ORIGINAL RESEARCH

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Telmisartan-induced eNOS gene expression is partially independent of its PPAR-gamma agonist property

Abstract

Purpose: Telmisartan, an angiotensin II receptor blocker (ARB), also acts as an activator of peroxisome proliferator-activated receptor-gamma (PPAR-gamma; PPAR- γ). Several studies have explored the PPAR- γ -endothelial nitric oxide synthase (eNOS) pathway associated with improvement of endothelial function by telmisartan. The ability of telmisartan to induce gene expression and protein level of eNOS and PPAR γ in adipocytes was investigated.

Methods: Expression of aP2, PPAR γ , eNOS and iNOS genes were measured using the quantitative real-time polymerase chain reaction. The changes, at the protein level, were explored by Western blot, which evaluated the native and phosphorylated eNOS forms, eNOS-Ser¹¹⁷⁷ and eNOS-Thr⁴⁹⁵.

Results: Adipocytes, exposed to telmisartan, exhibited an increase in PPAR γ gene expression but a decrease in protein level. Nonetheless, after the exposure to telmisartan, eNOS-Ser¹¹⁷⁷ phosphorylation, associated with eNOS activity increment, reached its highest value while eNOS-Thr⁴⁹⁵ phosphorylation, involved in the inhibition of eNOS activity, showed its lowest value.

Conclusion: The results suggest that telmisartan preserves eNOS activity via a mechanism that is partially independent of the PPAR γ -eNOS pathway in adipocytes.

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Age-related chronic pathologies such as atherosclerosis, diabetes and chronic kidney diseases are linked to oxidative stress [1,2]. Oxidative stress alters many endothelium functions associated with traditional risk factors, triggering early vascular inflammation and a predisposition to atherosclerosis. The anti-hypertensive drugs currently in use were designed to decrease blood pressure, but not to cope with disorders in the metabolism of carbohydrates and lipids. Combinations of these disorders are usually found in hypertensive patients, which increase the risk of these patients developing cardiovascular disease and diabetes. Nonetheless, some of these drugs, such as angiotensin type 1 receptor (AT1R) blockers (ARBs), have been linked to a decrease in oxidative stress [3] and dyslipidemia as well as to a decrease in insulin resistance. Several clinical studies report that ARBs, such as telmisartan, prevent the onset of type 2 diabetes mellitus [4], can prevent weight gain and obesity and can be beneficial in obesity-hypertension treatment [5].

Adipose tissue plays an important role in regulating body metabolism [6]. The identification of regulators of adipogenesis raises the prospect of preventing or reversing obesity and its secondary effects, including oxidative stress or insulin resistance, through pharmacological means. Increased oxidative stress causes insulin resistance by reducing insulin-stimulated glucose transport [7,8].

Endothelial nitric oxide synthase (eNOS) is activated through shear stress and diverse cellular events such as increased intracellular calcium or interactions with substrate and co-factors. eNOS regulation involves multiple levels of transcriptional, translational and post-translational regulation [1,9]. The latter controls lipid modifications, phosphorylation events and interactions with protein partners such as calmodulin, caveolin-3 and AT1 receptors [10].

eNOS activity is increased by phosphorylation of serine (Ser) at residues 1177, 633 and 615. Ser¹¹⁷⁷ constitutes the most important eNOS phosphorylation regulation site and is associated with eNOS activity in response to numerous stimuli. A number of kinases are implicated in Ser¹¹⁷⁷ phosphorylation [11], turning the eNOS synthesis regulation into a complex process. Factors such as blood flow-induced phosphatidylinositol 3-kinase, Akt-activation and eNOS-Ser¹¹⁷⁷ phosphorylation cascade result in reduced calcium dependence and a sustained release of endothelial nitric oxide (NO). In contrast, the phosphorylation of threonine at residue 495 (Thr⁴⁹⁵) acts as a negative regulator for eNOS activity [12,13]. Agonists of NO synthesis induce dephosphorylation of Thr⁴⁹⁵ in the Ca²⁺/Calmodulin binding domain, increasing eNOS activity. In contrast, it has been shown that Protein Kinase C (PKC) phosphorylates eNOS-Thr⁴⁹⁵, although the specific isoform of PKC

responsible is unknown [13]. In addition, experiments with mutants Thr⁴⁹⁵Ala, mimicking Thr⁴⁹⁵ dephosphorylation, show the importance of Thr⁴⁹⁵ cannot be explained by regulating the calmodulin binding domain [14].

In this study, we evaluated the telmisartan-derived antioxidant properties in 3T3-L1 cells differentiated to adipocytes, and whether these properties were related to differences in eNOS gene expression or protein phosphorylation. The relationship between these properties and PPAR- γ activity were also evaluated.

Materials and Methods

Adipocyte differentiation assay

Mouse 3T3-L1 pre-adipocytes were maintained in DMEM supplemented with 10% heat-inactivated FBS and antibiotics in a 5% CO₂ atmosphere at 37°C. Sub-confluent cells cultured in 60 mm dishes were maintained in DMEM medium or differentiation medium using a standard differentiation mixture as previously described [15, 16] (1 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10 μ g/ml insulin and 20% FBS). The first pre-adipocyte group was exposed to vehicle (0.01% DMSO) and constituted the non-differentiated control group ("control"). The second pre-adipocytes group was differentiated to adipocytes and exposed to vehicle, and is considered as a control for differentiation and maturation ("differentiated"). The third and fourth groups of pre-adipocytes were differentiated to mature adipocytes, switched to normal medium and exposed to telmisartan. Time-response experiments were done exposing cells to telmisartan (5 $\cdot$ 10⁻⁶ mol/L) [17] or vehicle (DMSO) for six or 12 hours.

Quantization of intracytoplasmic lipids

Cells were fixed by the addition of 10% formalin. After at least 5 min at RT, the fixation solution was replaced by 60% isopropyl alcohol and then cells were stained with Oil Red solution (Sigma O-0625) [18]. The extracted dye was spectrophotometrically quantified at 520 nm. Lipid accumulation was compared between cells exposed or not to telmisartan.

Reactive Oxygen Species (ROS) production in adipocytes

ROS production was measured by the lucigenin-derived chemiluminescence (LDCL) method. Lucigenin is widely used as a chemiluminescent detector of the superoxide anion radical *in vitro* enzymatic systems and intact cells [19]. Confluent cells in 60 mm dishes were harvested in trypsin, centrifuged and resuspended in 900 μ l Krebs' buffer, and 250 μ M lucigenin was

added. The reaction was monitored with Berthold Sirius luminometer over the course of 10 min.

Determination of nitrite/nitrate

Nitrite and nitrate concentrations were determined in cell culture supernatants using the NO quantization kit (Promega). First, the samples were deproteinized; culture medium samples (400 μ l) were mixed with 800 μ l of trichloroacetic acid (TCA) and centrifuged at 8000 rpm for 20 minutes at 4°C. The supernatant was used for determination of nitrite and all measurements were done in triplicate. A nitrite standard reference curve was prepared with nitrite stock solution and distilled water. Aliquots (50 μ l) of each experimental sample were incubated with sulfanilamide for 10 minutes, before adding a NED (N-1-naphthylethylenediamine dihydrochloride) solution. After 10 minutes, the absorbance was measured at 540 nm.

Gene expression assays

Expression levels of PPAR γ , adipocyte protein (aP2), eNOS and the products of inducible Nitric Oxide (iNOS) genes were determined by real-time polymerase chain reaction (RT-PCR) amplification. Total RNA was isolated with PureZOL (Bio-Rad) and cDNA synthesized using iScript (Bio-Rad) according to manufacturer instructions. PCR reactions were performed

in a CFX 96 (BioRad) device. Samples were measured in triplicate and the $\Delta\Delta$ Ct method was used for quantification after normalizing the Ct of each sample to the Ct obtained for the amplification of a 223 bp fragment of the glyceraldehyde phosphate dehydrogenase (GAPDH) gene. Primer specificity was confirmed by melting point analysis for each primer pair as well as by using standard agarose electrophoresis.

Western blot

Cells grown under the described conditions were lysed in a buffer containing 6 M urea, 25% glycerol, 0.1% SDS, 0.5% sodium deoxycholic acid, 1% Nonidet P-40 substitute (Sigma), 150 mM NaCl, 50 mM Tris/HCl (pH 7.8) and a protease inhibitor mixture (Roche) [20]. The protein concentration of the lysate was determined using the Bradford method (BioRad). Then, 20 μ g samples of protein in Leamli buffer were denatured for 5 min at 95°C and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gels electrophoresis (PAGE) on 5-7.5% polyacrylamide gels. The transfer was carried out on Polyvinylidene fluoride (PVDF) membranes that were blocked with 5% non-fat milk overnight and incubated with the following primary antibodies: β -actin (sc47778), PPAR γ (sc-7196), eNOS native (ab51086), p-NOS3-Ser1177 (sc21871) and p-NOS3-Thr495 (sc19827) (Santa Cruz Biotechnology) for 1 h. After a wash, the membranes were incubated with the appropriate secondary antibody (sc2030 and sc2005) for 1 h. The protein bands were detected with an ECL system (Amersham Pharmacia Biotech). The membranes were stripped and re-hybridized with another antibody. Densitometric analyses of immunoblots were performed using the ImageJ free software (<http://rsb.info.nih.gov/ij/>). Results were normalized using β -actin as the control.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Statistical significance of differences between mean values was evaluated by using the Mann-Whitney test. Two-sided P values <0.05 were considered to indicate statistical significance.

Results

Telmisartan induces slightly aP2 gene expression

To evaluate 3T3-L1 cells differentiation, the expression of aP2 was measured [21,22]. The relative expression of aP2 gene was significantly higher in adipocytes than pre-adipocytes (Figure 1). When switched to telmisartan, a slightly but significant increase in aP2 relative gene expression was also observed.

FIGURE 1. aP2 gene expression and 3T3-L1 differentiation. Undifferentiated pre-adipocytes (control) and adipocytes (differentiated) differed with respect to aP2 relative gene expression (* $p = 0.0437$). Differentiated adipocytes treated with 6 or 12 h of telmisartan also showed a slightly but statistically significant increase in the expression of aP2 in comparison with the control group (** $p = 0.0289$, *** $p = 0.0128$)

FIGURE 2. ROS production measured by lucigenin derived chemiluminescence (LDCL). Measurements of ROS production in 3T3L1 undifferentiated preadipocytes (control), and adipocytes untreated or treated with telmisartan for six and twelve hours. A significant increase in ROS production was seen in differentiated cells in comparison with respect to preadipocytes was observed (* $p = 0.02$). Telmisartan exposure for 6 h and 12 h associated with a significant decrease in ROS production with respect to untreated adipocytes as measured by the lucigenin derived chemiluminescence assays ($n = 6$, ** $p = 0.0286$, *** $p = 0.0286$)

Telmisartan reduces ROS production in adipocyte cells

To assess the involvement of oxidative stress in adipocyte differentiation, ROS production was evaluated. ROS production was markedly increased during differentiation of 3T3-L1 cells into adipocytes. As depicted in Figure 2, once differentiated, switched to non-differentiation medium and treated with telmisartan at indicated time-points, a significant reduction in ROS production was observed ($p = 0.0286$).

Nitric oxide metabolites and telmisartan treatment

In order to assess oxidative stress in adipocyte cells exposed to telmisartan, the production of nitrates and nitrites in the culture cell supernatants were measured. The results showed no statistically significant differences between any of groups (Figure 3). Therefore, there was no clear correlation between NO metabolites production and either cell differentiation or treatment.

FIGURE 3. Nitric oxide metabolites measurement. Nitrate and nitrite concentrations in culture cell supernatants of adipocytes. No statistically significant differences were observed between groups.

Nitric oxide gene expression increases during differentiation process

Nitric oxide has been reported as an important mediator of adipocyte physiology with lipogenic properties [23]. For this reason, the relative expression of eNOS and iNOS genes between groups was measured. In cells exposed to telmisartan for 6 or 12 h, the relative expression of eNOS and iNOS were statistically diminished with respect to untreated adipocytes (* $p = 0.0078$ and ** $p = 0.0208$, respectively) (Figure 4). Consistent with previous findings [23], no significant differences in the eNOS and iNOS gene expression in treated cells with respect to pre-adipocytes were observed.

Effect of telmisartan on eNOS native and phosphorylated forms

Since the relationship among the mRNA transcription, protein translation and eNOS activity is far from a simple one, we then explored whether these subtle changes in eNOS gene expression correlated with native and phosphorylated eNOS protein forms. No differences in native eNOS protein expression between pre-adipocytes, adipocytes and adipocytes exposed to telmisartan were observed (Figure 5a). Thus, analysis of the eNOS expression data and the native protein argue

FIGURE 4. *eNOS and iNOS relative gene expression.* Pre-adipocytes (control), non-treated adipocytes (differentiated) and telmisartan-treated adipocytes were evaluated for the eNOS and iNOS relative gene expression. Values were normalized to a GAPDH fragment as described in text. Telmisartan treatment lead to a significant reduction in the eNOS (6 h, * $p = 0.0078$) and iNOS relative gene expression (12 h, ** $p = 0.0208$) with respect to untreated adipocytes.

for the role of other transcriptional regulators to explain the increased oxidative stress observed in adipocytes with respect to pre-adipocytes as well as the significant decrease that was observed in cells treated with telmisartan. To evaluate this, the phosphorylation status of serine and threonine at positions 1177 and 495, respectively, were measured. As depicted in Figure 5b, significant increases in the phosphorylation of Ser¹¹⁷⁷ residue in adipocytes exposed to telmisartan were seen at 6 h ($p = 0.0002$) and 12 h ($p = 0.0224$), in comparison with pre-adipocytes ("control"). Notably, eNOS-Ser¹¹⁷⁷ phosphorylation was significantly increased at 6 h in comparison with the untreated adipocytes group ("differentiated") ($p = 0.0119$) but the increase seen by 12 h was not significant. Interestingly, a different pattern was seen for Thr⁴⁹⁵, where the phosphorylation level was increased in untreated adipocytes in comparison with pre-adipocytes ($p = 0.0002$) and adipocytes exposed for 6 h ($p = 0.02$) (Figure 5c). In addition, a statistically significant difference was observed between pre-adipocytes and telmisartan-treated cells at both 6 h ($p = 0.0007$) and 12 h in comparison with pre-adipocytes ($p = 0.0003$). Finally, eNOS-Thr⁴⁹⁵ phosphorylation was significantly higher in cells exposed to telmisartan for 12 h in comparison with those exposed for only 6 h ($p = 0.03$).

Telmisartan preserves PPAR γ gene expression

To determine whether the antioxidant effects of telmisartan depend on PPAR γ , PPAR γ gene expression was compared in pre-adipocytes, untreated adipocytes and adipocyte treated

with telmisartan [17]. As depicted in Figure 6a, a significant increase in the PPAR γ relative gene expression was seen in comparison with untreated adipocytes (differentiated) with respect to pre-adipocytes group (control) ($p = 0.0003$). In contrast, a significant decrease in the PPAR γ relative gene expression was observed for telmisartan-exposed adipocytes, for both 6 h and 12 h, in comparison with non-exposed adipocytes ($p = 0.0064$ and $p = 0.0001$, respectively). After 12 h of telmisartan exposure PPAR γ relative gene expression was lower than that obtained at 6 h ($p = 0.0002$).

Telmisartan preserves PPAR γ protein levels

PPAR γ protein levels were analysed by Western blot analysis. The results show no significant differences between treated and pre-adipocyte (control) groups, or between untreated and telmisartan-treated adipocytes after 6 h ($p = 0.06$), (Figure 6b).

Discussion

The results obtained in the present study support the partial independence of the PPAR γ -eNOS pathway to explain some of the antioxidant properties that have been attributed to telmisartan. Following telmisartan treatment, a time-dependent decrease of the PPAR γ gene transcription levels occurred in adipocytes, reaching its lowest level at 12 hours of treatment. In contrast, six hours after telmisartan exposure, the maximum levels of eNOS protein phosphorylation at Ser¹¹⁷⁷ were observed, whereas Thr⁴⁹⁵ phosphorylation was statistically lower in comparison with untreated adipocyte. After 12 hours,

FIGURE 5. Densitometric results of Western blots of native and phosphorylated eNOS. Representative images of Western blot and graphed densitometric analysis (%) of (a) native and phosphorylated at (b) Ser¹¹⁷⁷ and (c) Thr⁴⁹⁵ eNOS protein levels in the four experimental groups (from left to right of the images are two samples of cell lysates from pre-adipocytes, untreated adipocytes and adipocytes exposed to telmisartan for 6 and 12 hours respectively). Native eNOS (a) remained unchanged in all experimental groups. Phosphorylation at Ser¹¹⁷⁷ (b) increased significantly after 6 h of telmisartan exposure with respect to pre-adipocytes ($p^* = 0.0002$) and to non-treated adipocytes ($p^{**} = 0.0119$). The difference between pre-adipocytes and cells exposed to telmisartan for 12h was also significant ($p^{***} = 0.0224$). Interestingly, phosphorylation of Thr⁴⁹⁵ (c) decreased at 6h with respect to pre-adipocytes ($p^* = 0.0007$) and untreated adipocytes ($p^{**} = 0.02$).

phosphorylation at Ser¹¹⁷⁷ diminished and phosphorylation at the inactivating Thr⁴⁹⁵ residue increased; both changes were statistically significant. The telmisartan activity through PPAR γ pathway has been reported widely in several models, tissues and conditions [24-27], as well as the stimulation of eNOS by telmisartan through a PPAR γ and Rho-kinase pathway [28, 29]. As is known, adipocyte differentiation activates the transcription factor CCAAT/enhancer-binding protein β (C/EBP β) [30], which subsequently stimulates PPAR γ

expression, insulin or insulin-like growth factor-1, promoting adipocyte differentiation. Following differentiation, PI3-kinase and Akt are activated [31] and, in a similar way to eNOS phosphorylation at Ser¹¹⁷⁷, promote an increase in eNOS activity and NO bioavailability. Along with this, it has been shown that telmisartan also induces kinase activation to increase in Ser¹¹⁷⁷ whereas Thr⁴⁹⁵ phosphorylation status is unknown [32].

Interestingly, the differentiation of 3T3 cells has been associated with increased oxidative stress, which reduces NO bioa-

a

b

FIGURE 6. *PPAR γ relative gene expression and PPAR γ Western blot analyses.* (a) PPAR γ to GAPDH relative gene expression results. As shown, PPAR γ relative gene expression was statistically higher in non-treated adipocytes compared to both 6 and 12 h treated adipocytes and pre-adipocytes ($p^* = 0.0064$, $p^{**} < 0.0001$, $p^{***} = 0.0003$). After 12 h relative gene expression was lower than that obtained at 6 h ($p^\phi = 0.0002$) (b) Representative images of Western blot and graphed densitometry analysis (%) of PPAR γ protein (from left to right of the images are two samples of cell lysates from undifferentiated control 3T3-L1 cells (pre-adipocytes), differentiated control cells (adipocytes) and adipocytes switched to normal DMEM medium and exposed to telmisartan for 6 and 12 hours). There was a trend toward a significant decrease in the PPAR γ protein level in the 6 h group compared to untreated adipocytes ($p^* = 0.06$)

availability. NO metabolites were also analysed in this study in all four cell groups, but the results were inconclusive as it was not possible to discriminate the source of NO production

Several studies have shown that oxidative stress induces a compensatory increase in PPAR γ gene transcription, which is thought to occur because of a loss of PPAR γ activity. In our work, a compensatory increase in PPAR γ gene expression was observed in adipocytes in comparison with pre-adipocytes and treated adipocytes. Furthermore, the activity of PPAR γ can be

finely tuned through integration of diverse phosphorylation events [33, 34]. In this regard, a downward trend in PPAR γ protein levels in cells treated with telmisartan for 6 hours in comparison with the untreated group.

The antioxidant properties of telmisartan are related to several phosphorylation sites of eNOS [35]. The current knowledge of eNOS phosphorylation implicates a complex group of phosphorylation sites at Ser¹¹⁷⁷, Ser¹¹⁴, Ser⁶¹⁵, Ser⁶³³ and Thr⁴⁹⁵, with resultant effects on eNOS enzyme activity.

PI3-kinase and Akt induce eNOS phosphorylation [31] at Ser¹¹⁷⁷, increasing eNOS activity. The eNOS-Ser¹¹⁷⁷ phosphorylation has been associated with administration of PPAR γ agonists [36], insulin [37], bradykinin [38], Akt/PKB (protein kinase B) or AMPK(AMP- activated protein kinase) [39, 40], these latter molecules also involved in the PPAR γ pathway. Thiazolidinediones are agonists of PPAR γ and decrease serum triglycerides and fatty acids, resulting in glucose and insulin imbalance [41], whereas telmisartan, a partial agonist of PPAR γ , improves insulin sensitivity in adipose tissue [42]. Thus, this action of telmisartan is not dependent on PPAR γ activity.

On the other hand, the selective inhibition of endothelial PKC- β mediates the production of NO, and thus PKC, which phosphorylates eNOS-Thr⁴⁹⁵, leading to inhibition of the eNOS activity. As we commented before, Saitoh *et al.* [35] reported that telmisartan treatment decreased PKC activity and improved oxidative stress. Payne *et al.* [43] showed that peri-adventitial adipose-derived factors impair coronary endothelial NO production via a PKC- β -dependent, site-specific phosphorylation of eNOS at Thr⁴⁹⁵. In this sense, our Western blot analysis revealed a greater increase in eNOS phosphorylation at the inhibitory residue Thr⁴⁹⁵ in untreated murine adipocytes that was higher than that seen in the telmisartan-treated group. Knorr *et al.* [9] reported that the phosphorylation of eNOS-Ser¹¹⁷⁷ is a critical requirement for eNOS activation, but Mount *et al.* [11] suggested that the dephosphorylation of Thr⁴⁹⁵ is a prerequisite for the phosphorylation of Ser¹¹⁷⁷. Fleming *et al.* [13] suggested that the dual phosphorylation/dephosphorylation of Ser¹¹⁷⁷ and Thr⁴⁹⁵ determines the eNOS activity, being dephosphorylation of Thr⁴⁹⁵ previous to phosphorylation of Ser¹¹⁷⁷. Lin *et al.* [14] suggested that phosphorylation/dephosphorylation of Thr⁴⁹⁵ may be an intrinsic switch mechanism to determinate NO in cells. Harris *et al.* suggest that phosphorylation of Thr⁴⁹⁵ is enough to activate eNOS enzyme activity [12]. In our experiments, -Ser¹¹⁷⁷ phosphorylation increased and eNOS-Thr⁴⁹⁵ phosphorylation decreased with telmisartan treatment. Interestingly, this increased phosphorylation is not accompanied by increased PPAR γ expression.

Taking together, these findings are consistent with a telmisartan mechanism partially independent of PPAR γ . We can only speculate as to the mechanisms by which telmisartan reduces oxidative stress. It might be possible that telmisartan inhibits activation of PKC, leading to dephosphorylation of Thr⁴⁹⁵ and, consequently, to phosphorylation of Ser¹¹⁷⁷, which increases eNOS activity, thereby improving the oxidative state.

Conclusion

The present study gives experimental evidence that telmisartan treatment improves oxidative stress status in adipocytes. Our data point toward a previously unrecognized role of telmisartan in phosphorylation of eNOS-Thr⁴⁹⁵, which may be relevant for antioxidant properties of telmisartan. More studies are now needed to clarify this mechanism.

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