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ORIGINAL ARTICLE

Studying neuroprotective effect of Atorvastatin as a small molecule drug on high glucose-induced neurotoxicity in undifferentiated PC12 cells: role of NADPH oxidase

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Abstract Overproduction of reactive oxygen species (ROS) by NADPH oxidase (NOX) activation has been considered the essential mechanism induced by hyperglycemia in various tissues. However, there is no comprehensive study on the role of NOXs in high glucose (HG)-induced toxic effect in neural tissues. Recently, a therapeutic strategy in oxidative related pathologies has been introduced by blocking the undesirable actions of NOX enzymes by small molecules. The protective roles of Statins in ameliorating oxidative stress by NOX inhibition have been shown in some tissues except neural. We hypothesized then, that different NOXs may have role in HG-induced neural cell injury. Furthermore, we postulate that Atorvastatin as a small molecule may modulate this NOXs activity to protect neural cells. Undifferentiated PC12 cells were treated with HG (140 mM/24 h) in the presence and absence of Atorvastatin (1 μM/96 h). The cell viability was measured by MTT assay and the gene and protein expressions profile of NOX (1–4) were determined by RT-PCR and western blotting, respectively. Levels of ROS and malondialdehyde (MDA) were also evaluated. Gene and protein expression levels of NOX (1–4) and consequently ROS and MDA levels were elevated in HG-treated PC12 cells. Atorvastatin could significantly decrease HG-induced NOXs, ROS and MDA elevation and improve impaired cell viability. It can be concluded that HG could elevate NOXs activity, ROS and MDA levels in neural tissues and Atorvastatin as a small molecule NOX inhibitor drug may prevent and delay diabetic complications, particularly neuropathy.

Keywords Atorvastatin · High glucose · NADPH oxidase · Oxidative stress · PC12 cells

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Introduction

Diabetic neuropathy is considered to be one of the most widespread disabling complications of diabetes mellitus (DM), influencing as many as 50 % of patients with long-lasting DM. It can be associated with poor quality of life, reduced longevity and increased mortality. In addition, diabetic neuropathy is the major cause of non-traumatic amputation (Figueroa-Romero et al. 2008). Hyperglycemia, which is known to be a hallmark of diabetes, initiates neuronal tissue damage processes by activating polyol, advanced glycation end products (AGEs), hexosamine and diacylglycerol/protein kinase C (PKC) pathways resulting in aconsequent overproduction of ROS (Brownlee 2005).

There are compelling evidences proposing that ROS overproduction, which is a result of NOX activation is the essential mechanism induced by hyperglycemia where mitochondria has been considered to be the major source of its production in various tissues (Brownlee 2005; Kakehi and Yabe-Nishimura 2008).

Excessive ROS is shown to contribute to the formation of lipid, protein, and nucleic acid adducts and to activate

complex chain reactions; a process that is responsible for neuronal cell injuries and ultimately diabetic neuropathy (Edwards et al. 2008; Vincent et al. 2004).

NOX enzymes represent a family of multi-component membrane enzymes composed of seven members including NOX (1–5), Duox1 (Dual oxidase1), and Duox2. They would catalyze electrons transfer across the plasma membrane, resulting in superoxide production which could be converted into other forms of ROS. Beside their physiological roles, they contribute to many pathological conditions in particular cardiovascular and neurodegenerative diseases (Bedard and Krause 2007).

Among the seven known NOX subtypes, the expression of NOX1, NOX2, and NOX4 has been demonstrated in neural cells (Ibi et al. 2006; Tammariello et al. 2000; Tejada-Simon et al. 2005; Vallet et al. 2005). Moreover, the NOX3 expression has also been found in the fetal brain (Paffenholz et al. 2004). In addition, low levels of NOX5 have been found in the brain (Cheng et al. 2001).

Despite the fact that reported studies demonstrate the importance of NOX1, NOX2, and NOX4 enzymes in diabetic complications (Gorin and Block 2013), no comprehensive study exists on the role of NOX profile induced by toxic effect of high glucose in neuronal tissues and alteration of individual NOXs in such a condition.

Recently, a therapeutic strategy for treating oxidative stress-related pathologies, such as ischemia/reperfusion tissue injury, metabolic and neurodegenerative diseases has been introduced by blocking the undesirable actions of NOX enzymes (Jaquet et al. 2009). Another important drug development strategy hinges on exploring new application for previously approved drugs.

Statins, HMG-CoA reductase inhibitors are routinely used for lowering hyperlipidemia. They have demonstrated several pleiotropic effects which include restoring endothelial dysfunction, inhibiting proliferation of vascular smooth muscle, reducing platelet aggregation, and increasing stability of atherosclerotic plaques among many others (Istvan and Deisenhofer 2001; Rikitake and K-i 2009). Their neuroprotective effects have also been shown in many studies to be due to their modulation in isoprenylation and myelination processes, immune response, reduction in inflammatory and oxidative responses and increased expression of glutamatergic transporters (Bösel et al. 2005; Butterfield et al. 2011; Cui et al. 2010; Déry et al. 2009; Wood et al. 2010; Zacco et al. 2003). Furthermore, several previous reports have attributed the beneficial effects of Statins to reduction of oxidative stress and correcting the impaired function of nitric oxide in nerves and vasculature (Ii et al. 2005; Matsuki et al. 2010; Nangle et al. 2003). It has been recognized that Atorvastatin attenuates high glucose-induced superoxide formation that is mediated by NOX in coronary arteries (Christ et al. 2002). However it is not clear whether the neuroprotective effect of Statins is mediated by NOX inhibition.

Given the aforementioned background, this study is therfore designed to investigate the following issues: 1) role of NOX profile in high glucose-induced neurotoxicity, and 2) whether Atorvastatin is able to protect neural cells from high glucose-induced cell injury through modulating NOXs activity. We hypothesize that NOXs may have a major role in activating the pathways that lead to neural cell injury by increasing the glucose level. In addition the effect of NOXs on cell injury may be sub-type dependent. On the other hand, Atorvastatin and its proven anti-oxidative effects may modulate NOX activity and thus indirectly protect the neural cells from high glucose-induced cell injury. Therefore, this hypothesis was examined by cellular and molecular methods in vitro.

Materials and methods

Reagents and antibodies

Atorvastatin, Apocynin, DCF and MTT were purchased from Sigma (Sigma Aldrich, St Louis, MO, USA). Dulbecco′s modified Eagle′s medium (DMEM), fetal bovine serum (FBS), horse serum (HS), penicillin-streptomycin were obtained from Gibco (Carlsbad, CA, USA). Trizol reagent was from Invitrogen (Merelbeke, Belgium) and Oligo (dT) primer and molony murine leukemia virus reverse transcriptase (MMLV) was purchased from Fermentas (UK). Horseradish peroxidase linked anti-rabbit secondary antibody and anti-β-actin were obtained from Cell Signaling (Danvers, MA, USA). The enhanced chemiluminescence (ECL) detection kit was supplied by Amersham Biosciences (Amersham, Buckinghamshire, UK) and protein extraction kit from Santa Cruz Biotechnology (CA, USA). Antibodies against NOX2, 4 were obtained from Abcam (Cambridge, UK), NOX1 from Aviva system biology (San Diego, Ca, USA) and NOX3 from Sigma (Sigma Aldrich, St Louis, MO, USA).

Cell culture and drug treatment

PC12 cells were supplied by Pasteur Institute of Iran (Tehran, Iran). Cells were kept at 37 °C and 90 % air humidity with 5 % CO2. Cells were cultured in DMEM with 5 % (*v*/v) FBS, 10 % (v/v) HS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The culture medium was replaced every 48 h and the cells were passaged every 2–3 days.

The glucose concentration in DMEM was 25 mM and considered the normal glucose. The cells were used after 2 subcultures. In MTT assay, HG condition was estimated by exposing 96 well-culture plate (5000 cells/well) to 60 mM, 80 mM, 100 mM, and 140 mM D-glucose. Also, the cells were pretreated by Atorvastatin (0.001–1 μM) for 96 h and co-incubated with Atorvastatin and HG (140 mM) for 24 h. In experiments where Apocynin (Sigma, a10809) was used

together with HG, the cells were pretreated with Apocynin (10, 20, 40 μm) 45 min before glucose addition. Control cells were cultured in normal glucose (25 mM). Atorvastatin was dissolved in dimethyl sulfoxide (DMSO). The DMSO′s final concentration was less than 0.1 %.

Cell viability

Cell viability was determined by colorimetric MTT assay using modified 3-(4, 5- dimethylthiazol-2-yl) - 2, 5-diphenyl tetrazolium (MTT) reagent (Mosmann 1983). In this assay, tetrazolium salts were cleft to insoluble purple Formazan in metabolically active cells that could be quantified by spectrophotometer. PC12 cells were properly seeded at the density of 5000 per well in a 96-μ plate overnight. After the treatment of the cells as described before, MTT solution (0.5 mg/ml in PBS) was added and incubated for 2 h in 37 °C. Then, the medium was removed and 100 μl DMSO was added to solubilize the resulting Foramazin. The absorbance was measured at 570 and 630 nm in ELISA reader. The results were reported in percent with respect to the control.

Reverse transcription-polymerase chain reaction

Total cellular RNA was isolated using TRIZOL reagent according to the manufacturer′s directions and quantified by the spectrophotometer. First-strand cDNA was synthesized employing 1 μg RNA, 2 mM oligo dT, primer and 200 U MMLV at the total volume of 20 μL. The reaction mixture was incubated for 1 h at 42 °C and then was followed by incubation at 72 °C for 10 min. Aliquots of 5 μL of cDNA were exposed to PCR utilizing specific primers as follows: NOX1 sense primer, 5′-AGGCTCCAGACCTCCATTTG-3′, and antisense primer, 5′-ATGTTCAGCCCCAACCAAGA-3′; NOX2 sense primer, 5′-TGCCAGTGTGTCGGAATCTC-3′, and antisense primer,5′-TGTGAATGGCCGTGTGAAGT-3′; NOX3 sense primer, 5′-CAGGAA TTGGAGT CACCCCC-3′, and antisense primer, 5′-GGCAAACCACTCAAAGGCTG-3′; NOX4 sense primer, 5′-GTGTCTGCATGGTGGTGGTA-3′, and antisense primer, 5′-ACTGATACAGCCAGGAGGGT-3′. The PCR conditions for the primer sets were as follows: Hot start at 94 °C for 3 min; 30 amplification cycles, each consisting of 94 °C for 30 s, T_m for 1 min, 72 °C for 42 s, and the final extension step at 72 °C for 5 min.

Optimized T_m for the primers was: NOX1 48 °C, NOX2 55 °C, NOX3 59 °C, and NOX4 53 °C. PCR products were separated on 2 % agarose gels and visualized by Nancy staining.

Western blot analysis

Total protein was extracted by collecting the whole cell lysate using protein extraction kit (Santa Cruz Biotechnology), following the manufacturer′s protocols. Then, the cell lysates were incubated on ice for 30 min and centrifuged at 15,000 g for 30 min at 4 °C. The supernatant was collected and stored at −80 °C until analysis. The protein concentration was calculated using Bradford method (Bradford 1976). Proteins in equal amounts were loaded in 10 % SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Laemmli 1970) that was incubated with primary (NOX1–4) and secondary (anti-mouse HRP conjugated) antibodies. The bands were observable using ECL detection reagents. Finally, a densitometer program (TotalLab) was used to quantify the density of the bands. β-actin bands were used as the internal control.

Measuring intracellular ROS

The generation of ROS was assessed by the 2, 7-dichlorofluorescein diacetate (DCF-DA). The acetate ester form of 2′, 7′ dichlorodihydrofluoresceindiacetate (H_2DCFDA-AM) is a membrane permanent molecule that penetrate the cell membrane. Cellular esterases act on the molecule to form the non-fluorescent moiety H_2DCFDA, which is ionic in nature and can be trapped inside the cell. H_2DCFDA oxidizes by ROS and is converted into the fluorescent molecule, DCF. PC12 cells were plated in a 24-well microplate, pretreated with Atorvastatin for 96 h, and treated with high glucose for 24 h. The media were replaced with serum free DMEM and placed back in the incubator overnight. Cells were loaded with DCF-DA (5 μM) for 30 min at 37 °C and were then washed. The oxidation product was evaluated using 485/20 nm excitation and 528/20 nm emission wavelengths by the microplate reader.

Measuring lipid peroxidation

MDA is a naturally occurring end product of lipid peroxidation. Lipid peroxidation is a well-established mechanism of cellular injury induced by ROS. Levels of MDA were measured by thiobarbituric acid reactive substances (TBARS) assay (Draper and Hadley 1990). The supernatant of the cells was used for analysis and measurements were performed using 530/25 nm excitation and 575/15 nm emission wavelength by the microplate reader.

Statistical analysis

Data were presented as means ± SEM. Statistical differences were determined by non-paired student′s t-test between two groups and ANOVA between multiple groups. P-value < 0.05 was considered the significance criterion.

Results

Effect of glucose on cell viability

Undifferentiated PC12 cells were used throughout the study. PC12 cells were exposed to the increasing concentrations of glucose (60–140 mM) for 24 h using MTT assay in order to investigate the effect of HG on neural cell viability. HG in 100, 120, and 140 mM could significantly decrease cell viability compared to controls in a dose dependent manner (Fig.1). The 140 mM was selected as HG condition throughout the studies.

Effects of atorvastatin on PC12 cell viability

PC12 cells were pretreated with different concentrations of Atorvastatin for 96 h to evaluate its possible toxic effect on cell viability. As demonstrated in Fig. 2, Atorvastatin (0.01–4 μM) could not significantly affect cell viability.

Effect of Atorvastatin on HG-induced PC12 cytotoxicity

Prior study of Atorvastatin neuroprotection against glutamate-induced cytotoxicity has suggested that significant protection can be observed in 96 h pre-treatment (Bösel et al. 2005). In current study we have also observed the significant protective effect of Atorvastatin on HG-induced PC12 cytotoxicity could only be seen at 96 h of pretreatment incubation, but not in 24, 48, and 72 h (data not shown).

HG remarkably reduced cell viability compared to controls ($P < 0.001$). However, incubation of the cells with Atorvastatin (0.001–1 μM) for 96 h reversed cell toxicity in a concentration-dependent manner, which was significant at 1 μM ($P < 0.001$) and was selected and used throughout the whole study. Apocynin, a NOX inhibitor (Petrônio and Zeraik 2013) was also used as the positive control by its pretreatment for 45 min at different HG concentrations (10, 20, and 40 μM). Apocynin could significantly ($P < 0.001$) protected the cells against HG (10 μM) induced cell death.

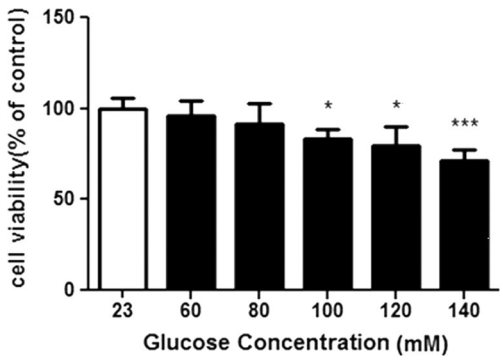

Fig. 1 Measuring PC12 cells viability by MTT assay. Cells were exposed to various HG (high glucose) concentrations for 24 h and compared with control. Results are reported as mean ± SEM ($n = 8$). $^{*}P < 0.05$ and $^{***}P < 0.001$, vs control

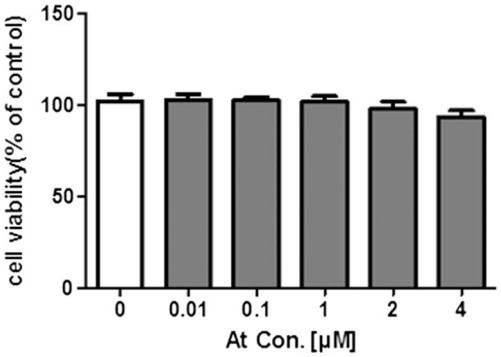

Fig. 2 Effect of Atorvastatin (At) on cell viability in PC12 cells. Cells were treated with different concentrations of Atorvastatin for 96 h. Cell viability was assessed by MTT assay. Results are reported as mean ± SEM (n = 8)

Mannitol (140 mM) was used to mimic HG osmolality condition and did not show any significant change in cell viability. This result indicated that the high glucose influence on cell death was not secondary to osmotic loading **(**Fig.3**)**.

Effect of Atorvastatin on NOX profile gene expression

Effects of Atorvastatin (1 μM) on the expressions of NOX (1–4) mRNA in PC12 cells were examined using semiquantitative RT-PCR assays. Densitometric analysis showed that NOX (1–4) expressions were significantly increased in HG-treated cells for 24 h compared to control groups ($P < 0.001$). These overexpressions were reversed by Atorvastatin significantly ($p < 0.001$). Apocynin also significantly inhibited NOX (1–4) overexpressions compared to HG groups ($p < 0.001$) **(**Fig. 4**)**.

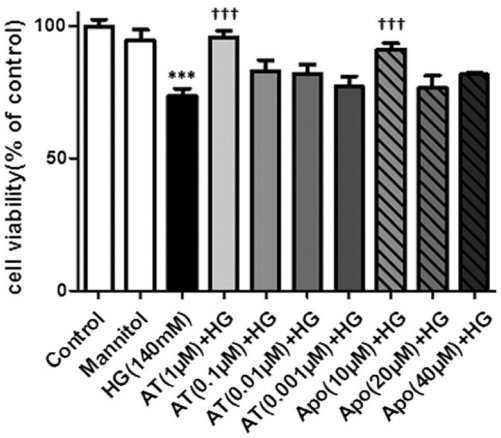

Fig. 3 Concentration-response of Atorvastatin (At) and Apocynin (Apo) on cell viability in HG condition in PC12 cells. Cells were pre-treated with various Atorvastatin concentrations for 96 h and then exposed to HG (140 mM for 24 h). Apocynin (Apo) was used as the Atorvastatin control in different concentrations and treated 45 min prior HG treatment (140 Mm for 24 h) and mannitol used as the glucose osmolarity control. Cell viability was measured by MTT assay. Results are reported as mean ± SEM (n = 8). $^{***}P < 0.001$, vs control. ††† $P < 0.001$, vs HG

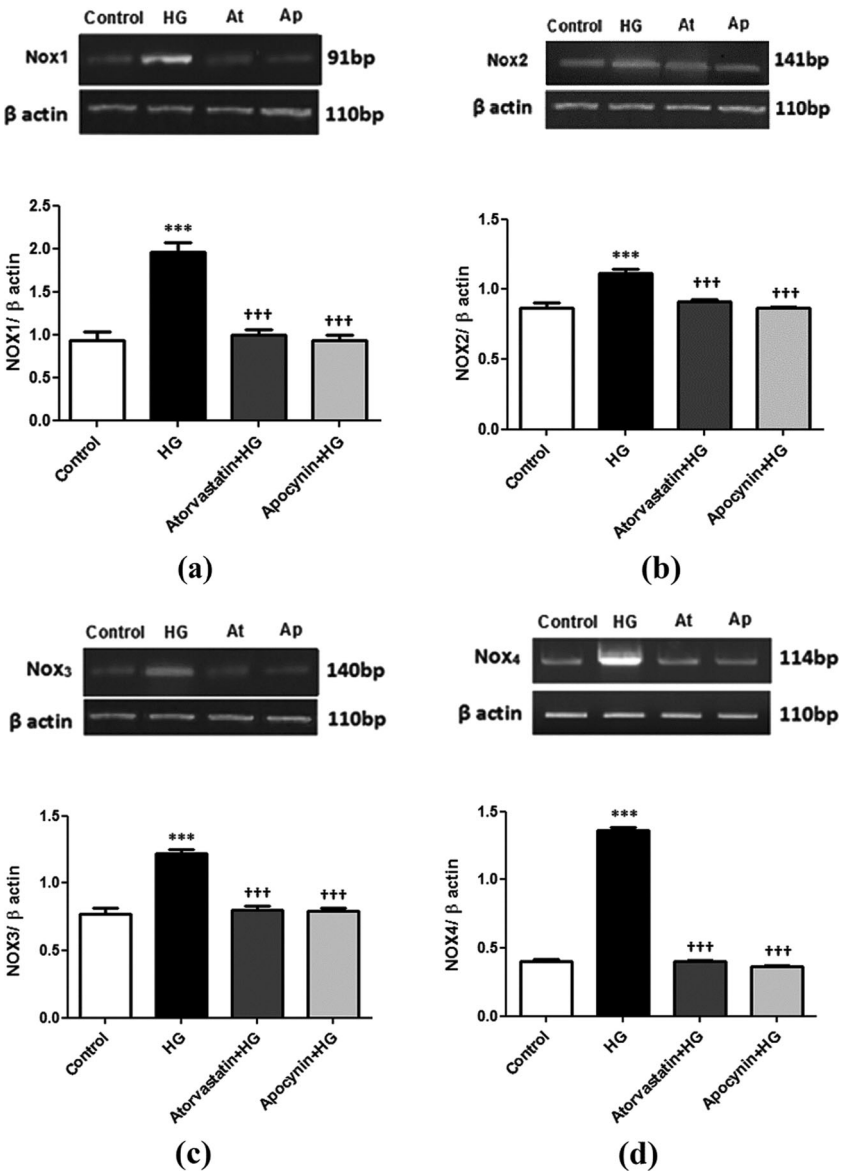

Fig. 4 RT-PCR analysis to determine Atorvastatin effect on gene expression of NOX1 (**a**), NOX2 (**b**), NOX3 (**c**), and NOX4 (**d**) in PC12 cells. Cells were pre-treated with Atorvastatin (1 μm) for 96 h and then exposed to HG (140 mM) for 24 h. Apocynin (10 μM) was used as the Atorvastatin control and added 45 min before HG treatment. The densities of NOX1 (**a**), NOX2 (**b**), NOX3 (**c**), and NOX4 (**d**) ratio to β-actin were determined. Results are reported as mean ± SEM ($n = 3$). ***$P < 0.001$, vs control and ††† $P < 0.001$, vs HG

Effect of Atorvastatin on NOX profile protein expression

Western blot analysis was performed to determine the effect of Atorvastatin (1 μM) on NOX (1–4) protein expressions. PC12 cells were pre-treated with Atorvastatin for 96 h followed by exposing to HG for 24 h. Similarly, in other group, the cells were pretreated for 45 min with Apocynin. HG significantly increased NOX (1–4) protein expressions [especially NOX1 ($P < 0.001$), NOX2 ($P < 0.01$), and NOX4 ($P < 0.001$)] compared to controls. Atorvastatin could attenuate these increases in NOX (1–4), which were more significant in NOX1 ($P < 0.001$), NOX2 ($P < 0.01$), and NOX4 ($P < 0.001$) compared with HG groups. Apocynin was used as the positive control for Atorvastatin and could significantly reversed NOX (1–4) elevations (Fig.5).

Effect of Atorvastatin on intracellular ROS generation

The effect of pretreatment of Atorvastatin on HG-induced ROS production was investigated. The treatment of PC12 cells with 140 mM glucose increased ROS levels ($P < 0.001$) which was significantly reversed by Atorvastatin (1 μM) for 96 h ($P < 0.01$). Apocynin was also used as the positive control and similarly decreased ROS elevation ($P < 0.001$). (Fig.6).

Effect of Atorvastatin on lipid peroxidation

MDA levels were significantly increased after the exposure of PC12 cells to HG concentration ($P < 0.001$); however, in the cells pre-treated with Atorvastatin (1 μM), MDA elevation was significantly inhibited ($P < 0.01$). Apocynin was used as

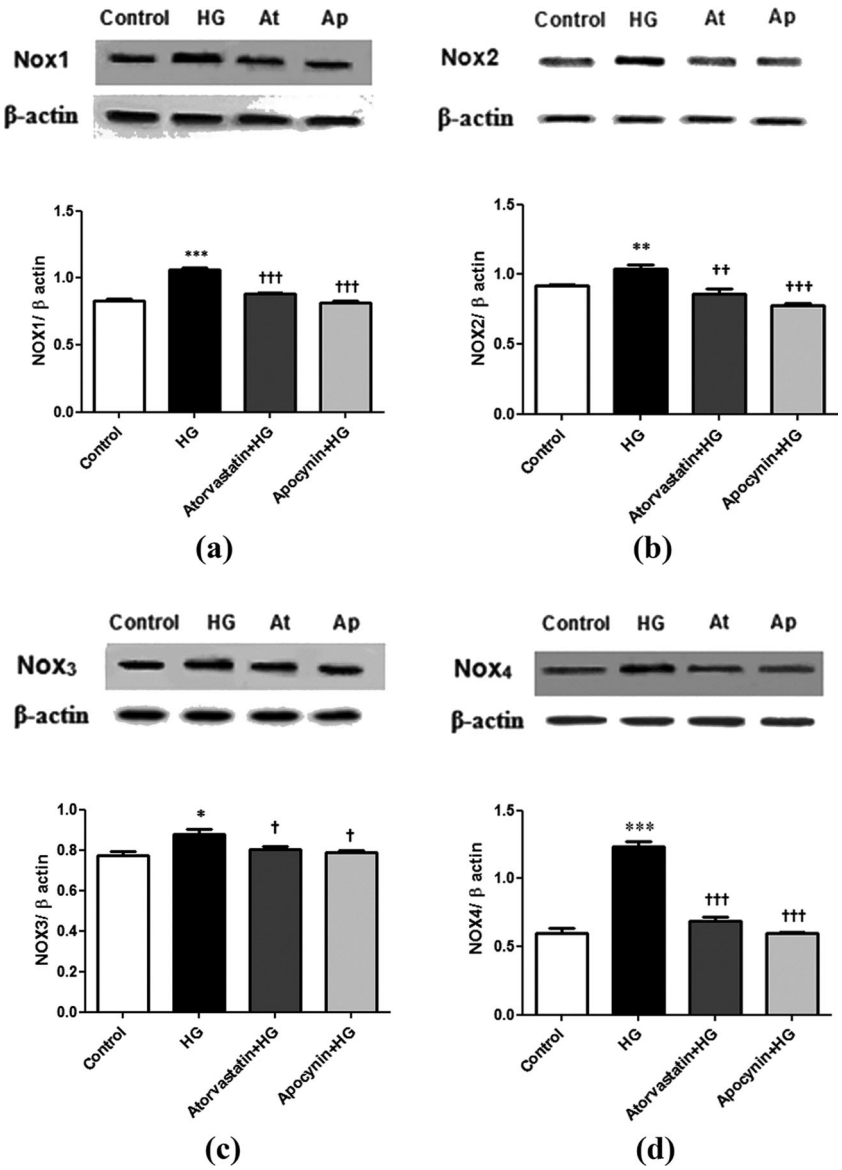

Fig. 5 Western blotting analysis to determine Atorvastatin effect on protein levels of NOX1 (**a**), NOX2 (**b**), NOX3 (**c**), and NOX4 (**d**) in PC12 cells. Cells were pre-treated with Atorvastatin (1 μM) for 96 h and then exposed to HG (140 mM) for 24 h. Apocynin (1 μM) was used as the Atorvastatin control and added 45 min before HG treatment. The densities of NOX1 (**a**), NOX2 (**b**), NOX 3 (**c**), and NOX4 (**d**) ratio to β-actin were determined. Results are reported as mean ± SEM (n = 3). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, vs control, † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$, vs HG

the positive control and significantly decreased MDA-induced HG condition ($P < 0.01$) (Fig.7).

Discussion

The current study revealed that NOXs have a major role in facilitating the neural cell injury and death. Also, our results indicated that the effect of NOXs on HG-induced cell injury is type dependent with NOX1 and NOX4 being the most significant contributors. In addition, Atorvastatin has been shown to modulate NOXs activity and indirectly protect the neural cells from HG-induced cell injury.

In addition, our study corroborates the fact that mRNA levels of NOX (1–4) were overexpressed in HG-exposed PC12 cells, while at protein expression levels, they were more pronounced in NOX1, NOX2 and NOX4.

Consistent with our results, the elevation in NOX1 levels which is accompanied by enhanced ROS production and endothelial dysfunction in aortic vessels of diabetic rats has been reported (Wendt et al. 2005). Moreover, role of NOX1 in diabetes-accelerated atherosclerosis has also been reported (Gray et al. 2013).

Importantly, HG-induced ROS generation that is mediated by NOX1 was associated with decreased angiotensin-converting enzyme 2 (ACE2) and Ag(1–7) [angiotensin(1–7)], which are considered to be two protective factors regulating vascular smooth muscle as well as endothelial cell function (Lavrentyev and Malik 2009).

It is demonstrated that NOX2 is required for HG-stimulated ROS production and cell injury in cardiomyocytes and increased expression of transforming growth factor beta (TGF-β), as a pro-fibrotic factor which operates downstream of hyperglycemia. Similarly NOX2 up-regulation in the

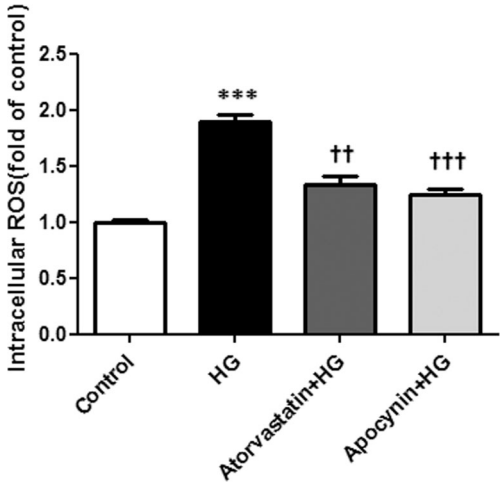

Fig. 6 Measuring intracellular ROS production by DCF assay. Cells were pre-treated with Atorvastatin (1 μM) for 96 h and then exposed to HG (140 mM) for 24 h. Apocynin (1 μM) was used as the Atorvastatin control and added 45 min before HG treatment. ROS generation was compared in fold with the control. Results are reported as mean ± SEM (n = 8). $^{***}P < 0.001$, vs control, †† $P < 0.01$ and ††† $P < 0.001$ vs HG

myocardium of diabetic rats and mice have also been reported (Gorin and Block 2013).

It is also known that high glucose condition induces lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) expression via NOX1 and NOX2 activations in PC12 cells (Zhang et al. 2012).

Moreover NOX4 elevation of mRNA and protein expression in response to HG is shown to be possibly mediated by angiotensin II (AngII) in mesangial cells. On the other hand, HG has been shown to induce Ag II production by activating local RAS (renin angiotensin system) in glomerular endothelial cells and also in PC12 cells. (Gorin and Block 2013; Shahveisi et al. 2014).

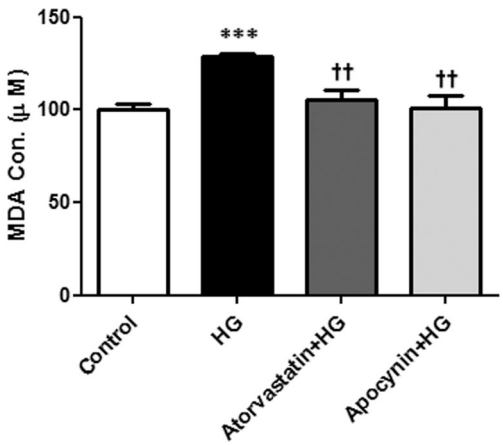

Fig. 7 Measuring MDA levels by TBARS assay. Cells were pre-treated with Atorvastatin (1 μM) for 96 h and then exposed to HG (140 mM) for 24 h. Apocynin (1 μM) was used as the Atorvastatin control and added 45 min before HG treatment. Results are reported as mean ± SEM (n = 8). $^{***}P < 0.001$, vs control. †† $P < 0.01$, vs HG

Interestingly, in human podocytes, NOX4 gene silencing caused the reduced production of ROS and down-regulation of pro-inflammatory monocyte chemoattractant protein-1 (MCP-1) and nuclear factor kappa B (NF-kB) and pro-fibrotic proteins (collagen IV and fibronectin) which are indicated in diabetic nephropathy (Jha 2014).

Consequently, it is speculated that similar to other tissues, elevated NOX1, NOX2 and NOX4 as a result of HG condition may affect neuronal cells in the current study.

In agreement with previous studies, the current work indicates that HG could elevate ROS production in PC12 cells (Aminzadeh et al. 2014; Eslami et al. 2014; Lelkes et al. 2001). ROS generation by enhancing glycolytic process could be considered as a major factor in progressive nerve damage (Greene et al. 1999; Pop-Busui et al. 2006). NOXs, as the major contributors of ROS formation, mediate the transfer of electrons across plasma membranes to oxygen and generate superoxide and other downstream ROS (Kakehi and Yabe-Nishimura 2008; Lambeth 2007; Vincent et al. 2004). High glucose has previously shown to induce apoptosis in PC12 cells (Sharifi et al. 2009; Sharifi et al. 2007). Excessive NOX activity is an important factor which causes apoptotic cell death and contributes to a large number of pathological conditions, including neurodegeneration (Abramov et al. 2004; Abramov and Duchen 2005; Meischl et al. 2006; Ryter et al. 2007).

The three major mitogen activated protein kinases (MAPK) including c-Jun NH2-terminal kinases (JNK), P38, and extracellular signal-regulated kinase (ERK) are involved in NOX-mediated redox signaling and lead to apoptosis (Jiang et al. 2011; Plotnikov et al. 2011).

Our results also indicate that HG could increase MDA levels as previously shown by other studies (Eslami et al. 2014; Lelkes et al. 2001). MDA, the end product of lipid peroxidation, is one of the most reliable biomarkers for assessing the severity of oxidative stress (Pizzimenti et al. 2010; Siddique et al. 2012).

Our findings show that Atorvastatin can significantly decrease mRNA and protein expression of NOX (1–4); however, these inhibitions were more remarkable in NOX1, NOX2, and NOX4 protein levels.

In line with current results, the protective effect of Fluvastatin in cardiac neuropathy of diabetic rats was described, which indicated the attenuation of elevated oxidative stress and reduction of myocardial $p22^{phox}$ mRNA expression (Matsuki et al. 2010). Similarly, it is also observed that Atorvastatin could decrease superoxide formation by inhibiting NOX activation in the endothelial cells of coronary arteries which were incubated in HG condition (Christ et al. 2002). Moreover, another study reported the protective role of Simvastatin in diabetic retinopathy by inhibiting NOX-mediated activation of STAT3 (signal transducer and activator of transcription 3) (Al-Shabrawey et al. 2008).

Our study also reveals that Atorvastatin could inhibit HG-induced ROS and MDA levels elevation in PC12 cells. In agreement with our findings, several animal and human studies using Statins have shown the reduction of oxidative stress by various mechanisms which include inhibiting NADPH oxidase (Takayama et al. 2004), stimulating activity of antioxiadant enzyme thioredoxin (Haendeler et al. 2004), protecting oxidation of LDL by stimulating the activity of paraoxonase (PON) (Deakin et al. 2003), and restoring antioxidants such as vit E, vit C, ubiquinone, and glutathione (Yilmaz et al. 2004).

Impaired vascular function is associated with pathology of diabetic neuropathy. Increased ROS formation by NADPH oxidase leads to reduce NO and its related endothelium-dependent vasodilation in arterioles feeding nerves. The role of NOXs in endothelial dysfunction has been also proven in retinopathy and macrovascular complications of diabetes (Gorin and Block 2013; Kakehi and Yabe-Nishimura 2008). Based on exploration of the major detrimental role of NOXs signaling in diabetic complications, as well as many other similar pathological conditions, design and development of agents inhibiting this pathway has been attracted considerable attention in drug discovery.

Previous studies depicted the pivotal role of NOX1, NOX2, and NOX4 in other diabetic complications, but there was no study performed in diabetic neuropathy.

In conclusion, our findings determined the major role of NOX1, NOX2, and NOX4 in undifferentiated PC12 cells exposed to HG as an in vitro model for studying diabetic neuropathy. Atorvastatin exerts an inhibitory effect on NOX1, NOX2, and NOX4 that could lead to further speculation for using Statins in preventing and delaying diabetic complications, particularly neuropathy.

For future studies, we propose to investigate the neuroprotective effects of Atorvastatin in primary neural cells as well as animal model of diabetic neuropathy invivo.

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