Protective effect of telmisartan against oxidative damage induced by high glucose in neuronal PC12 cell

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HIGHLIGHTS

• We investigated the effects of telmisartan on high glucose-induced oxidative damage in PC12 cells.
• Telmisartan counteracts high glucose-triggered oxidative damage and cell death in neuronal PC12 cells.
• The antioxidant action of telmisartan and its inhibitory effect on NADPH oxidase may, at least in part, have mediated the inhibition of cell death.

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ABSTRACT

Telmisartan is an angiotensin II type 1 receptor blocker and partial agonist of peroxisome proliferator-activated receptor gamma (PPAR-γ). Here, we investigated the protective capacity of telmisartan against high glucose (HG)-elicited oxidative damage in PC12 cells. The activity of lactate dehydrogenase (LDH), NADPH oxidase (NOX), superoxide dismutase (SOD), catalase (CAT) as well as the levels of malondialdehyde (MDA), glutathione (GSH), intracellular reactive oxygen species (ROS), cell viability and DNA fragmentation were measured in HG-treated PC12 cells with and without telmisartan co-treatment. Moreover, the direct antioxidant effect of telmisartan was determined by 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay and protein expression of Bax, Bcl-2, cleaved caspase-3 and NOX subunit p47phox by western blotting. Telmisartan exhibited antioxidant activity in the ABTS assay with the IC50 value of 37.5 μM. Pretreatment of PC12 cells with telmisartan, prior to HG exposure, was associated with a marked diminution in cleaved caspase-3 expression, DNA fragmentation, Bax/Bcl-2 ratio, intracellular ROS and MDA levels. Additionally, the cell viability, GSH level, SOD and CAT activity were notably elevated by telmisartan, whereas the activity and the protein expression of NADPH oxidase subunit p47phox were attenuated. Interestingly, co-treatment with GW9662, a PPAR-γ antagonist, partially inhibited the beneficial effects of telmisartan. These findings suggest that telmisartan has protective effects on HG-induced neurotoxicity in PC12 cells, which may be related to its antioxidant action and inhibition of NADPH oxidase. Furthermore, the results show that PPAR-γ activation is involved in the neuroprotective effects of telmisartan.

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1. Introduction

There is a large body of evidence highlighting that chronic high glucose (HG) condition leads to neuronal cell oxidative damage via NADPH oxidase-dependent generation of ROS [12,13]. ROS can attack proteins, lipid membranes and nucleic acids, causing oxidative injury to cells [20]. As well, excessive formation of ROS by HG increases apoptosis, a possible mechanism of glucose neurotoxicity [25,26].

High-glucose milieu in diabetes not only generates more ROS but also attenuates anti-oxidant protective mechanisms through glycation of the antioxidant enzymes [12,20]. It is now accepted that an effective approach to prevent and treatment of diabetic complications such as neuropathy must focus on both early glycemic control and reducing factors related to oxidative stress [2]. Currently, no selective and effective therapeutic agent has been identified for clinical use to prevent or treat HG-induced neuronal cell injury and dysfunction [9]. Therefore, it is crucial to explore tools by which one can reduce oxidative stress in neurons to ameliorate the impaired neuronal function produced by hyperglycemia.
Telmisartan has been widely used as a highly selective angiotensin II type 1 (AT₁) receptor (AT₁R) blocker (ARB) for the treatment of hypertension. Beyond its antagonistic activity at AT₁ receptors, it has additional favorable metabolic effect [4,30]. Recent studies demonstrated that telmisartan provided a significant antidiabetic effect and ameliorated hyperglycemia in rats with streptozotocin-induced or spontaneous diabetes [7,11,15]. The neuroprotective effect of telmisartan has been observed against many types of damage stimuli [10]. It is also recognized to attenuate oxidative stress in human umbilical vein endothelial cell (HUVEC) and neuronal cultures [3,22]. However, whether telmisartan has a neuroprotective role in high glucose condition has not been elucidated. To the best of our knowledge, this is the first study to investigate the protective effect of telmisartan in neuronal PC12 cells.

The PC12 cell line has been extensively used as a cell model to study glucose neurotoxicity [25]. Previous studies in our laboratory have demonstrated the cytotoxicity of high glucose in PC12 cells [25,26]. The present study was designed to examine the protective effect of telmisartan on PC12 cells maintained under HG concentration. We investigated whether the neuroprotective effects of telmisartan are related to its antioxidant properties using an ABTS radical cation-scavenging assay. The alterations of antioxidant defenses (GSH level, SOD and CAT activities), intracellular ROS level, NADPH oxidase activation, MDA level, cell viability, and apoptotic neuronal cell death induced by high glucose were determined. We also used the PPAR-γ antagonist GW9662 to examine the possible involvement of PPAR-γ activation in the protective effect of telmisartan.

2. Methods

2.1. Cell culture and drug treatment

PC12 cells were cultured as described previously [25]. After cultured in serum-free medium for 24 h, the cells were pre-incubated with 0.1–50 µM of telmisartan (Sigma, T8949) for 2 h and then co-treated with telmisartan and high glucose (75 mM) for 72 h. In experiments where GW9662 (Sigma, M6191) was used together with telmisartan, cells were pre-treated with GW9662 (20 µM) for 1 h before the addition of telmisartan. Control cells were cultured with normal glucose (23 mM), and not treated with telmisartan. Mannitol (75 mM) was also used as negative control for osmolarity. Telmisartan and GW9662 were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in experimental conditions was 0.1%. The selected concentrations of telmisartan and GW9662 were according to the previous study [23].

2.2. Determination of cell viability, LDH release and intracellular ROS level

Cell viability was measured using MTT assay as previously described [18,25]. The LDH activity was quantified using a LDH diagnostic kit (Stanbio Laboratory, USA). In brief, at the end of the drug treatment, the medium was collected and 100 µL of it was added to 1 mL of LDH reagent. The absorbance was recorded at 340 nm. The production of intracellular ROS was measured by using 2,7-dichlorofluorescein diacetate (DCFH-DА) [29]. After treatment, cells were washed and incubated with DCFH-DА for 20 min. Then the relative levels of fluorescence were quantified by a fluorescence microplate reader.

2.3. MDA, GSH, SOD, and CAT assay

After drug treatment, the cells were washed and homogenized. The homogenate was centrifuged and the protein content of supernatant was measured using the Bradford method [1]. The CAT activity was quantified according to the method of Shen et al. [27]. Briefly, 1.0 mL of hydrogen peroxide (19 mM) and 50 µL of the supernatant were mixed with 1.95 mL of phosphate buffer (0.05 M, pH 7.0). The CAT activity was then estimated from the decrease in absorbency at 240 nm for 2 min. The SOD activity was measured using a SOD activity assay kit (BioVision, Mountain View, CA). Briefly, 20 µL of the supernatant was added to 200 µL tetrazolium and 20 µL of enzyme solution. After incubating at 37 °C for 20 min, the absorbance was read at 450 nm. The GSH level was measured according to the method of Gulati et al. [8]. Briefly, 100 µL of the supernatant was mixed with phosphate buffer and its absorbance measured at 412 nm. MDA content was measured as previously described [21]. Briefly, 100 µL of the supernatant was mixed with 1.5 mL of acetic acid (20%), 1.5 mL of thiobarbituric acid (0.8%), and 200 µL of sodium dodecyl sulphate (8%). Each reaction mixture was heated for 60 min at 95 °C and cooled to room temperature. Then, 5 mL of n-butanol was added. After mixing and centrifugation at 3000 × g for 10 min, the organic layer was collected and the absorbance measured at 532 nm.

2.4. ABTS radical cation-scavenging assay

The antioxidant activity of telmisartan was determined by the method of Ghanta et al. [6] and expressed as IC₅₀, which was defined as the concentration of test material required to cause a 50% decrease in initial ABTS radical cation concentration. Briefly, 10 µL of telmisartan (0.1–70 µM) was mixed with 990 µL of ABTS solution. The absorbance at 734 nm was recorded. Trolox was used as the standard.

2.5. Quantification of DNA fragmentation

To examine DNA fragmentation, we used Cell Death Detection ELISAPlus kit (Roche Applied Sciences, Germany) and performed assay according to the manufacturer’s instructions, except that cells were cultured in 96-well plates at a density of 2 × 10⁴ cells/well for the ELISA analysis.

2.6. Western blot analysis

Western blot analysis was performed using whole-cell lysate from PC12 cells as previously described [25]. Briefly, proteins samples were separated by SDS-PAGE and transferred onto a PVDF membrane which was incubated with primary antibodies including NADPH oxidase p47phox subunit (Santa Cruz, sc-14015), Bcl-2 (BioVision, 3033-100), Bax (BioVision, 3032-100), caspase-3 (BioVision, 3015-100) and secondary antibody (BioVision, 6401-05). Bands were visualized using ECL detection reagents. The resultant bands were quantified by densitometric analysis using the Image master program and normalized against β-actin protein.

2.7. Estimation of NADPH oxidase activity in PC12 cells

The activity of NADPH oxidase was quantified using lucigenin-enhanced chemiluminescence with a commercial kit (Genmed Sciences Inc.) according to manufacturer’s protocols.

2.8. Statistical analysis

Data were presented as mean ± S.E.M, and were analyzed by ANOVA followed by post hoc Dunnett’s t-test. The criterion for significance was a P value < 0.05.
3. Result

3.1. Effect of telmisartan on HG-induced cytotoxicity as measured by cell viability and LDH release

HG significantly reduced cell viability and increased LDH release compared to control cells (P < 0.01). However, incubation of cells with telmisartan (0.1–50 μM) concentration-dependently reversed HG-induced cell death (Fig. 1A) and remarkably blocked LDH leakage (Fig. 1B). Mannitol (75 mM), which was used to create a high osmotic pressure mimicking HG condition, did not show significant change in cell viability. This result indicated that the effect of high glucose on proliferation was not secondary to osmotic load. Co-treatment with GW9662 (20 μM) partially prevented the protective effects of telmisartan in MTT assay. Under the condition of normal glucose (23 mM d-glucose), DMSO (0.1%), GW9662 (20 μM) or telmisartan (50 μM) itself had no significant effect on the PC12 cells viability and also on none of the other following measurements in 3–8 experiments (data not shown).

3.2. Effects of telmisartan on MDA, GSH, SOD, CAT and ROS

The GSH level, SOD activity, and CAT activity significantly decreased after exposure of PC12 cells to HG (*P< 0.05 vs. control, Table 1). However, intracellular MDA (Table 1) and ROS (Fig. 1C) were significantly raised. Following drug treatment, the MDA and ROS level significantly decreased (*P< 0.05), whereas CAT, SOD activity and the GSH level significantly increased (*P< 0.05) compared with HG-treated PC12 cells.

3.3. ABTS radical cation-scavenging capacity of telmisartan

As shown in Fig. 2, telmisartan at different concentrations (0.1–70 μM) was found to effectively scavenge ABTS radical cations (2.3–100% inhibition), with an average IC50 value of 37.5 μM. IC50 value for Trolox, used as the reference compound, was 21.3 μM.

3.4. Effect of telmisartan on the DNA fragmentation

When PC12 cells were exposed to HG, there was a significant increase in DNA fragmentation vs. untreated controls (Fig. 1D),
which was efficiently prevented by telmisartan (0.1–50 μM). However, this effect of telmisartan was antagonized by GW9662.

3.5. Expression of NADPH oxidase p47phox subunit, Bcl-2, Bax and caspase-3 proteins

Treating PC12 cells with HG led to a significant increase of the Bax/Bcl-2 ratio (Fig. 3A) and cleaved caspase-3 expression (Fig. 3D) as compared with the control group. Pre- and co-treatment with 10 μM telmisartan decreased significantly the Bax/Bcl-2 ratio and caspase-3 expression, as compared with HG group. GW9662 reduced this effect of telmisartan (Fig. 3D). As shown in Fig. 3B and C, high glucose significantly up-regulated p47phox expression as well as NADPH oxidase activity in PC12 cells. Pre-incubation of PC12 cells with telmisartan attenuated these changes.

4. Discussion

This study found that telmisartan can exert a protective effect against ROS-mediated oxidative damage and apoptosis induced by high glucose in neuronal PC12 cells. Glucose induction of ROS is critical to the pathogenesis of diabetic neuropathy [20]. In addition, Hydroxyl radical is one of the highly ROS that causes the oxidative damage associated with diabetes [16]. Our results revealed that glucose enhanced ROS concentration in PC12 cells while simultaneously treating with telmisartan and glucose effectively

Table 1

<table>
<thead>
<tr>
<th>% of Control</th>
<th>MDA</th>
<th>GSH</th>
<th>SOD</th>
<th>CAT</th>
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<tr>
<td>Control</td>
<td>100 ± 7.4</td>
<td>100 ± 8.1</td>
<td>100 ± 6.8</td>
<td>100 ± 4.6</td>
</tr>
<tr>
<td>HG (100 mM)</td>
<td>159.3 ± 9.1*</td>
<td>47.5 ± 3.5*</td>
<td>43.6 ± 4.4*</td>
<td>55.2 ± 3.7*</td>
</tr>
<tr>
<td>HG + TL (0.1 μM)</td>
<td>152.7 ± 8.9</td>
<td>53.6 ± 3.4</td>
<td>47.9 ± 3.8</td>
<td>59.9 ± 4.4</td>
</tr>
<tr>
<td>HG + TL (1 μM)</td>
<td>138.4 ± 6.5</td>
<td>63.9 ± 4.3</td>
<td>58.7 ± 3.5</td>
<td>64.5 ± 4.7</td>
</tr>
<tr>
<td>HG + TL (10 μM)</td>
<td>126.5 ± 6.9</td>
<td>74.2 ± 4.2</td>
<td>68.3 ± 3.6</td>
<td>71.1 ± 4.9</td>
</tr>
<tr>
<td>HG + TL (50 μM)</td>
<td>119.4 ± 5.7</td>
<td>79.6 ± 6.1</td>
<td>76.1 ± 5.1</td>
<td>75.4 ± 6.8</td>
</tr>
</tbody>
</table>

*Cultured PC12 cells were exposed to 75 mM glucose (HG). Telmisartan (TL) was added 2 h prior to glucose addition and the levels of antioxidant enzymes, MDA and GSH were measured 72 h later. Data are means ± SEM, n = 3–8.

* P < 0.05 vs. control.

† P < 0.05 vs. HG.

Fig. 3. Effects of telmisartan (TL) on the ratio of protein expression of Bax/Bcl-2 (A), upregulation of NADPH oxidase p47phox subunit protein expression (B), NADPH oxidase activity (C) and the expression of caspase-3 (D) in PC12 cells exposed to HG in the presence or absence of telmisartan (10 μM) or 20 μM GW9662 (GW) for 72 h. Data are mean ± SEM, n = 3. ** P < 0.01 as compared to HG group. † P < 0.05 compared to telmisartan (10 μM) treatment.
attenuated ROS generation. This finding corroborates with previous studies which have shown that telmisartan acted as an efficient scavenger of ROS particularly hydroxyl radicals [3].

To evaluate the antioxidant capacity of telmisartan, the ABTS cation radical-scavenging assay, was employed [6]. The results clearly showed that telmisartan displays antioxidant properties, which is consistent with previous studies that have demonstrated an excellent antioxidant activity for telmisartan [3]. Moreover, it has been suggested that telmisartan, an AT1 receptor antagonist, inhibits intracellular oxidative stress, at least in part, in a receptor-independent manner, possibly owing to its lipophilic and antioxidant structure [24]. The chemical structure of telmisartan contains the benzimidazolico and benzoic groups that probably confer selective scavenging properties for hydroxyl radicals [3]. On the other hand, some studies have previously shown that PC12 cells derived from rat pheochromocytoma predominantly express AT2 receptor [14,19]. Based on these observations, it is very likely that in PC12 cells, the neuroprotective effects of telmisartan are independent of AT1 receptor inhibition.

In the present study, we also found that NADPH–oxidase activity and protein expression of p47phox was significantly higher in GTreated than control PC12 cells. p47phox is a cytosolic subunit of NOX and several reports support its importance in diabetes. For instance, it has been shown that deletion of p47 (phox) attenuates diabetes–induced glomerular injury and beta cell dysfunction [17]. Furthermore, superoxide production and neuronal death were also blocked in studies using mice or cultured neurons deficient in the p47phox subunit of NADPH oxidase [28]. Our results demonstrated that telmisartan pre- and co-treatment suppressed the upregulated p47phox, and NADPH oxidase activity. In agreement with our present findings, previous observations have also shown that ARBs decrease NADPH oxidase activation associated with oxidative stress and neuronal apoptosis [23].

ROS also is a well-known initiator of apoptosis in many cell types [20]. In this study DNA fragmentation assay, a hallmark of apoptosis, Bax/Bcl-2 index and cleaved caspase-3 expression were used for measurements of apoptotic cell death. Telmisartan prevented HG-induced increase in caspase-3 expression, DNA fragmentation in PC12 cells and decreased the ratio of Bax/Bcl-2. This ratio is supposed to dictate the relative sensitivity or resistance of cells to a wide variety of apoptotic stimuli [26]. Interestingly, telmisartan has the unique character of having both ARB and PPAR-γ agonistic effect [10], and Fuenzalida et al. reported that PPARγ up-regulates Bcl-2 in neurons [5]. Also, Tamami Haraguchi et al. suggested that telmisartan reduces neuronal apoptosis via a PPARγ-dependent caspase-3 inhibiting mechanism [10]. We used the PPAR-γ antagonist GW9662 to examine the possible involvement of PPAR-γ activation in the protective effect of telmisartan. Results demonstrated that the beneficial effects of telmisartan were weakened by co-treatment with GW9662. Previous reports are in line with our current data [5,10], which indicate that telmisartan diminishes HG-elicited apoptosis in neuronal PC12 cells, possibly, via down-regulation of Bax/Bcl-2 and caspase-3 expression.

To mitigate cumulative burden of oxidative stress, cells generally utilize antioxidant defense systems and scavenge ROS. SOD and CAT are two important antioxidant enzymes [20]. In this regard, our present study indicated that PC12 cells treated with high glucose showed a marked rise in oxidative stress as evidenced by excessive ROS and MDA production, together with depletion of “endogenous antioxidant reserve,” including GSH contents, SOD and CAT activity level. However, co-treatment with telmisartan significantly attenuated oxidative damage to PC12 under high glucose condition, as reflected by the augmentation of antioxidant defense system (CAT, SOD and GSH) with accompanying decrease in MDA and ROS levels. These results confirm the in vitro antioxidant activity of telmisartan.

In summary, our results indicate that telmisartan plays a protective role against HG-induced cell death in PC12 cells in a dose-dependent manner. This is possibly accomplished through diminution of NADPH oxidase activation and ROS formation in parallel with the increases in the GSH level, SOD and CAT activity. Furthermore, the results show that PPAR-γ activation is involved in the neuroprotective effects of telmisartan.

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References


