The role of local renin-angiotensin system on high glucose-induced cell toxicity, apoptosis and reactive oxygen species production in PC12 cells

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ARTICLEINFO	ABSTRACT
Article type: Original article *Article history:* Received: Sep 28, 2013 Accepted: Dec 3, 2013 *Keywords:* Apoptosis High glucose toxicity Oxidative stress PC12 Renin-angiotensin system	***Objective(s):*** Hyperglycemia, oxidative stress and apoptosis have key roles in pathogenesis of diabetic neuropathy. There are local renin-angiotensin systems (RASs) in different tissues such as neural tissue. Local RASs are involved in physiological and pathophysiological processes such as inflammation, proliferation and apoptosis. This study aimed to investigate the role of local renin-angiotensin system on high glucose-induced cell toxicity, apoptosis and reactive oxygen species (ROS) production in PC12 cells, as a cell model of diabetic neuropathy. ***Materials and Methods:*** PC12 cells were exposed to a high glucose concentration (27 mg/ml), captopril (ACE inhibitor), telmisartan and losartan (AT1 antagonists), and also PD123319 (AT2 antagonist) were administered before and after induction of high glucose toxicity. Then cell viability was assessed by MTT assay and apoptotic cells and intracellular ROS production were detected by annexin V-propidium iodide and DCFDA, respectively, using flow cytometry. ***Results:*** High glucose concentration decreased cell viability, and increased apoptotic cells. Intracellular ROS production was also increased. In PC12 cells pretreatment and treatment by the drugs showed a significant improvement in cell viability and reduced apoptosis in captopril, telmisartan and PD123319 but only captopril and telmisartan were able to reduce ROS production. Losrtan significantly lowered ROS but didn't show any improvements in cell viability and apoptotic cells. ***Conclusion:*** The results of the present study showed that RAS inhibitors reduced cell toxicity and apoptosis and ROS production was induced by high glucose. It may be suggested that local RAS has a role in high glucose toxicity.

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Introduction

Diabetes mellitus (DM) with a prevalence of 5.1% of all world population is characterized by high blood glucose levels (1-3). DM has several long-term complications including cerebrovascular, coronary, and peripheral vascular diseases, nephropathy, retinopathy and neuropathy (4, 5). Diabetic neuropathy is one of the most frequent and severe complications of diabetes which could affect sensory, motor, and autonomic nerves (6). Despite numerous efforts to uncover the precise mechanism(s) of diabetic neuropathy, the results were disappointing due to the etiological and pathological complexities. Hyperglycemia plays a key role in the development of diabetic complications (7); on the other hand a significant improvement in neuropathy following reduction of glucose level has been reported (8). The exact mechanism(s) involved in high glucose-induced toxicity is still unknown. However, it is suggested that insufficiency of arterial blood flow due to a high glucose-induced atherosclerosis (vascular aspect) may be involved (8, 9). In addition, intracellular alteration of biochemical reactions induced by direct effect of high glucose on neuronal cells (non-vascular aspect) may also contribute (6, 8-10). Some of this biochemical

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alterations including increased formation of advanced glycation end products, activation of protein kinase C (PKC) through increased diacylglycerol production, enhanced polyol pathway flux and increased hexosamine pathway flux have been reported as underlying mechanisms (9). Increased glucose concentrations activate the reactive oxygen species (ROS) production through two main sources, the first is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase located on plasma membrane, and the second are mitochondria through a dysfunction of electron transport chain (2, 11-13). A large growing body of evidence reports an increased oxidative injuries induced by hyperglycemia in many diabetic tissues such as neural tissue (14-16). In fact, oxidative stress is an upstream crucial trigger which promotes numerous detrimental pathways ending to apoptosis and cell death (9, 13). Several studies have reported that apoptosis is the other mechanism involved in diabetic neural injury (2, 17, 18).

Aside from classical renin-angiotensin system (RAS), a hormonal one maintaining blood pressure, electrolytes and water balance in the body, there are local or even intracellular renin-angiotensin systems in different tissues (5, 19-21). Researchers refer many physiological and pathophysiological functions to local RAS including inflammation, fibrosis, proliferation, differentiation or even apoptosis (22). The local RAS physiologically has been found in almost all body parts including: adrenal gland (5, 21), pancreas (23), heart (24), blood vessels (25), kidney (26), nervous system (19, 22), reproductive system (27), and many tissues such as adipose tissue (28). Several evidences have been reported an over-activation of RAS in diabetic complications, as it has been well-documented that the renin-angiotensin system (RAS) plays a major role in the pathophysiology of diabetic nephropathy (26).

Angiotensin II has two main receptors named angiotensin receptor type 1 (AT_1) and type 2 (AT_2) (9, 29). Actually the most known actions of angiotensin such as vasoconstriction, renal sodium reabsorption and also aldosterone and vasopressin secretion are mediated by AT_1 but the roles of AT_2 receptor remain mysterious and controversial (29). AT_2 receptor has been abundantly found in fetus organs, uterus and neural tissues (29). Surprisingly, functions such as cellular proliferation, differentiation, and apoptosis that have been attributed to AT_2 receptors are corresponded well to the local RAS (9, 29). So, it seems that this receptor play an important role in local RASs. There is several evidence of activation of local RAS in most of diabetic tissues as well as neural tissue (8, 9, 26). Recently angiotensin converting enzyme (ACE) inhibitors associated with angiotensin II receptor antagonist are recognized as first line treatment for nephropathy(9, 30). In addition, it seems that RAS contributes to almost all biochemical events which are promoted by hyperglycemia and finally result in neural cell death (9). Not only hyperglycemia, but also angiotensin II, the main effector of RAS, is among strong inducers of oxidative stress (9, 31, 32). It is conceivable that locally increased ROS induced by angiotensin II (Ang II) could add to high glucose-induced ROS production and cause even more damages. Interestingly, in a study on proximal tubule cells in rat, it was found that the effect of hyperglycemia on stimulation of angiotensinogen gene expression was through increasing ROS production (33). It was suggested that besides locally Ang II elevation following hyperglycemia, increased ROS formation may in turn act as a second messenger for local Ang II production (13). As previously mentioned, one possible mechanism to develop diabetic neuropathy is reduction of blood supply due to vasoconstriction of microvasculature; thus, ACE inhibitors such as captopril or AT_1 antagonists such as telmisartan and losartan as vasodilator agents can be curative in this situation (11, 30). Although, some studies have shown a vascular effect of these drugs, but nonvascular actions of these agents in high glucose-induced neuropathy is still unclear (9).

The required components of an intrinsic RAS have been found in rat's adrenal medulla as well as PC12 cells (21, 34). The PC12 cells, pheochromocytoma cells are derived from rat's adrenal medulla, have a neural crest origin and abundantly express AT_2 angiotensin receptor (34-37).

In the present study, the role of local RAS on high glucose-induced cell toxicity, apoptosis and ROS production was investigated in PC12 cells.

Materials and Methods
Materials
PC12 cells were obtained from Pasteur Institute (Tehran, Iran). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), 2',7'-dichlorofluorescein diacetate (DCF-DA), captopril, telmisartan, losartan, PD-123319, dimethyl sulfoxide, and D-glucose were purchased from Sigma-Aldrich company (St. Louis, MO, USA). DMEM, fetal bovine serum, trypsin, penicillin, and streptomycin were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA) and annexin V-FITC & PI kit was purchased from BioVision (Mountain View, CA, USA).

Cell culture
PC12 cells were routinely cultured in Dulbecco's modified Eagle's medium, containing 4.5 mM-D-(+)-glucose, 10% heat-inactivated fetal bovine serum, 100U/ml penicillin, and, 100 U/ml streptomycin, incubated at a temperature of 37°C in a humidified atmosphere of 95% air and 5% CO_2, and were passaged when reached to 70% confluency.

Cell treatment
PC12 cells were pretreated for 3 hr prior to and after high glucose-induced toxicity with a glucose
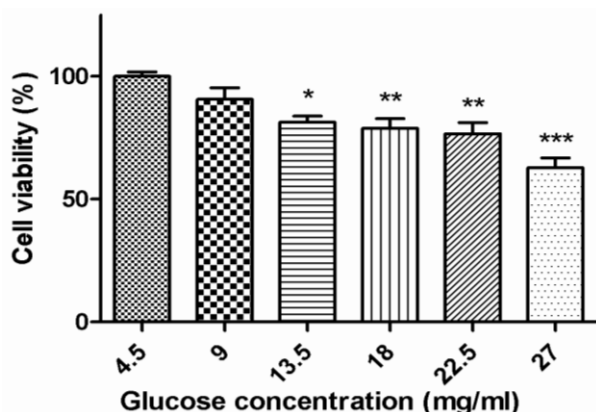
Figure 1. The effect of different concentrations of glucose on cell viability of PC12 cells after 144 hr. Cell viability was performed by MTT assay. The concentrations of glucose are 4.5 (normal), 9, 13.5, 18, 22.5, and 27 mg/ml, respectively
Results are expressed as Mean ± SEM of three different experiments, each performed in triplicate
*$P<0.05$, **$P<0.01$ and ***$P<0.001$ *vs* normal glucose (NG)
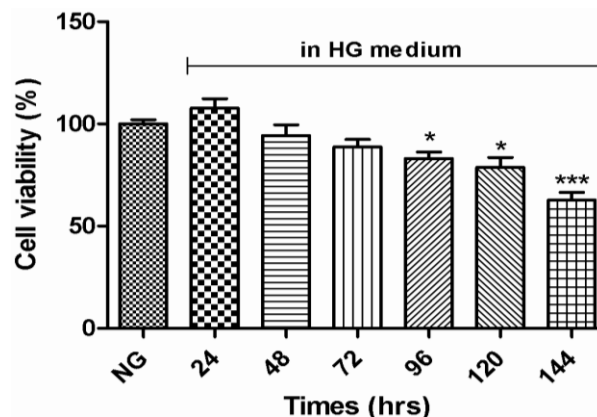

Figure 2. The effect of high glucose (HG) toxicity (27 mg/ml) in different times on PC12 cells. Cell viability was performed by MTT assay. The concentration of glucose in control was 4.5 mg/ml and the rest of experiments were exposed to 27 mg/ml for 24 to 144 hr. Results are expressed as Mean ± SEM of three different experiments, each performed in triplicate
*$P<0.05$, and ***$P<0.001$ *vs* normal glucose (NG)

concentration of 27 mg/ml by following drugs: captopril (10 μM), telmisartan (10 μM), losartan (10 μM), PD-123319 (10 μM), and two combination groups include telmisartan plus PD-123319 (each 10 μM), and, losartan plus PD-123319 (each 10 μM).

Determination of cell viability

To evaluate cell viability, PC12 cells were seeded (at 1×10^3 cells per well) in a 96-well culture plate and cell viability was measured by MTT colorimetric assay. Thus, after treatment, 11 μl MTT solution (5mg/ml) was added into each well and cells were incubated at 37°C for 4h in a humidified atmosphere containing of 5% CO_2 and 95% air. After removing the medium, formazan crystals were solubilized by adding 100 μl of dimethylsulfoxide (DMSO). The absorption was measured at 570 nm (620 nm as a reference wavelength) by means of an ELISA reader.

Apoptosis analysis

Apoptosis was evaluated by annexin V-FITC and propidium iodide (PI) staining followed by flow cytometry. Cells were detached and Annexin V&PI double staining was performed according to manufacturer's instructions and then measured by flow cytometry.

Measurement of intracellular reactive oxygen specious

After treatment, the PC12 cells once washed with prewarmed PBS, then they were incubated with 10 μM DCFH-DA at 37°C in the dark. 30 min later, the cells were rewashed and resuspended in PBS for analysis by FACScalibur flow cytometer (BD, USA) with excitation and emission wave lengths of 475 and 525 nm, respectively. For each analysis 10,000 events were recorded.

Data Analysis

All results derived from at least three independent experiments are expressed as mean ± SEM. The significance of difference was analyzed by a one-way ANOVA followed by Tukey as post-test using GraphPad Prism 5 and$P<0.05$ was considered to be statistically significant.

Results
Effect of high glucose on PC12 cell viability

MTT assay showed that treatment with high glucose concentrations decreased cell viability in a time- and concentration-dependent manner. Figure 1 shows that all concentrations of glucose decreased cell viability. Concentrations of 13.5, 18, 22.5 and 27 mg/ml showed significant difference ($P<0.05$-$P<0.001$). Figure 2 shows that 27 mg/ml of glucose had cytotoxic effects in a time dependent manner and it decreased cell viability at 96, 120 and 144 hrs ($P<0.05$-$P<0.001$).

Effect of the drugs on glucose-induced toxicity

The cell viability at 27 mg/ml of glucose (high glucose; HG) was significantly lower than normal glucose (NG) concentration after 144 hrs ($P<0.001$). Viability was increased in experimental groups: captopril ($P<0.05$), telmisartan ($P<0.001$), PD123319 ($P<0.05$), Telmisartan/PD123319 ($P<0.001$), and losartan+PD123319 ($P<0.01$), respectively (Figure 3).

Effects of drug treatment on Apoptotic and Necrotic cells

The flow cytometric results of annexin V-FITC and propidium iodide staining assay showed that apoptotic and necrotic cells were increased in high glucose condition (27 mg/ml) by ($P<0.001$) and ($P<0.01$) respectively. Pretreatment followed by
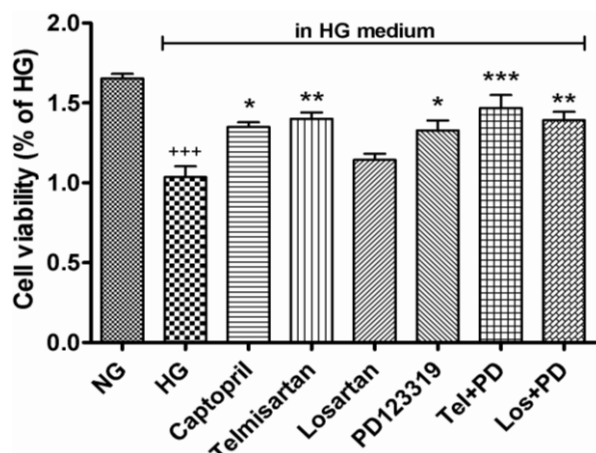
Figure 3. The effects of the drugs on high glucose toxicity of PC12 Cells after 144 hrs. Cell viability was assessed by MTT assay. The glucose concentrations in control group is 4.5 mg/ml (NG) and were 27 mg/ml (HG) in all other groups and the concentrations of all drugs were 10 µM. Viability in all experimental groups compared with High Glucose (HG) group. Results are expressed as Mean ± SEM of three different experiments, each performed in triplicate
*$P<0.05$, **$P<0.01$ and ***$P<0.001$ *vs* high glucose (HG) and +++$P<0.001$ *vs* normal glucose (NG)

Figure 4. The effect of the drugs on apoptotic and necrotic cells. The glucose concentrations in control group was 4.5 mg/ml (NG) and were 27 mg/ml (HG) in all other groups and the concentrations of all drugs were 10 µM. Apoptotic and necrotic cells were detected by annexin V-FITC and propidium iodide staining assay. Results are expressed as Mean ± SEM of three different experiments
*$P<0.05$, **$P<0.01$ and ***$P<0.001$ *vs* high glucose (HG); ++$P<0.01$ and +++$P<0.001$ *vs* normal glucose (NG)

treatment with captopril ($P<0.05$), telmisartan ($P<0.01$), PD123319 ($P<0.01$), telmisartan/PD123319 ($P<0.001$) and losartan/PD123319 ($P<0.01$) significantly decreased apoptotic cells in high glucose conditions. As shown in Figure 4, telmisartan and telmisartan/PD groups had significant reduction ($P<0.05$) in necrotic cells.

Effects of the drugs on high glucose-induced elevation of ROS Production

DCF-DA used as a probe to evaluate the intracellular ROS alterations by flow cytometery. It was revealed that high glucose concentration increased the ROS production after 1 h ($P<0.001$). As the Figure 5 shows it was increased to the most high level after 2 hrs ($P<0.001$) and then it was

returned to the base up to 5 hrs.

The Figure 6 shows the effects of the drugs on decreasing ROS production at 27 mg/ml of glucose after 2 hrs. The ROS production in high glucose (HG) condition was increased compared to NG condition ($P<0.001$). Treatment by quercetin (rutin) as an antioxidant decreased the ROS production ($P<0.001$). After 3hs pretreatment and then 2hs treatment with the drugs in high glucose medium (27 mg/ml), tel/PD ($P<0.001$), telmisartan ($P<0.001$), los/PD ($P<0.01$), captopril ($P<0.01$) and losartan ($P<0.05$), all treatments reduced ROS production as amount of 69, 66, 40, 34 and 32%, respectively. Telmisartan in comparison with losartan had a better effect in lowering ROS which statistically significant (Figures 6, 7, $P<0.01$).
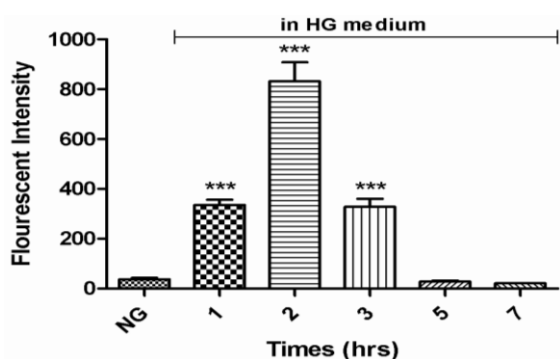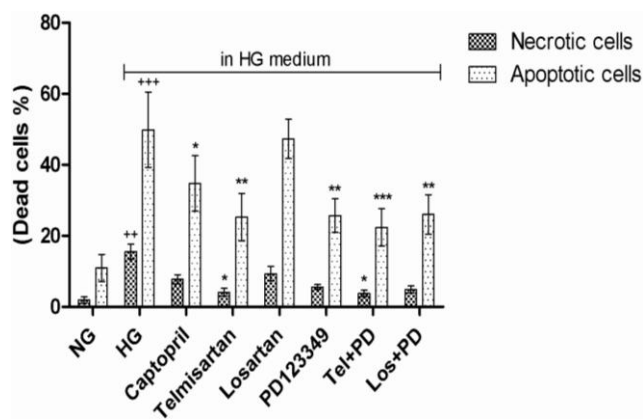

Figure 5. Flow cytometric results of DCF-DA staining for measuring ROS production in PC12 cells. Intracellular ROS production was measured according to changes in the fluorescence intensity of DCF, the oxidized derivative of DCF-DA. Alterations in ROS production was assessed after exposure to 27 mg/ml glucose in different times (hrs)
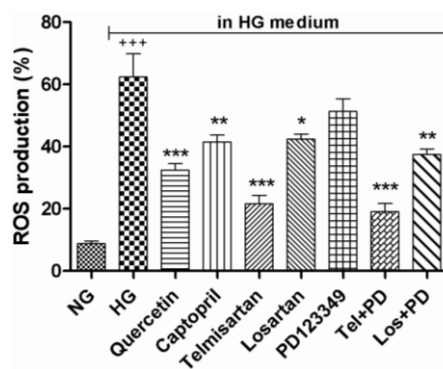*$P<0.05$, **$P<0.01$ and *** $P<0.001$ *vs* normal glucose (NG)

Figure 6. The effect of the drugs and quercetin on high glucose induced ROS production in PC12 cells. The cells pretreated with drugs for 3 hrs before exposure to 27 mg/ml of glucose with a treatment for 2 hrs. The concentrations of all drugs were 10 µM. Intracellular ROS production was measured according to changes in the fluorescence intensity of DCF, the oxidized derivative of DCF-DA. The values represent five independent experiments
*$P<0.05$, **$P<0.01$ and *** $P<0.001$ *vs* high glucose (HG) and +++$P<0.001$ *vs* normal glucose (NG)
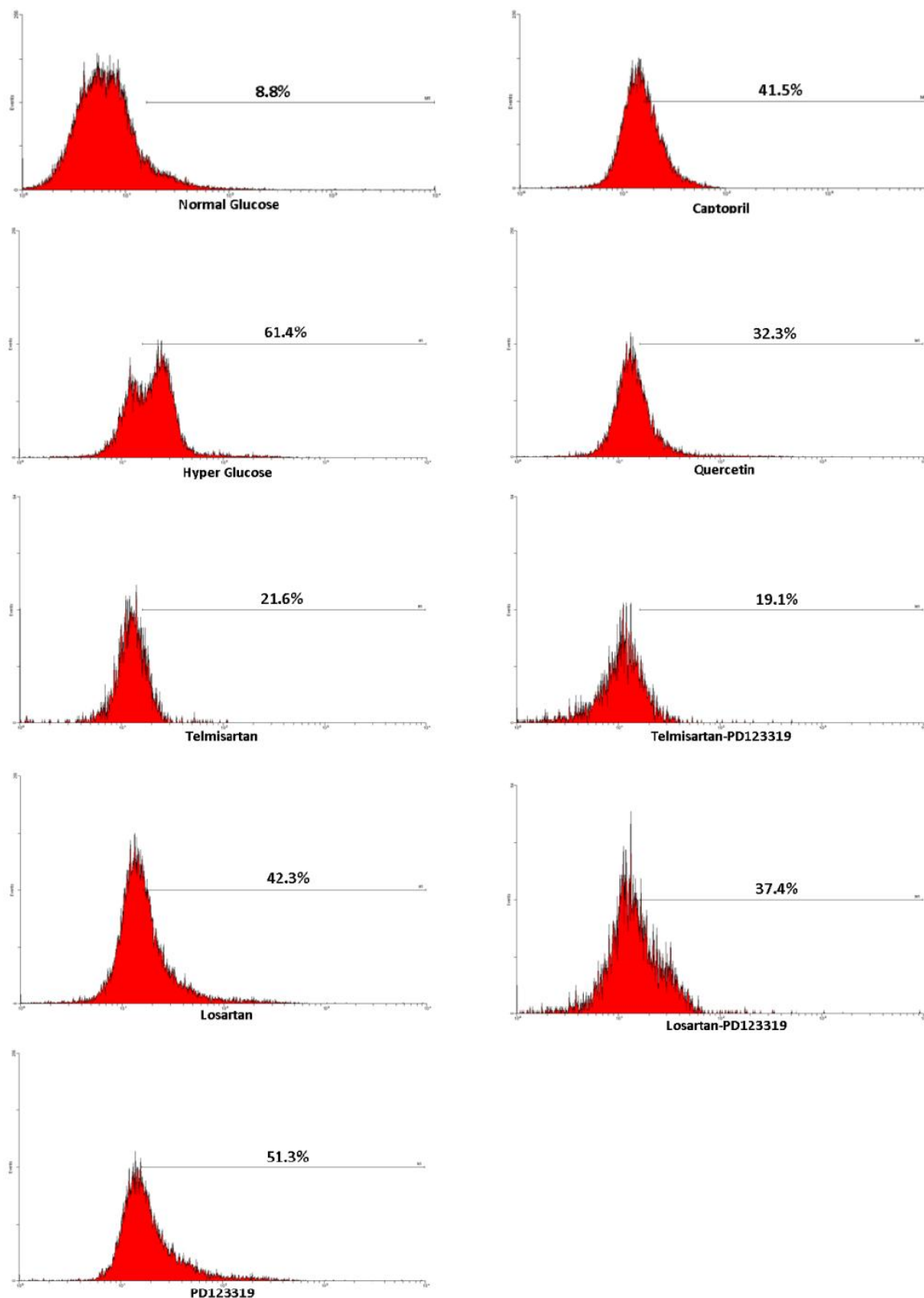
Figure 7. Flow cytometry histograms of DCFH-DA staining for measuring ROS after 2 hr exposure to 27 mg/ml of glucose plus drugs. The cells were pretreated with drugs for 3 hr. The concentrations of all drugs were 10 μM. Intracellular ROS production was measured according to changes in the fluorescence intensity of DCF, the oxidized derivative of DCF-DA. The values represent five independent experiments

Discussion

The results of present study showed that high levels of glucose had both time and concentration dependent toxicity effects in PC12 cells. The cytotoxic effects of glucose were prevented by RAS inhibitors. The results also showed that the cytotoxic effects of glucose were accompanied with increasing of ROS production, the later was also attenuated by RAS inhibitor drugs.

Hyperglycemia clearly plays an important role in the development and progression of diabetic neuropathy which characterized by an increasing loss of nerve fiber functions (1, 2). PC 12 cells or pheochromocytoma cells have neural crest origin and thus frequently have been used as a cell model for studying of high glucose toxicity; the condition which mimics the effects of hyperglycemia to induce neuropathy (2, 3, 6, 12, 18). Despite a long time needed for diabetic neuropathy to occur in human, diabetic neuropathy symptoms appear in STZ-induced diabetic rats just after 8-10 weeks (38, 39); also, direct exposure of high glucose concentrations to susceptible cells such as PC12 cells can accelerate this deleterious process. It has been well-documented in PC12 cells that 4-7 days exposure to high glucose concentrations could decrease cell viability significantly; thereby, this high glucose cytotoxicity is considered as an acceptable model to simulate diabetic neuropathy in many publications (2, 3, 6, 12, 18). It has been well documented that hyperglycemia in diabetes mellitus as well as high glucose toxicity lead to neuronal damage, apoptosis and cell death (1, 2). The results of the present study also showed that the cell viability in high glucose concentrations was time and concentration dependently lower than normal concentration of glucose. The flow cytometry results of annexin V-FITC and propidium iodide staining also showed that apoptotic PC12 cells were increased in high glucose concentrations.

Several mechanisms including mitochondrial-dependent and -independent mechanisms are involved in diabetic neuropathy (40). It was shown that mitogen-activated protein kinases (MAPK) and p53-mediated pathways have important roles in apoptosis and cell death due to hyperglycemia (40). An increased formation of advanced glycation end products and activation of protein kinase C (PKC) through increased diacylglycerol production have been reported to contribute in high glucose toxicity (41, 42). Enhanced polyol pathway flux, and increased hexosamine pathway flux have also been suggested to be involved in neuronal damage due to high glucose levels (40-42). It has also been well documented that oxidative stress, directly, or, as a second messenger, is one of the main mechanism involved in development of diabetic neuropathy(2,13, 43).

The results of the present study added another

evidence to confirm that free radicals have an important role in neural damage and cell death as well as apoptosis in hyperglycemia. These results revealed that high glucose concentration increased the ROS production. It has been previously reported that hyperglycemia promotes oxidative stress through cell-membrane enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and mitochondrial pathways in diabetic neurons resulting in neuronal damage and dysfunction (8, 17). It has also been reported that hyperglycemia can induce apoptosis in neural cell such as PC12 cells by increasing production of reactive oxygen species such as hydrogen peroxide (3,6, 12).

In addition to classic renin angiotensin system that is well known for its hemodynamic and cardiovascular functions, the presence of local renin angiotensin systems and their physiological roles within different tissues and organs including nervous system have been reported (5, 19, 22-28). It has been well documented that local RASs are involved in several cell processes such as growth, differentiation, and even apoptosis at cellular levels (9, 22). It was suggested that the local RAS contributes to virtually all biochemical events including ROS production and apoptosis which are triggered by hyperglycemia (9,24, 26). In the present study, the role of RAS in cell death and apoptosis due to hyperglycemia was evaluated. Our finding showed that all RAS inhibitors including captopril, telmisartan and PD123319 increased the cell viability in high glucose toxicity. All these drugs also reduced the apoptotic cells.

The neuroprotective effects of ACE inhibitor as well as the angiotensin receptor antagonists (11,30, 44) confirm the results observed in the present study. These drugs have been shown to treat diabetic neuropathy mostly with vascular aspect (30). Coppy and his colleagues showed that administration of enalapril, as an ACE inhibitor, or L-158809, as an angiotensin II receptor blocker, reduced neurovascular deficits such as blood flow and motor nerve conduction velocity in diabetic conditions (11). Malik *et al* assessed the effect of trandolapril, an ACE blocker, on human diabetic neuropathy and found a significant increase in peroneal motor nerve conduction velocity, M-wave amplitude and sural nerve action potential amplitude; also a significant decrease in F-wave latency (44).

Several mechanisms have been suggested for the protective effects of RAS inhibitors against hyperglycemic conditions (30,45, 46). It has been suggested that, not only hyperglycemia, but also Ang II is an inducer of oxidative stress (9, 31). It has also been reported that oxidative stress can in turn induce expression of Ang II and its receptors, in a positive feedback manner (9, 47). In fact, ROS as a second messenger may serve as a link between RAS, hyperglycemia and apoptosis in pathophysiological events of diabetic neuropathy. Reduction of ROS

production by RAS inhibitors which were seen in the present study confirmed this idea.

On the other hand, the insignificant effects of losartan on improving cell viability and reducing apoptosis which was observed here might be attributed to the presence of a limited number of AT_1 receptors on PC12 cells (36).

The results of ROS production showed a decrease in high glucose-induced ROS formation after treating by all drugs except PD123319. Several experiments have suggested a receptor-independent intracellular radical scavenging activity for ACE inhibitors and AT_1 receptor blockers (48). According to some studies, the AT_1 receptors are absent in PC12 cells (37, 49). Thus, such AT_1 independent antioxidant activity was observed in this study at least in case of losartan is in agreement with above reports; although, it needs further investigation in future. In addition to its AT_1 blocker activity, telmisartan also serve as partial agonist of peroxisome proliferator-activated receptor gamma (PPAR-γ) (50, 51). Furthermore, the effectiveness of telmisartan seems to be through activation of different receptors, such as PPAR-γ, which has been suggested as a possible mechanism for its neuroprotective effects (52, 53). It has been previously reported that the anti-inflammatory and antioxidant activities of telmisartan is mainly mediated activating of the PPAR-γ receptor (50,52,53). Thiazolidinediones as PPAR-γ agonists for many years have been used in diabetic treatments (54, 55). These drugs were even proposed as neuroprotective factors in several conditions such as ischemia and glucose cytotoxicity (56, 57). The presence of PPAR-γ receptor on PC12 cells (58) and the stronger free radical scavenging effects of telmisartan in comparison with losartan which is seen in the present study might be another evidence for the effects of telmisartan by activating of PPAR-γ receptors.

Overall, the results of the present study showed that telmisartan, PD123319 and captopril antagonized high glucose-induced cell death and apoptosis but only telmisartan and captopril performed these effects through reducing ROS-induced cell damage.

Conclusion

The results of the present study showed that telmisartan, PD123319 and captopril antagonized high glucose-induced cell death and apoptosis. It was also shown that losartan, telmisartan and captopril reduced ROS production. So, it seems that AT_2 receptor is important in neuroprotection against high glucose toxicity by a mechanism other than protection against oxidative stress. As previously has been reported, NADPH oxidase has a pivotal role in Ang II-induced ROS production (13); this mechanism and/or other mechanism(s) may contribute in ROS generation. It is also suggested that both losartan and telmisartan have a non AT_1 receptor mediated antioxidant effects. The better antioxidant and cell protective effects of telmisartan observed in the present study are suggested to be through other pathways such as PPAR-γ receptors.

Acknowledgment

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