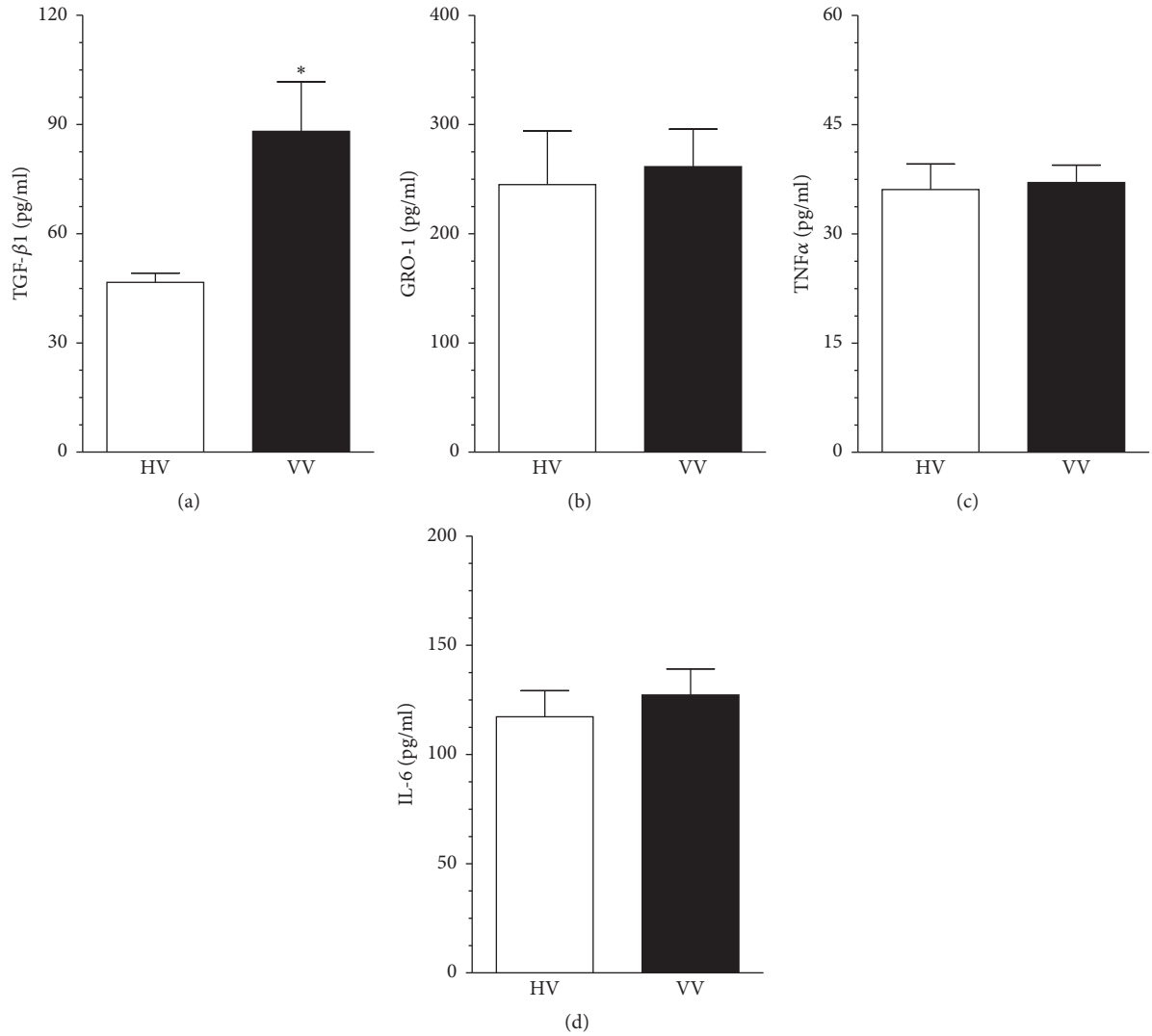


Figure 2: Concentration of TGF-β1 (a), GRO-1 (b), TNFα (c), and IL-6 (d) in serum from the healthy volunteers (HV) and from the patients with varicose veins (VV). The measurements were made using appropriate ELISA kits. Results (expressed as means ± SEM) derive from experiments performed with sera from 24 individuals per group. Asterisk indicates significant difference as compared with HV.

In order to finally confirm the causative role of TGF-β1, SA-β-Gal and ROS were retested in the endothelial cells subjected to serum from the VV patients in which the cytokine was neutralized using the specific antibody. The study showed that both parameters that were initially elevated in response to serum from VV patients declined to the level characterizing the control group (HV) when the TGF-β1 was inactivated (Figures 3(d) and 3(e)).

3.2. Serum from Varicose Patients Increases the Secretion of Proinflammatory Agents by Senescent Endothelial Cells. Secretory phenotype (SASP) is one of the most elementary features of senescent cells [16].

In this project we induced senescence of endothelial cells by serial passaging and then compared secretory properties of young and senescent cells exposed to sera from varicose patients and from the healthy controls. The study was focused on the release of eight arbitrarily selected mediators of vascular inflammation, that is, ICAM-1, VCAM-1, E-selectin, P-selectin, uPA, PAI-1, ET-1, and TFPI. Experiment showed that senescence of HUVECs maintained in 20% serum from HV displayed upregulated secretion of all tested agents as compared with their young counterparts. The release of ICAM-1 was increased by 30 ± 15% ($P < 0.01$; Figure 4(a)), VCAM-1 by 374±90% ($P < 0.0001$; Figure 4(b)), E-selectin by 263 ± 54% ($P < 0.0001$; Figure 4(c)), P-selectin by 215 ± 69% ($P < 0.0001$; Figure 4(d)), uPA by 191 ± 35% ($P < 0.001$; Figure 4(e)), PAI-1 by 133 ± 27% ($P < 0.0001$; Figure 4(f)), ET-1 by 656 ± 103% ($P < 0.0001$; Figure 4(g)), and TFPI by 163 ± 28 ($P < 0.0001$; Figure 4(h)).

When the reaction of the senescent cells to serum from both groups of donors was compared, it turned out that the conditioned medium generated by cells exposed to serum from VV patients contains remarkably higher concentrations of ICAM-1 (by 31 ± 15%, $P < 0.02$; Figure 4(a)), VCAM-1 (by 122 ± 77%, $P < 0.01$; Figure 4(b)), P-selectin (by 489 ± 208%,

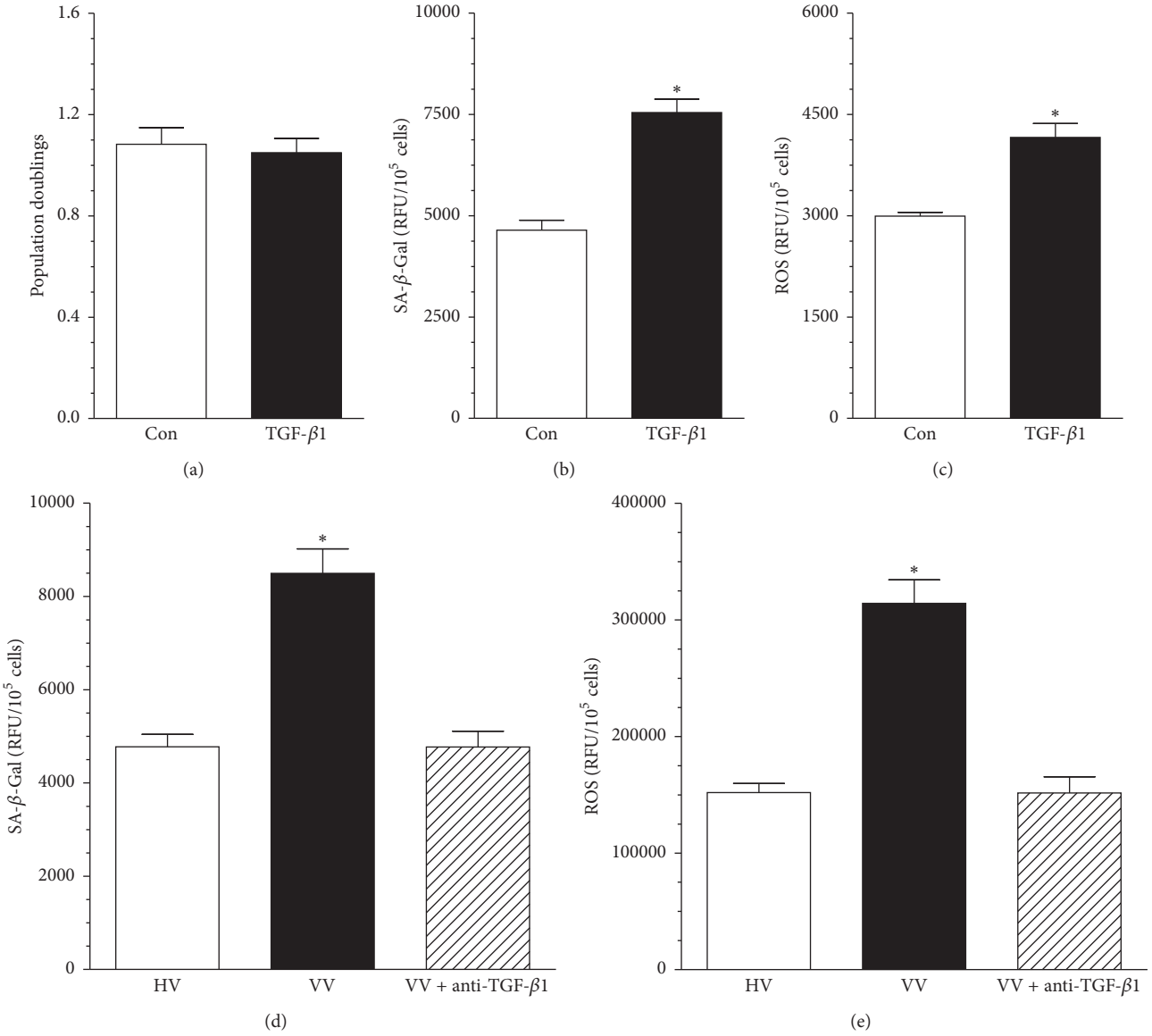
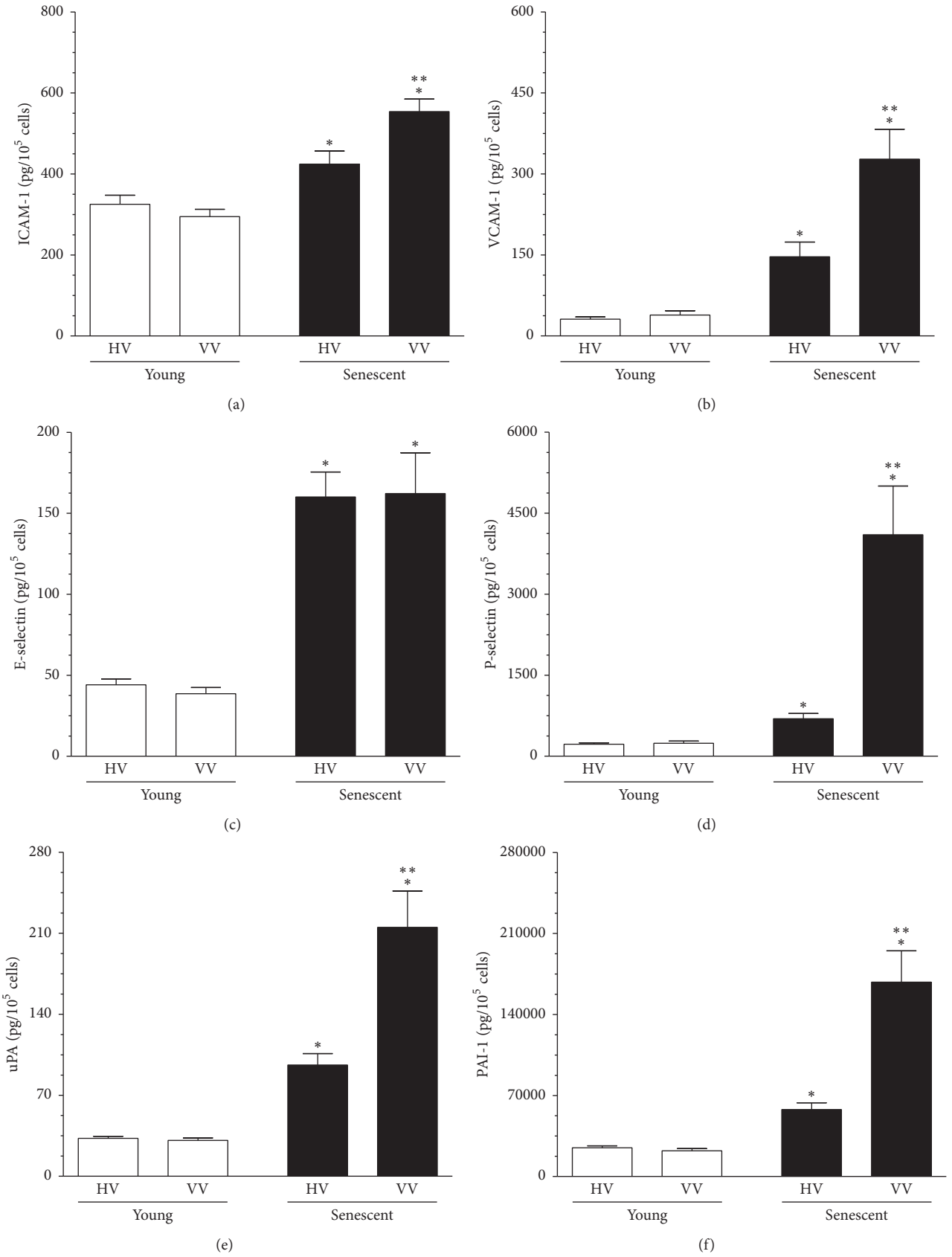


Figure 3: The role of TGF-β1 in the dysfunction of endothelial cells subjected to serum from varicose patients (VV). Effect of exogenous, recombinant TGF-β1 on proliferation (a), senescence (b), and oxidative stress (c) in HUVECs. The cells were subjected to exogenous protein used at the dose corresponding to its serum (for VV patients) level for 72 h. Asterisks indicate significant differences as compared with the control (Con) group (cells maintained in standard conditions). Effect of TGF-β1 neutralization in serum from VV patients on senescence (d) and oxidative stress (e) in HUVECs. Asterisks indicate significant differences as compared with the cells exposed to HV serum. Results (expressed as means ± SEM) derive from experiments performed in octuplicate. RFU: relative fluorescence units.

$P < 0.0001$; Figure 4(d)), uPA (by 124 ± 59%, $P < 0.002$; Figure 4(e)), PAI-1 (by 190 ± 52; $P < 0.0001$; Figure 4(f)), and ET-1 (by 70 ± 34%, $P < 0.02$; Figure 4(g)). The concentrations of E-selectin (Figure 4(c)) and TFPI (Figure 4(h)), in turn, did not differ ($P > 0.05$) between the groups.

In addition, significant differences were also observed in case of two agents released by young endothelial cells: the concentration of ET-1 in the medium produced by cells exposed to serum from the VV patients was increased by 196 ± 26 ($P < 0.0001$; Figure 4(g)), while the concentration of TFPI produced by these cells was decreased by 14 ± 4% ($P < 0.01$; Figure 4(h)), as compared with cells subjected to serum from HV.

3.3. Serum from Varicose Patients Contains Altered Level of Several Proinflammatory Agents. Concentration of proinflammatory agents tested before in the culture conditions was analyzed again directly in samples of serum from patients with varicose veins and from healthy donors. Study showed that the concentration of five out of eight factors in the serum from VV patients was increased, whereas the concentration of two was decreased and of one remained unchanged. More specifically, the concentration of ICAM-1 increased by 56±8% ($P < 0.0001$; Figure 5(a)), of VCAM-1 by 7 ± 4% ($P < 0.05$; Figure 5(b)), of P-selectin by 111 ± 23% ($P < 0.0001$; Figure 5(d)), of uPA by 72±21% ($P < 0.0001$; Figure 5(e)), and of ET-1 by 48 ± 15 ($P < 0.001$; Figure 5(g)). At the same time,



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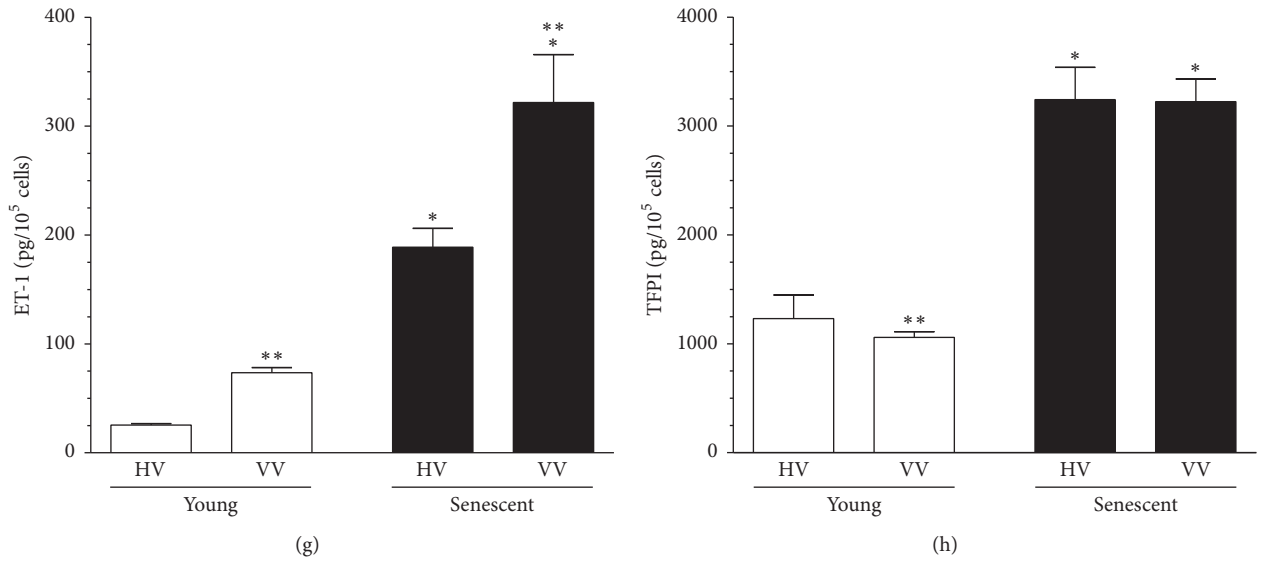


Figure 4: Concentration of proinflammatory agents in conditioned medium generated by young and senescent endothelial cells subjected to serum from the healthy volunteers (HV) and from the patients with varicose veins (VV). Endothelial cells were subjected to 20% serum for 72 h and then they were washed and exposed for next 72 h to serum-free medium to generate conditioned medium in which proinflammatory agents were measured. Results (expressed as means ± SEM) derive from experiments performed with sera from 24 individuals per group. Single asterisks indicate significant differences as compared with young endothelial cells. Double asterisks indicate significant differences as compared with HV.

the concentration of PAI-1 decreased by 17 ± 8% ($P < 0.02$; Figure 5(f)) and of TFPI by 24±6% ($P < 0.0001$; Figure 5(h)). The concentration of E-selectin was unchanged (Figure 5(c)).

3.4. Varicosity Predisposes to a Positive Correlation between a Serum Level of Proinflammatory Agents and Calendar Aging. It is believed that human aging is associated with a chronic inflammatory response which is manifested by age-dependent increase in the local and systemic concentration of several proinflammatory molecules (so-called *inflamm-aging*) [17]. In this project we aimed to compare an age-dependency of the production of proinflammatory agents in serum from healthy individual and varicose patients. An analysis conducted using control serum from HV showed that aging correlates positively with the concentration of uPA ($P < 0.02$, $r = 0.4786$; Figure 6(e)) and negatively with the level of P-selectin ($P < 0.0001$, $r = -0.6627$; Figure 6(d)). As per the rest of tested agents, there was no relationship ($P > 0.05$) between agents' level and donors' age.

When analogical assessment was performed with sera from VV patients, the results were different. Specifically, aging appeared to correlate positively with the concentration of ICAM-1 ($P < 0.03$, $r = 0.3581$; Figure 7(a)), VCAM-1 ($P < 0.03$, $r = 0.3550$; Figure 7(b)), and ET-1 ($P < 0.02$, $r = 0.3918$; Figure 7(g)). At the same time, there was no relationship ($P > 0.05$) for remaining factors.

4. Discussion

Over the past two decades, a body of evidence has accumulated that senescence of cells forming blood vessels (endothelial cells, smooth muscle cells, and fibroblasts) contributes to the development of various vascular pathologies, including atherosclerosis [18, 19], hypertension [20], and impaired healing of venous ulcers [21]. At the same time, the relationship between vascular dysfunction and cellular senescence is clearly bidirectional, which confirmed reports showing accelerated senescence of endothelial cells subjected to atherosclerosis-related disturbances in blood flow [22] or exposed to foam cell-derived lipid peroxidation product, 4-hydroxynonenal [23]. In this paper we described as first that serum obtained from patients with varicose veins causes a senescence-related dysfunction of vascular endothelial cells.

Our considerations on the prosenescence effect of varicose veins stem from the histopathological evaluations by Aunapuu and Arend [24], who found that endothelial cells from varicose patients display specific discontinuity and denudation that may be a manifestation of cellular senescence. In order to verify such a possibility we exposed endothelial cells (HUVECs) to serum from patients with varicose veins and from healthy individuals and found that cells growing in the presence of varicose serum displayed improved proliferative capacity but also increased activity of SA-β-Gal, a marker of senescence [25], and increased level of ROS, being the most significant culprits of endothelial cell senescence [26].

Taking into account above-mentioned desquamation of endothelial cells [24], one may assume a scenario that destroyed integrity of cell monolayer in varicose patients may be underlined by oxidative stress-related senescence of these cells, followed by a compensatory intensification of mitotic divisions [27]. Increased expandability of endothelial cells probably reflecting their reaction to the harmful activity of the pathological serum seems to be, however, a blind

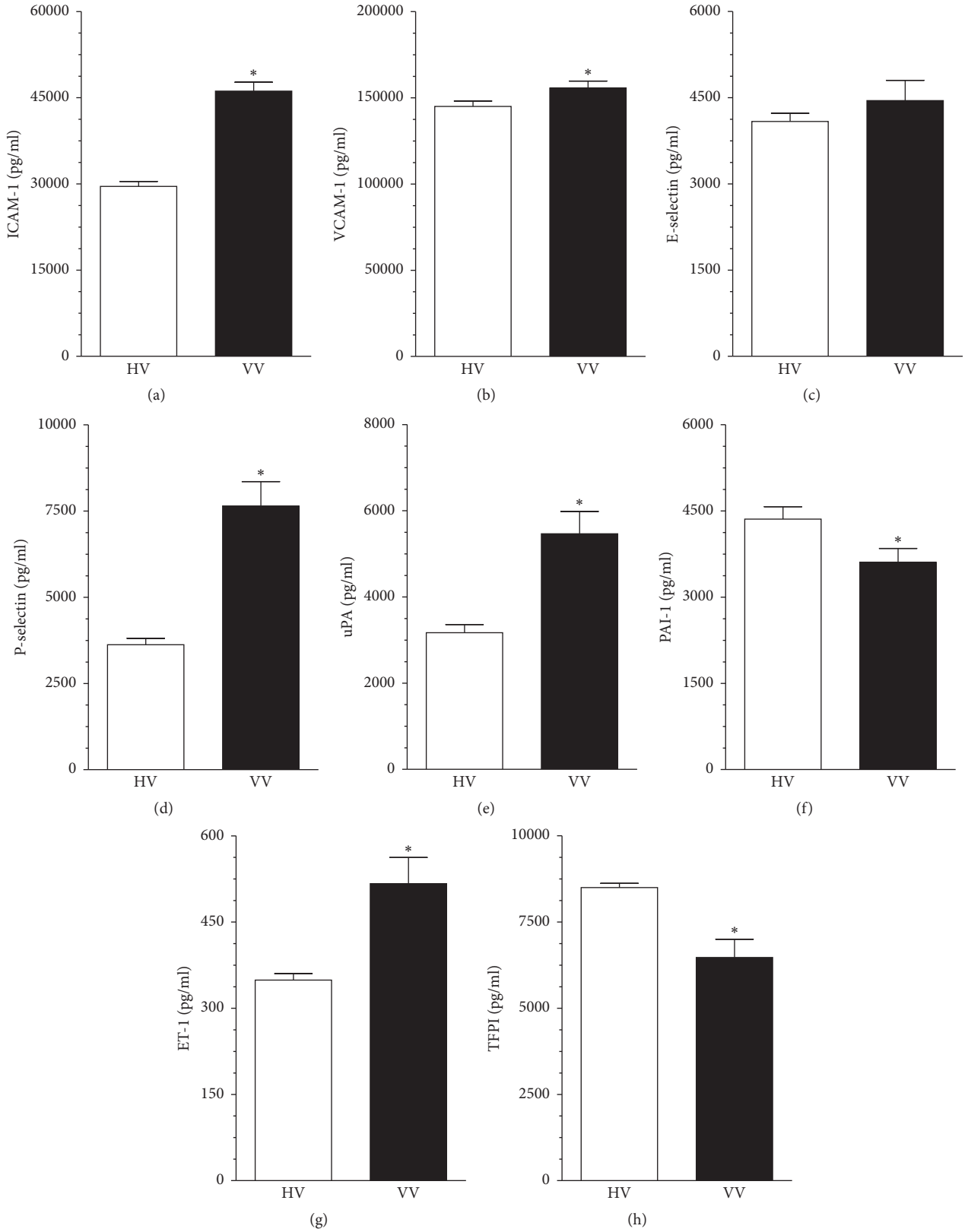
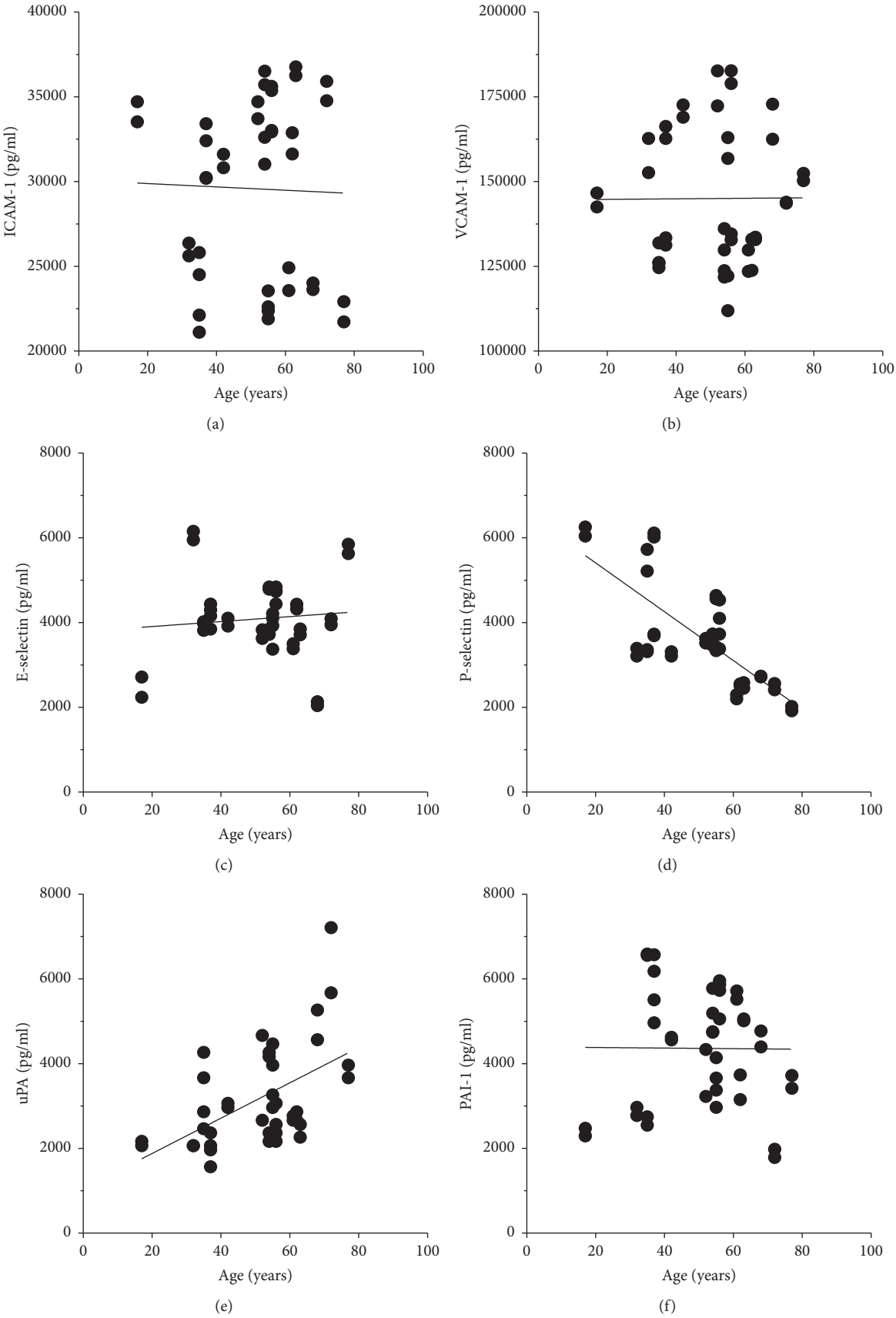


Figure 5: Concentration of proinflammatory agents in serum from the healthy volunteers (HV) and from the patients with varicose veins (VV). The measurements were made using appropriate ELISA kits. Results (expressed as means ± SEM) derive from analysis of sera obtained from 40 individuals per group. Asterisks indicate significant differences as compared with HV.



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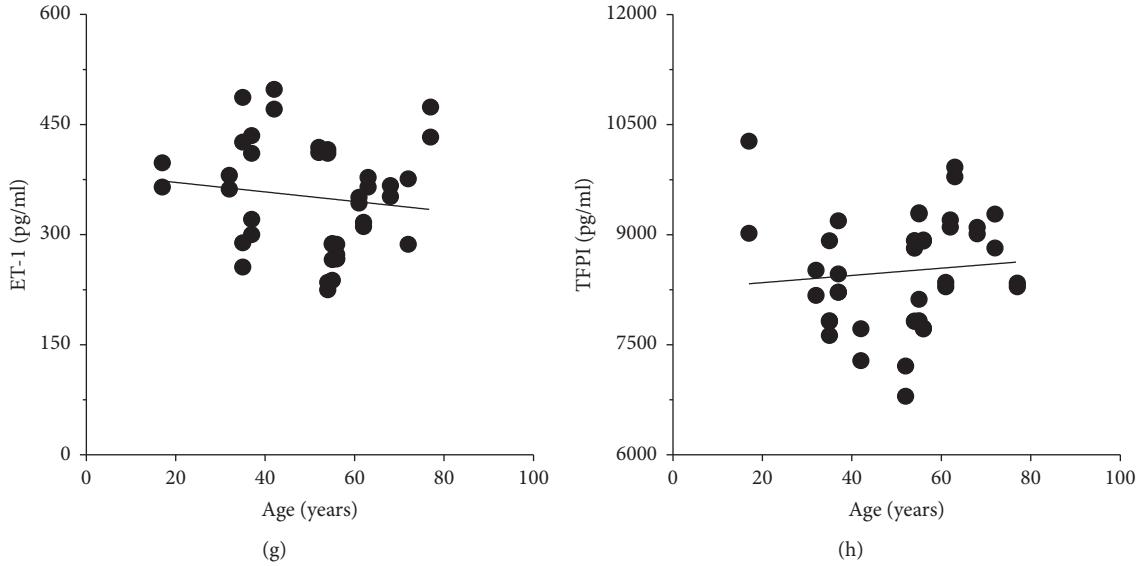


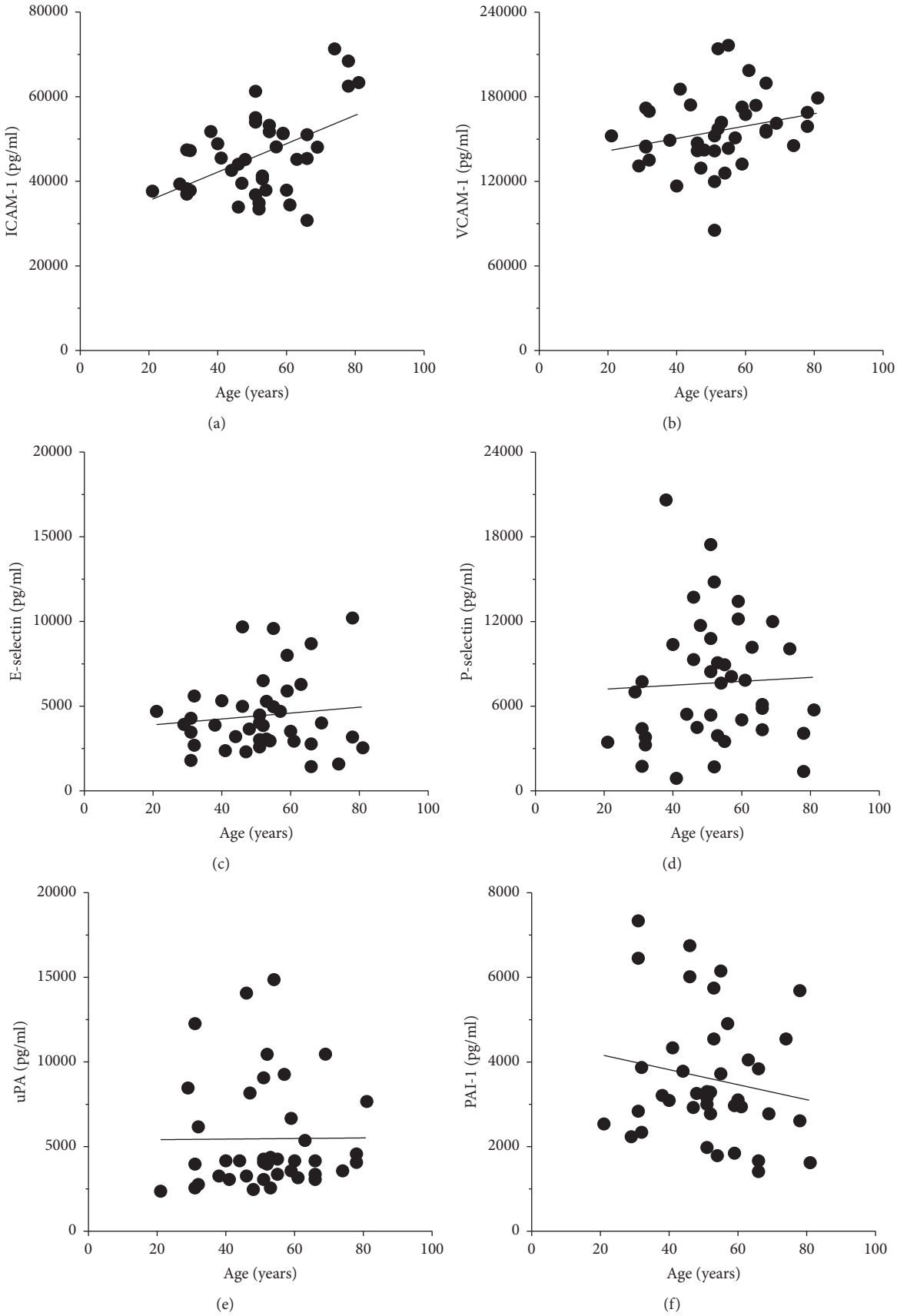
Figure 6: Effect of the healthy volunteers' age on the serum concentration of proinflammatory agents. Correlative analysis was performed with the Spearman test using serum samples from 40 individuals. Exact statistical significance P and correlation coefficient r-values can be found in Section 3.

alley, as it leads to beneficial effect (a restoration of the cell integrity) only temporarily. In long-term perspective, such compensation will result in a premature exhaustion of limited number of achievable divisions, contributing further to the progression of the disease [28]. Such situation has already been described, for example, by Sone and Kagawa, who found that the compensatory proliferation of pancreatic cells driven by insulin resistance resulted in an elevated fraction of senescent cells, which led to even deeper deterioration of insulin activity and diabetes [29].

Comparative analysis of the tested sera as well as intervention experiments with both exogenous protein and specific neutralizing antibody allowed identifying TGF-β1 as a plausible mediator by which varicose serum induces senescence and oxidative stress in endothelial cells. This finding is in line with results of Takehara et al., who found that this cytokine is capable of inducing senescence in endothelial cells [12]. Similar effect of TGF-β1 has also been found in fibroblasts [30], epithelial cells [31], and mesothelial cells [11]. Moreover, TGF-β1 has been recognized to induce ROS in a mitochondria-dependent mechanism [32].

In order to further verify our conception that varicosity potentiates endothelial cell dysfunction in the senescence-related mechanism, we compared directly the reaction of young and senescent HUVECs to sera from both groups of donors. As a measure of endothelium dysfunction we used the cells' ability to hypersecrete mediators of vascular inflammation, including adhesion molecules (ICAM-1, VCAM-1, and E- and P-selectin), vasoconstrictive protein (ET-1), serine protease (uPA), serine protease inhibitor (PAI-1), and the coagulation inhibitor (TFPI). In this context we observed that the release of all tested proteins by senescent cells was remarkably greater as compared with their young counterparts. This finding, confirming the presence of SASP in cultured endothelium is in keeping with reports by other groups, describing, for example, the overproduction of PAI-1 [33], ICAM-1, and VCAM-1 [34] by senescent endothelial cells. Our study enriched, however, the list of factors released at higher amounts to environment by these cells in E-selectin, P-selectin, uPA, and TFPI. Our observations also agree with a general idea of inflammatory phenotype characterizing endothelial cell senescence, depicted at the molecular level in an elegant study by Prattichizzo et al. [35]. More importantly, however, the production of six out of eight tested agents, that is, ICAM-1, VCAM-1, P-selectin, uPA, PAI-1, and ET-1, by senescent cells exposed to serum from varicose patients appeared to be much more pronounced as compared with the control group. Interestingly, further analysis of the concentration of the proinflammatory agents directly in the serum from the varicose patients and from the healthy individuals revealed that the level of five out of six proteins, the secretion of which was upregulated by senescent endothelial cells (apart from PAI-1), is also increased in the first group, which may suggest that senescent endothelial cells may be causatively linked with this phenomenon.

Taking into account the above-mentioned findings one may envisage that pathologies resulting from the presence of varicose veins, in particular thrombotic disorders, may be evoked, at least to some extent, by the proinflammatory behaviour of senescent endothelial cells, efficiently translating to serum characteristics. This argumentation has support in a paper by Tian and Li, who summarized available data to suggest that senescence of endothelial cells may be involved in the development of several vascular pathologies [36]. As per agents tested in our project, the above hypothesis agrees with results of other groups that showed either increased concentration of ICAM-1 and VCAM-1 in serum from varicose patients [37] or the relationship between elevated ICAM-1, VCAM-1, and P-selectin and the development of venous thrombosis [38, 39] and pulmonary embolism [40].



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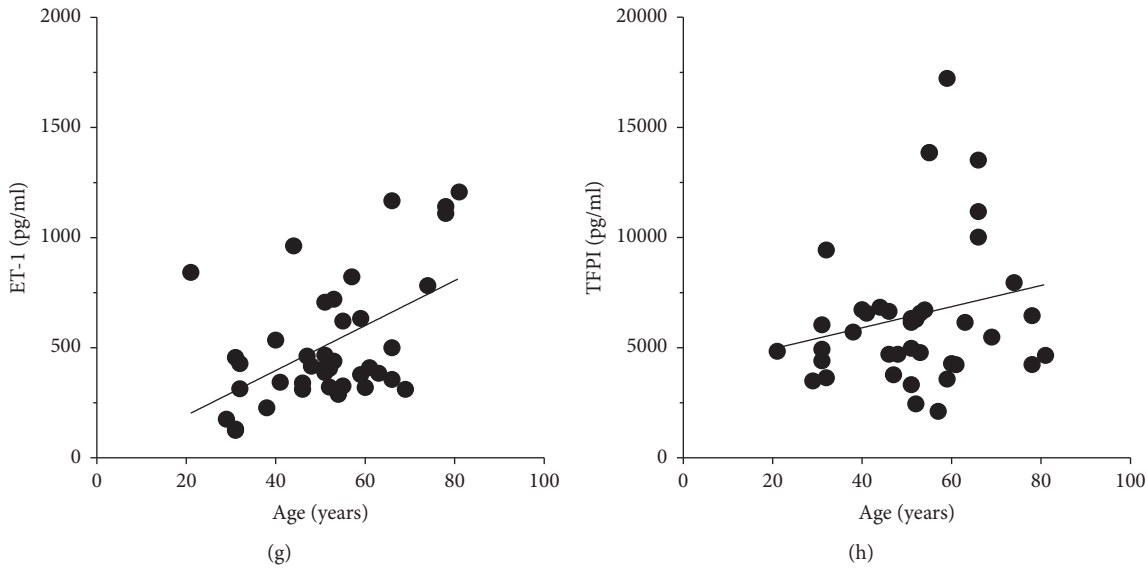

Figure 7: Effect of the varicose patients' age on the serum concentration of proinflammatory agents. Correlative analysis was performed with the Spearman test using serum samples from 40 individuals. Exact statistical significance P and correlation coefficient r-values can be found in Section 3.

In the last part of the project we examined if the presence of varicose veins may exacerbate a systemic (serum-dependent) phenomenon of inflamm-aging [17]. This hypothesis was based on the facts that (i) serum from varicose patients induces senescence of endothelial cells, (ii) senescent endothelial cells subjected to these sera overproduce proinflammatory agents, and (iii) senescent endothelial cells accumulate in tissues during aging [41]. To clarify this issue we correlated levels of eight proinflammatory agents with calendar age of serum donors. As per the healthy individuals only the concentration of uPA correlated positively with aging (uPA exerts atherosclerotic capabilities [42]), whereas the concentration of P-selectin did correlate negatively. Importantly, however, when the analysis was repeated using the sera from varicose patients, aging appeared to correlate positively with other group of agents, that is, ICAM-1, VCAM-1, and ET-1, whereas the correlations noticed for the healthy donors disappeared. It should be stressed at this moment that the age-dependent decrease in P-selectin should be considered as the beneficial process for healthy people, as this molecule mediates various elements of proinflammatory cascade and contributes to vascular disorders [43]. The lack of the correlation regarding P-selectin in varicose patients, in combination with positive relationships for ICAM-1, VCAM-1, and ET-1 (all are actively involved in atherosclerosis [44, 45]), implies that varicosity makes a serum more proinflammatory, and thus it may be partly responsible for the development of certain age-related pathologies.

5. Conclusions

In conclusion, our study provides evidence that serum from patients with varicose veins is capable of generating the proinflammatory local (endothelium-related) and systemic environment. This activity of varicose serum seems to be primarily associated with TGF-β1-dependent activation of endothelial cell senescence.

Competing Interests

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

Acknowledgments

The study was partly supported by a grant from the National Science Centre, Poland (Decision no. DEC-2011/03/B/NZ3/01214).

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