Biochemical and Biophysical Research Communications 411 (2011) 202–207

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Anti-oxidant and anti-inflammatory mechanisms of amlodipine action to improve endothelial cell dysfunction induced by irreversibly glycated LDL

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ARTICLE INFO

Article history:
Received 14 June 2011
Available online 25 June 2011

Keywords:
Amlodipine
Endothelial dysfunction
Glycated LDL
NADPH oxidase
NF-κB
p38 MAPK

ABSTRACT

Amlodipine, alone or in combination with other drugs, was successfully used to treat hypertension. Our aim was to evaluate the potential of amlodipine (Am) to restore endothelial dysfunction induced by irreversibly glycated low density lipoproteins (AGE-LDL), an in vitro model mimicking the diabetic condition. Human endothelial cells (HEC) from EA.hy926 line were incubated with AGE-LDL in the presence/absence of Am and the oxidative and inflammatory status of the cells was evaluated along with the p38 MAPK and NF-κB signalling pathways. The cellular NADPH activity, 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine levels in the culture medium and the adhesion of human monocytes to HEC were measured by chemiluminescence, UHPLC, Western Blot and spectrofluorimetric techniques. The gene expression of NADPH subunits ($p22^{phox}$, NOX4), eNOS and inflammatory molecules (MCP-1, VCAM-1) were determined by Real Time PCR, while the protein expression of $p22^{phox}$, MCP-1, iNOS, phospho-p38 MAPK and phospho-p65 NF-κB subunit were measured by Western Blot. Results showed that in HEC incubated with AGE-LDL, Am led to: (i) *decrease of the oxidative stress*: by reducing $p22^{phox}$, NOX4, iNOS expression, NADPH oxidase activity, 4-HNE and 3-nitrotyrosine levels; (ii) *decrease of the inflammatory stress*: by the reduction of MCP-1 and VCAM-1 expression, as well as of the number of monocytes adhered to HEC; (iii) *inhibition of ROS-sensitive signalling pathways*: by decreasing phosphorylation of p38 MAPK and p65 NF-κB subunits. In conclusion, the reported data demonstrate that amlodipine may improve endothelial dysfunction in diabetes through anti-oxidant and anti-inflammatory mechanisms.

1. Introduction

Diabetes mellitus is considered a worldwide epidemic and its atherosclerotic complications are a main cause of morbidity and mortality [1]. Often, diabetes is associated with hypertension, thus leading to an increased number of cardiovascular events in the affected subjects.

Endothelial dysfunction, characterized by an increased pro-oxidant and pro-inflammatory stress of the endothelial cell (EC), is a disturbance common to atherosclerosis, diabetes and hypertension. NADPH oxidase (NADPHox) is considered one of the main sources of superoxide anions in the vasculature [2]. EC contain this protein complex, of which $p22^{phox}$ is the main regulatory subunit [2]. When superoxide anions are produced together with nitric oxide (NO), they can react to form a highly reactive peroxynitrite molecule. Peroxynitrite is an important mediator of peroxidative modifications, such as low density lipoprotein (LDL) oxidation [3].

Atherosclerosis is a chronic inflammatory disorder characterized by accumulation of macrophages and T lymphocytes in the arterial intima. The macrophages accumulated in the atherosclerotic plaques are derived mainly from blood monocytes that adhere to EC. The monocyte chemoattractant protein-1 (MCP-1) is a key chemokine involved in the recruitment of circulating monocytes to the site of inflammation. Increased levels of circulating MCP-1 are detected in diabetes [4–6]. Vascular cell adhesion molecule-1 (VCAM-1), a protein involved in monocytes rolling on EC, is also increased in atherosclerosis and diabetes [7].

The accelerated atherosclerosis in diabetes might be explained by the alteration of vascular cells' function as a consequence of their direct interaction with glycated proteins and lipids which appear due to the high glucose levels in plasma [8]. Apolipoprotein B (ApoB), the specific protein of LDL is among the plasma proteins known to be glycated in diabetes. Its level in diabetic patients is about twofold higher than in healthy individuals [9].

Amlodipine (Am), a dihydropyridine L-type calcium channel blocker (CCB), was successfully used to stop atherosclerosis in hyperlipidemic animal models [10,11] and to limit the number of cardiovascular events in diabetic hypertensive patients. Studies regarding Am effects in pro-diabetic conditions are few.

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doi:10.1016/j.bbrc.2011.06.137

Our hypothesis was that the positive effects of Am in hypertensive diabetic patients are due to additional actions of this molecule besides lowering blood pressure. The aim was to investigate Am capacity to correct EC dysfunction induced by exposure of human EC (HEC) to irreversibly glycated LDL (AGE-LDL), an in vitro model mimicking diabetic condition. To assess the molecular mechanisms of Am action, the p38MAPK and NF-κB signalling pathways were investigated.

2. Material and methods

2.1. Reagents

Protease inhibitor cocktail, sodium orthovanadate, sodium fluoride, and total RNA isolation kit were from Sigma–Aldrich Co., USA. Reagents for cDNA amplification were from Promega Corporation, USA and Invitrogen Ltd., UK. Amlodipine was from LKT Laboratory, Inc., USA. All reagents for UHPLC (4-HNE determination) were from Merck KGaA., Germany. Antibodies for iNOS, MCP-1, phospho-p65 (pp65), 3-nitrotyrosine, β-actin and all the secondary antibodies were from Abcam, UK. The antibodies for phospho-p38 (pp38) and total p38 were from Cell Signaling Technology, Inc., USA, and anti-$p22^{phox}$ was from Santa Cruz Biotechnology, Inc., USA. The enhanced chemiluminescence detection (ECL) kit was from Pierce, USA and the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) from Invitrogen Ltd., UK.

2.2. LDL isolation and glycation

Plasma was separated from the blood of healthy donors from the Haematology Centre, Bucharest. Native LDL (nLDL) was isolated by ultracentrifugation as described [12]. Non-enzymatically irreversibly glycated LDL (AGE-LDL) was prepared and characterized as in [13].

2.3. Cell culture and experimental procedure

Human umbilical vein endothelial cells from the EA.hy926 cell line (HEC) were purchased from ATCC, USA. The cells were cultured in Dulbecco's modified Eagle Medium (DMEM) with 5 mM glucose and 10% fetal calf serum (FCS). At confluence, the cells were starved in DMEM with 0.2% FCS, then incubated 24 h with 100 μg protein/ml nLDL or AGE-LDL, with/without 10 μM Am in medium. After 24 h, the cells and the culture media were harvested and further analysed.

2.4. Lipid peroxides assessment

Lipid peroxides were determined in the culture media of HEC exposed to AGE-LDL with/without Am or nLDL as thiobarbituric acid reactive substances (TBARS) and expressed as nmoles malondialdehyde (MDA)/ml [14]. In addition, 4-hydroxynonenal (4-HNE) was measured by UHPLC as previously described [15].

2.5. NADPH oxidase activity assay

After 24 h exposure of HEC to AGE-LDL with/without Am or nLDL, NADPHox activity was determined in cell suspensions as previously reported [16] and expressed as arbitrary units (A.U.) relative to cells incubated with nLDL.

2.6. Quantification of $p22^{phox}$, NOX4, eNOS, MCP-1 and VCAM-1 gene expression

Total RNA was isolated and reverse transcribed using MMLV-RT. The complementary DNA (cDNA) was amplified using specific primers for human $p22^{phox}$, NOX4, eNOS, MCP-1, VCAM-1 and β-actin (as the reference gene), in an Applied Biosystem Real-Time PCR StepOne Plus. The sequences of the primers are given in Table 1 (see Supplementary data). The amplification products were detected using SyBr green. The quantification of PCR products was performed using the "Fit Point Method" and expressed as arbitrary units (A.U.). The gene expression was expressed relative to the values in nLDL-exposed HEC.

2.7. Evaluation of $p22^{phox}$, iNOS, pp38MAP kinase and pp65 protein expression

After exposure of HEC to AGE-LDL with/without Am or nLDL, the cells were processed as in [17] in the presence of protease inhibitor cocktail and phosphatase inhibitors (1 mM NaF and 2 mM Na_3VO_4). Specific primary antibodies for human $p22^{phox}$, iNOS, pp38MAPK, p38MAPK, pp65 or β-actin were used. The relative protein expression was determined by densitometric analysis of the peaks using TotalLab 120 software (Nonlinear Dynamics Ltd., UK). The protein was expressed relative to cells exposed to nLDL.

2.8. MCP-1 quantification in the culture medium

After exposure of HEC to AGE-LDL with/without Am or nLDL, the culture media were collected and secreted MCP-1 was assayed by Western Blot as in [17]. MCP-1 protein was expressed relative to the total protein in the culture medium.

2.9. Determination of 3-nitrotyrosine in the culture medium

Media from the cells incubated with AGE-LDL with/without Am or nLDL were concentrated and assayed by Dot Blot. The 3-nitrotyrosine level was expressed as arbitrary units (A.U.).

2.10. NOx assay

Nitrite/nitrate (NOx) levels were quantified in the media of the cells incubated with AGE-LDL with/without Am or nLDL using a fluorimetric kit (BioVision, USA).

2.11. Protein assay

Protein concentration of each sample was measured with a modified Lowry method, or BCA, using bovine serum albumin as standard.

2.12. Monocyte adhesion to HEC assessment

Monocyte adhesion to HEC was measured as previously reported [18]. The number of fluorescent monocytes that adhered to HEC was expressed as relative fluorescent units (RFU).

2.13. Statistical analysis

Statistical evaluation was done by *t*-test with two-tailed distribution. $P \leqslant 0.05$ was considered statistically significant. Data were expressed as means ± SD.

3. Results

3.1. Amlodipine decreases the oxidative stress in HEC incubated with AGE-LDL

NADPH oxidase activity was determined in HEC incubated either with nLDL or AGE-LDL with/without Am. Results showed

that after 24 h incubation, NADPHox activity was increased by 30% in AGE-LDL-exposed HEC compared to nLDL-incubated cells (1.30 ± 0.17 vs 1.00 ± 0.04 A.U., $p < 0.05$). Addition of Am to the incubation medium of AGE-LDL treated cells significantly decreased the activity of NADPHox (0.66 ± 0.14 A.U., $p < 0.01$) (Fig. 1A).

To determine the molecular mechanism by which Am decreases NADPHox activity, we evaluated the gene and protein expression of $p22^{phox}$ and NOX4, two NADPHox subunits, by Real-Time PCR and Western Blot. Incubation of HEC with AGE-LDL led to an increase of NOX4 gene expression compared to nLDL (1.30 ± 0.3 vs 1.00 ± 0.09 A.U). Addition of Am to AGE-LDL exposed cells led to a 50% decrease of the NOX4 gene expression (0.59 ± 0.08 A.U.) (Fig 1B). AGE-LDL induced only a slight increase of $p22^{phox}$ gene expression compared to nLDL (1.17 ± 0.05 vs 1.00 ± 0.04 A.U., $p < 0.05$). Am addition to the incubation medium of AGE-LDL exposed cells decreased $p22^{phox}$ gene expression by 40%. The protein expression of $p22^{phox}$ was also increased in HEC by AGE-LDL compared to nLDL. Am addition to cells incubated with AGE-LDL decreased $p22^{phox}$ protein expression by 40% (Fig. 1C and D).

The antioxidant capacity of Am was also tested by measuring lipid peroxides in the media of HEC incubated with AGE-LDL and the drug. Results showed that exposure of HEC to AGE-LDL led to an increase of TBARS levels in the culture media compared to incubation with nLDL (0.97 ± 0.01 vs 0.69 ± 0.03 nmol MDA/ml). In addition, 4-HNE was higher in the media of AGE-LDL-exposed cells compared to nLDL-treated cells (683.17 ± 44.09 vs 253.49 ± 69.05 pM). Addition of Am to AGE-LDL-incubated cells induced a decrease of both TBARS (0.56 ± 0.02 nmol MDA/ml) and 4-HNE (220.67 ± 18.73 pM) (Fig. 1, Supplementary data).

3.2. Amlodipine decreases nitrotyrosine levels and iNOS protein expression in AGE-LDL-exposed HEC

AGE-LDL produced a notable increase of the 3-nitrotyrosine levels in the culture media of HEC relative to nLDL-exposed cells (12813.00 ± 123.04 vs 2787 ± 93.34 A.U., $p < 0.01$) (Fig. 3A). iNOS protein expression significantly increased in AGE-LDL-incubated cells compared to nLDL-exposed cells (1.60 ± 0.3 vs 1.00 ± 0.1 A.U.) (Fig. 2B). Addition of Am significantly decreased the nitrotyrosine levels in the culture media of cells incubated with AGE-LDL (8548.5 ± 256.68 A.U., $p < 0.01$). The protein expression of iNOS in AGE-LDL exposed cells treated with Am was also reduced (0.58 ± 0.37 A.U., $p < 0.01$) (Fig. 2A and B). The gene expression of the endothelium constitutive NO synthase (eNOS) was decreased in AGE-LDL-treated cells compared to nLDL-exposed cells. Am addition significantly increased eNOS gene expression (from 0.74 ± 0.08 to 1.08 ± 0.16 A.U., $p < 0.05$) (Fig. 2C). The bioavailability of NO, measured as NOx/3-nitrotyrosine ratio, was dramatically decreased in the media of AGE-LDL-treated cells (1.00 ± 0.03 vs 0.21 ± 0.03 A.U., $p < 0.01$). Am addition had only a limited effect, increasing NO bioavailability to 0.3 ± 0.05 A.U., $p < 0.05$ (Fig. 2D).

3.3. Amlodipine decreases the inflammatory stress induced by AGE-LDL in HEC

To investigate the anti-inflammatory effects of Am in HEC incubated with AGE-LDL, MCP-1 gene expression was evaluated by quantitative RT-PCR. Relative to nLDL, AGE-LDL induced a 100% increase of MCP-1 mRNA (from 1.00 ± 0.07 to 2.03 ± 0.16 A.U., $p < 0.01$). The addition of Am to the medium of HEC incubated with AGE-LDL produced a statistically significant reduction of MCP-1 mRNA (1.50 ± 0.22 A.U., 26%, $p < 0.01$) (Fig. 3A). We also evaluated MCP-1 protein secreted by HEC incubated with AGE-LDL in the presence/absence of Am. Western Blot analysis showed that Am statistically decreased secreted MCP-1 by 50% ($p < 0.01$) in HEC incubated with AGE-LDL (Fig. 3B).

Vascular adhesion molecule-1 (VCAM-1) gene expression was investigated by Real-Time PCR in HEC incubated with AGE-LDL with/without Am. Results showed that AGE-LDL caused a statistically significant increase of VCAM-1 mRNA compared to nLDL-exposed cells (1.26 ± 0.03 vs 1.00 ± 0.02, $p < 0.01$). Addition of Am to HEC incubated with AGE-LDL induced a 40% decrease of VCAM-1 mRNA (0.79 ± 0.04, $p < 0.01$) (Fig. 3C).

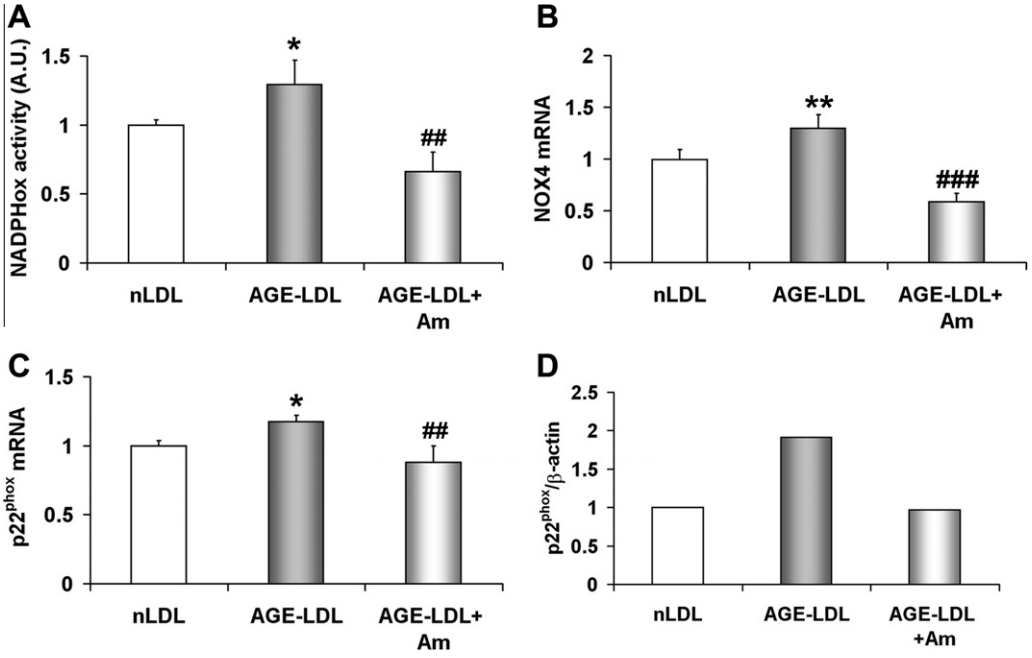

Fig. 1. NADPH oxidase activity and expression in human endothelial cells incubated with AGE-LDL is decreased by amlodipine (Am). (A) NADPHox activity in cells exposed for 24 h to 100 μg/ml AGE-LDL in the presence/absence of 10 μM Am, relative to cells incubated with nLDL; (B and C) NOX4 and $p22^{phox}$ mRNA quantification in AGE-LDL-treated cells with/without Am relative to mRNA in nLDL-treated cells; (D) $p22^{phox}$ protein in nLDL or AGE-LDL-treated cells in the presence/absence of Am, with β-actin as the reference protein; $^{*}p < 0.05$ AGE-LDL vs nLDL, $^{**}p < 0.01$ AGE-LDL vs nLDL, $^{\#\#}p < 0.01$ AGE-LDL + Am vs AGE-LDL, $^{\#\#\#}p < 0.001$ AGE-LDL + Am vs AGE-LDL.

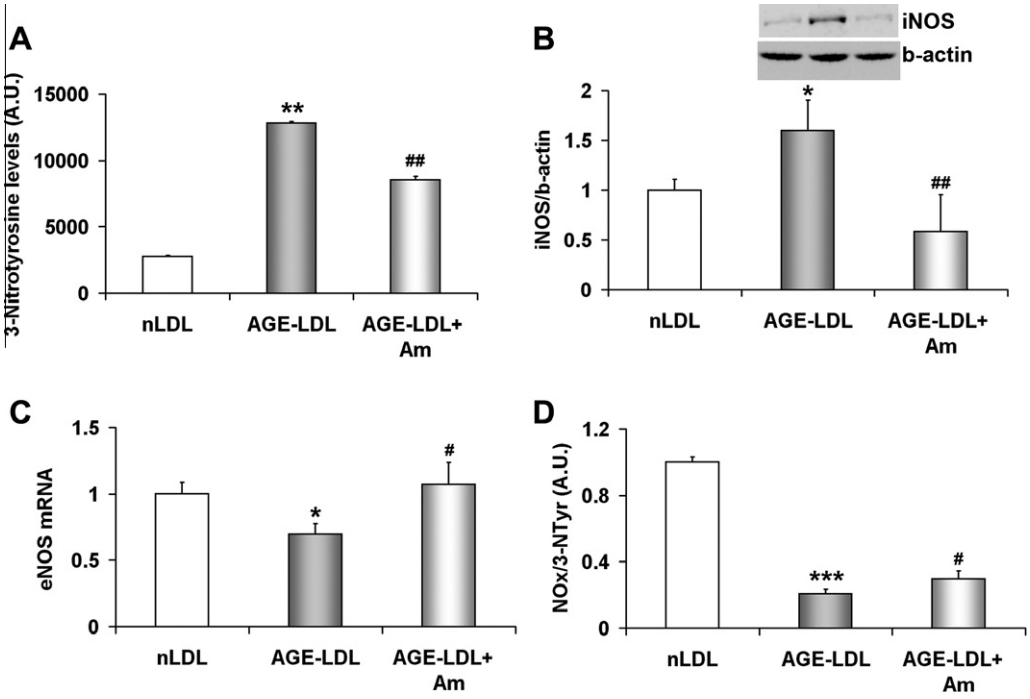

Fig. 2. Peroxinitrites are decreased by amlodipine (Am) in the culture medium of human endothelial cells incubated with AGE-LDL. (A) Densitometric analysis of the Dot Blots for 3-nitrotyrosine in the media of cells incubated with AGE-LDL with/without 10 μM Am, relative to cells incubated with nLDL; (B) iNOS protein in nLDL and AGE-LDL-treated cells with/without Am and densitometric analysis of the blots, with β-actin as the reference protein; (C) eNOS mRNA in AGE-LDL treated cells with/without Am, relative to mRNA from nLDL-treated cells; (D) NO bioavailability measured as NOx/3-nitrotyrosine in the culture media; $^{*}p < 0.05$ AGE-LDL vs nLDL, $^{**}p < 0.01$ AGE-LDL vs nLDL, $^{***}p < 0.001$ AGE-LDL vs nLDL, $^{\#}p < 0.05$ AGE-LDL + Am vs AGE-LDL, $^{\#\#}p < 0.01$ AGE-LDL + Am vs AGE-LDL.

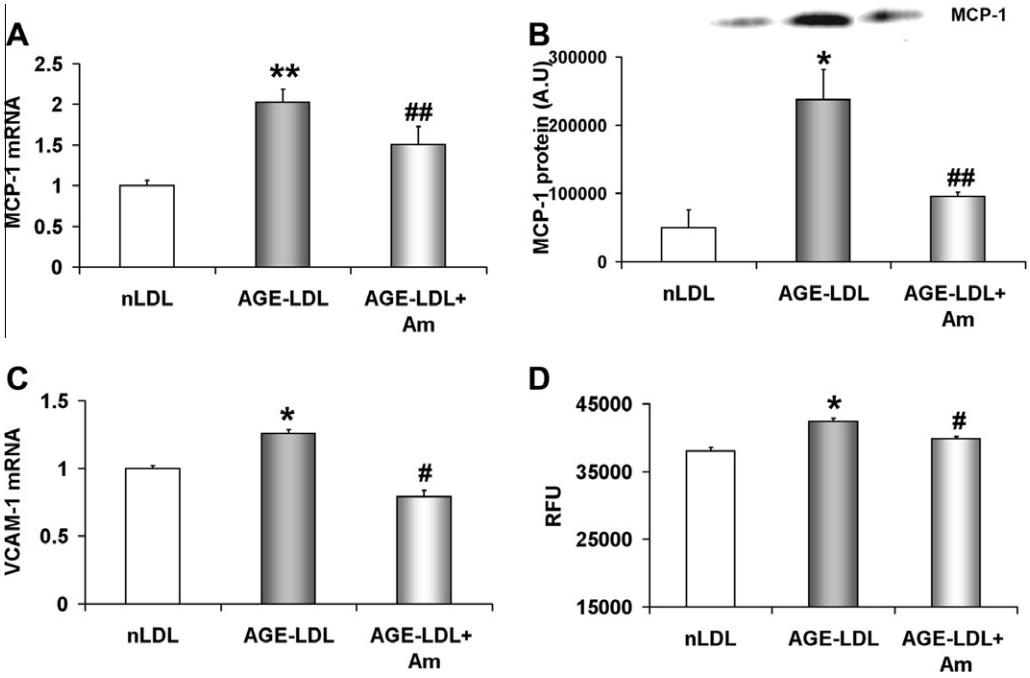

Fig. 3. Amlodipine (Am) decreases the inflammatory stress in human endothelial cells (HEC). (A) MCP-1 mRNA in AGE-LDL-treated cells with/without Am, relative to mRNA from nLDL-treated cells; (B) MCP-1 protein expression in the medium of cells incubated with nLDL or AGE-LDL in the presence/absence of Am by Western Blot and densitometric analysis of the blots; (C) VCAM-1 mRNA in cells incubated with AGE-LDL with/without Am, relative to nLDL-treated cells; (D) Monocytes adhering to HEC incubated with nLDL, AGE-LDL or AGE-LDL + Am detected spectrofluorimetrically and expressed as relative fluorescence units (RFU); $^{*}p < 0.05$ AGE-LDL vs nLDL, $^{**}p < 0.01$ AGE-LDL vs nLDL, $^{\#}p < 0.05$ AGE-LDL + Am vs AGE-LDL, $^{\#\#}p < 0.01$ AGE-LDL + Am vs AGE-LDL.

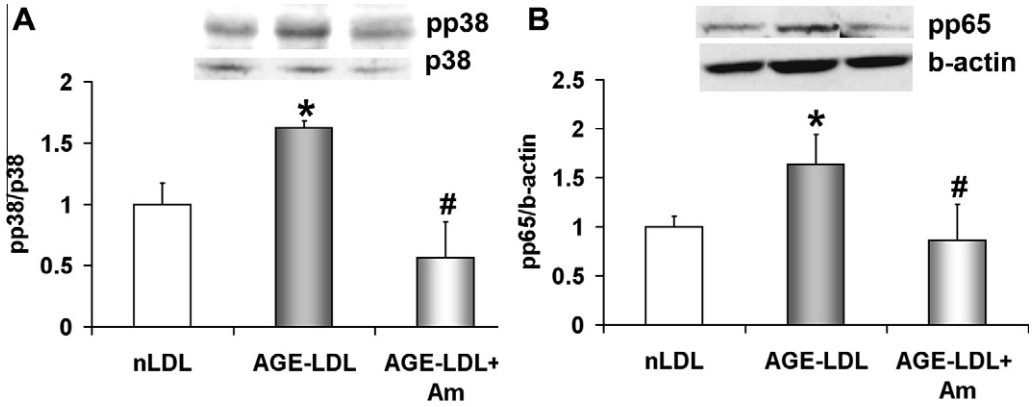

Fig. 4. Amlodipine (Am) decreases the phosphorylation of pro-inflammatory signalling molecules in human endothelial cells incubated with AGE-LDL. (A) pp38MAPK and (B) pp65 (NF-κB subunits) in cells incubated with nLDL, AGE-LDL or AGE-LDL + Am by Western Blot and densitometric analysis; $^{*}p < 0.05$ AGE-LDL vs nLDL, $^{\#}p < 0.05$ AGE-LDL + Am vs AGE-LDL.

3.4. Amlodipine decreases monocyte adhesion to HEC exposed to AGE-LDL

To validate the modulation of MCP-1 and VCAM-1 by Am, we performed a functional test to evaluate the adhesion of monocytes to HEC incubated with AGE-LDL with/without Am. AGE-LDL exposure led to a statistically significant increase of the monocytes adhering to HEC compared to nLDL-incubated cells (38010.50 ± 576.29 vs 42420.50 ± 450.43 RFU, $p < 0.05$). Am decreased the number of the monocytes adhering to HEC incubated with AGE-LDL (39899.50 ± 273.65, $p < 0.05$) (Fig. 3D).

3.5. Amlodipine decreases p38 MAPK and p65 phosphorylation

To assess whether p38 MAPK and NF-κB subunits' phosphorylation is modulated by Am, we performed Western Blot analysis. Compared to nLDL-incubated cells, an increase of p38 MAPK (60%) and p65 (64%) phosphorylation in AGE-LDL exposed HEC was observed. Addition of Am to AGE-LDL-exposed HEC decreased the phosphorylation level of both phosphorilated proteins (by 65% and 47%, respectively), suggesting that the inhibition of the two pathways might be (at least in part) responsible for the beneficial effect of the drug (Fig. 4A and B).

4. Discussion

Amlodipine, a third generation CCB, was successfully used to decrease blood pressure in hypertensive patients alone or in combination with other drugs [19,20]. Diabetes, a disease characterised by hyperglycemia and dyslipidemia, is associated with increased oxidative and inflammatory stress. Molecular studies of the effect of Am in pro-diabetic conditions are scarce and the mechanisms of action of this drug have not been clarified.

In a previous study, we had demonstrated that NADPH oxidase activity is increased in HEC exposed to AGE-LDL [17]. In the present study, we have evaluated the benefits of Am on EC dysfunction. The present data show that addition of Am decreases NADPHox activity in HEC incubated with AGE-LDL. A number of studies have evaluated the effect of Am on NADPHox activity in different hypercholesterolemic and hypertensive animal models, with contradictory results [21–23]. Our results show that this drug induces a significant reduction of NADPHox subunit (NOX4 and $p22^{phox}$) expression, in good agreement with results reported in hypertensive rats [22]. In addition, we bring evidence of the antioxidant capacity of Am, revealed by the decrease of 4-HNE and TBARS in the culture media of HEC incubated with AGE-LDL in the presence of the drug.

It is known that superoxide generation is central to the formation of reactive intermediates including peroxynitrites, powerful pro-oxidant molecules [24]. In the present study, we demonstrate increased nitrotyrosinilation of proteins in the culture media of HEC incubated with AGE-LDL. Am added to the incubation media decreased the nitrotyrosinylation of the proteins and increased the bioavailability of NO (evaluated as NOx/3-nitrotyrosine ratio). In addition, a decrease of iNOS protein expression in the cells exposed to AGE-LDL and Am was detected, a result that concurs with decreased NADPHox activity and the ensuing reduction of peroxinitrite formation. These results are in good agreement with those obtained by Mason et al., who demonstrated that Am increases NO production in HEC in parallel with the re-establishment of the imbalance between NO and ONOO [25].

HEC incubation with AGE-LDL leads to a slight reduction of eNOS gene expression, while Am addition reverses this effect. The constitutively expressed eNOS is essential for the control of vascular tone, in contrast to the iNOS isoform, which is capable of synthesising high amounts of NO in a short time and thus accelerating the formation of peroxynitrites [26]. The restoration of the iNOS–eNOS imbalance is thus an important atheroprotective mechanism of Am. The potential of Am to normalize eNOS expression was also demonstrated in vivo in hypertensive animals [27].

Recruitment of monocytes to the vessel wall is a critical step in the development of atherosclerosclerosis [28]. It has been demonstrated that activation of NADPHox leads to the stimulation of endothelial MCP-1, thus establishing a direct link between the inflammatory and oxidative stress [29]. The decreased gene expression of VCAM-1 and MCP-1, together with the diminishing of the secreted MCP-1 and the reduced number of monocytes adhering to HEC demonstrates an important athero-protective mechanism of Am. Our results confirm and extend the studies of Yu et al. demonstrating that Am leads to a decrease of the monocytic THP-1 cells adhering to HEC [30].

In a previous article, we reported that AGE-LDL induces pro-oxidant and pro-inflammatory effects in HEC by activation of the NF-κB and p38MAPK signaling pathways [17]. To determine which signaling pathway is modulated by Am, we evaluated the phosphorylation of p38MAPK and p65 in HEC incubated with AGE-LDL with/without the drug. Our results reveal that in AGE-LDL treated cells, Am decreases both p38MAPK and p65 phosphorylation. An inhibitory action of Am on p38MAPK phosphorylation was also reported in EA.hy 926 cells exposed to high glucose concentrations [31] .

Existing data demonstrate that EC are not equipped with L-type calcium channels. We therefore assume that the beneficial effects of Am presented in this report are related to the positioning of this drug in the plasma membrane near the receptors for native and modified LDL, eNOS, etc. The dihydropyridine ring of Am resides at the same depth as the sterol nucleus of cholesterol, possibly impeding and/or reversing the adverse effects of excessive cholesterol on the membrane structure and function [32]. It was demonstrated that Am induced dissociation of the caveolin/eNOS heterocomplex by a mechanism independent of the intracellular calcium [33].

The results of this study indicate that amlodipine inhibits NADPH oxidase and ROS-sensitive inflammatory signaling molecules and induces positive effects on the endothelial-dependent mechanisms of NO biosynthesis by modulating eNOS and iNOS expression and their balance, thus decreasing cytotoxic peroxynitrites formation.

In conclusion, our data demonstrate that amlodipine can ameliorate endothelial dysfunction induced in diabetes through mechanisms involving p38 MAPK and NF-κB signalling pathways.

Conflict of interest

All the authors declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgments

The authors thank Daniela Rogoz, Ioana Manolescu, Cristina Dobre for their excellent technical assistance and Stefan Nicolau, MD student at McGill University, Montreal, Canada for editorial assistance. The project was funded by a Grant from the Romanian Ministry of Education and Research (PNII no. 1027/2009).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.137.

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