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 PPARγ in adipocytes was investigated.

Methods: Expression of aP2, PPARγ, 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 PPARγ 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 PPARγ-eNOS pathway in adipocytes.
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 antihypertensive 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 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10 µg/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 (5·10-6 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% isopropanol 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 µl Kreb’s buffer, and 250 µM 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 µl) were mixed with 800 µl of trichloroacetic acid (TCA) and centrifuged at 8000 rpm for 20 minutes at 4ºC. 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 µl) 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 PPARγ, 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 ΔΔCt 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 µg samples of protein in Laemmli buffer were denatured for 5 min at 95ºC and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gels electrophoresis (PAGE) on 5-7.5% polyacrilamide 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), PPARγ (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 (Amerham Pharmacia Biotech). The membranes were stripped and re-hybridized with another antibody. Densitrometric 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 ± SEM. Statistical significance of differences between mean values was evaluated by using the Mann-Whitney test. Two-sides 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)
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 3T3L1 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.

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 genes 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...
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 PPARγ gene expression**

To determine whether the antioxidant effects of telmisartan depend on PPARγ, PPARγ 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 PPARγ 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 PPARγ 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 PPARγ relative gene expression was lower than that obtained at 6 h (p = 0.0002).

**Telmisartan preserves PPARγ protein levels**

PPARγ 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 PPARγ-eNOS pathway to explain some of the antioxidant properties that have been attributed to telmisartan. Following telmisartan treatment, a time-dependent decrease of the PPARγ 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,
phosphorylation at Ser^{1177} diminished and phosphorylation at the inactivating Thr^{495} residue increased; both changes were statistically significant. The telmisartan activity through PPARγ pathway has been reported widely in several models, tissues and conditions [24-27], as well as the stimulation of eNOS by telmisartan through a PPARγ and Rho-kinase pathway [28, 29]. As is known, adipocyte differentiation activates the transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) [30], which subsequently stimulates PPARγ 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 Ser^{1177}, 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 Ser^{1177} whereas Thr^{495} phosphorylation status is unknown [32].

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

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**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) Ser^{1177} and (c) Thr^{495} 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 Ser^{1177} (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 Thr^{495} (c) decreased at 6h with respect to pre-adipocytes (p*= 0.0007) and untreated adipocytes (p** = 0.02).
vailability. 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 PPARγ gene transcription, which is thought to occur because of a loss of PPARγ activity. In our work, a compensatory increase in PPARγ gene expression was observed in adipocytes in comparison with pre-adipocytes and treated adipocytes. Furthermore, the activity of PPARγ can be finely tuned through integration of diverse phosphorylation events [33, 34]. In this regard, a downward trend in PPARγ 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.

**FIGURE 6.** PPARγ relative gene expression and PPARγ Western blot analyses. (a) PPARγ to GAPDH relative gene expression results. As shown, PPARγ 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* = 0.0002) (b) Representative images of Western blot and graphed densitometry analysis (%) of PPARγ 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 PPARγ protein level in the 6 h group compared to untreated adipocytes (p* = 0.06).
PI3-kinase and Akt induce eNOS phosphorylation [31] at Ser\textsuperscript{1177}, increasing eNOS activity. The eNOS-Ser\textsuperscript{1177} phosphorylation has been associated with administration of PPAR\textgamma 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\textgamma pathway. Thiazolidinediones are agonists of PPAR\textgamma and decrease serum triglycerides and fatty acids, resulting in glucose and insulin imbalance [41], whereas telmisartan, a partial agonist of PPAR\textgamma, improves insulin sensitivity in adipose tissue [42]. Thus, this action of telmisartan is not dependent on PPAR\textgamma activity.

On the other hand, the selective inhibition of endothelial PKC-\beta mediates the production of NO, and thus PKC, which phosphorylates eNOS-Thr\textsuperscript{495}, 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-\beta-dependent, site-specific phosphorylation of eNOS at Thr\textsuperscript{495}. In this sense, our Western blot analysis revealed a greater increase in eNOS phosphorylation at the inhibitory residue Thr\textsuperscript{495} 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-Ser\textsuperscript{1177} is a critical requirement for eNOS activation, but Mount et al. [11] suggested that the dephosphorylation of Thr\textsuperscript{495} is a prerequisite for the phosphorylation of Ser\textsuperscript{1177}. Fleming et al. [13] suggested that the dual phosphorylation/dephosphorylation of Ser\textsuperscript{1177} and Thr\textsuperscript{495} determines the eNOS activity, being dephosphorylation of Thr\textsuperscript{495} previous to phosphorylation of Ser\textsuperscript{1177}. Lin et al. [14] suggested that phosphorylation/dephosphorylation of Thr\textsuperscript{495} may be an intrinsic switch mechanism to determine NO in cells. Harris et al. suggest that phosphorylation of Thr\textsuperscript{495} is enough to activate eNOS enzyme activity [12]. In our experiments, Ser\textsuperscript{1177} phosphorylation increased and eNOS-Thr\textsuperscript{495} phosphorylation decreased with telmisartan treatment. Interestingly, this increased phosphorylation is not accompanied by increased PPAR\textgamma expression.

Taking together, these findings are consistent with a telmisartan mechanism partially independent of PPAR\textgamma. 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 Thr\textsuperscript{495} and, consequently, to phosphorylation of Ser\textsuperscript{1177}, 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-Thr\textsuperscript{495}, which may be relevant for antioxidant properties of telmisartan. More studies are now needed to clarify this mechanism.

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References


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