

Chronic Treatment With Losartan Results in Sufficient Serum Levels of the Metabolite EXP3179 for PPAR γ Activation

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Abstract—The losartan metabolite EXP3174 exhibits angiotensin II receptor 1 (AT1R)-blocking properties, whereas the metabolite EXP3179 potently induces the activity of the insulin-sensitizing peroxisome proliferator-activated receptor γ (PPAR γ) as a partial agonist in vitro. We investigated whether chronic treatment with losartan leads to sufficient serum levels of EXP3179 to activate PPAR γ in monocytes derived from losartan-treated patients. Hypertensive patients (n=15) treated with losartan (100 mg/daily for at least the past 2 months) and untreated control patients (n=7) were included. Monocytes were extracted by negative isolation using a Dynal Monocyte Kit, followed by analysis of PPAR γ target gene expression (CD36, ABC transporter G1 [ABCG1]) by quantitative real-time RT-PCR. Serum was prepared before, 2, 4, and 6 hours after losartan (100 mg) ingestion for HPLC-based determination of losartan, EXP3174, and EXP3179. Chronic treatment with losartan resulted in basal levels of losartan, EXP3174, and EXP3179 of 348.3 ± 101.8 ng/mL, 115.3 ± 56.1 ng/mL, and 176.2 ± 143.4 ng/mL, respectively. Levels of both EXP3174 and EXP3179 were time-dependently increased in serum with a maximum 2 hours after drug intake (1706.0 ± 760.1 ng/mL, 808.9 ± 618.2 ng/mL, respectively). In consonance with detectable PPAR γ -activating EXP3179 serum levels, monocytic PPAR γ target gene expression was significantly upregulated in patients treated with losartan by 3.75 ± 0.95 - and 252.02 ± 46.86 -fold for CD36 and ABCG1 ($P=0.043$, $P=0.0045$ versus control patients, respectively). This is the first clinical description of monocytic PPAR γ -target gene regulation by chronic treatment with losartan, which likely is mediated by its metabolite EXP3179. Our data show that sufficient serum levels of EXP3179 are present under losartan treatment. PPAR γ activation by AT1R-blockers may translate into synergistic beneficial actions in monocytes. (*Hypertension*. 2009;54:00-00.)

Key Words: peroxisome proliferator-activated receptor gamma ■ angiotensin receptor blocker ■ losartan ■ CD36 ■ ABCG1 ■ monocytes ■ hypertension

Most angiotensin II-induced actions have been attributed to be mediated through the angiotensin II receptor 1 (AT1R). This led to the use of AT1R-blockers (ARBs) as antihypertensive drugs, which exhibit a variety of pleiotropic protective vascular effects. Along with this, the Losartan Intervention For End point reduction in hypertension (LIFE) study demonstrated a significantly greater benefit for cardiovascular morbidity and mortality and a 25% lower rate of new-onset diabetes in patients treated with losartan compared to β -blocker treatment.¹ Interestingly, blood pressure reduction in LIFE was identical in both groups, strongly indicating that the antidiabetic action of the ARB was independent of blood pressure lowering. This is further supported by data from the VALUE (Valsartan Antihypertensive Long-term

Use Evaluation) trial in which valsartan was compared to amlodipine.² Despite a more prominent blood pressure-lowering effect in the amlodipine group, valsartan lowered the risk of new-onset diabetes by 23%. Thus, these data suggest cardiovascular protective and antidiabetic properties of losartan which are, at least in part, independent from its blood pressure lowering actions. The molecular mechanisms for the beneficial effects of losartan beyond AT1R blockade are still incompletely understood.

Losartan is metabolized via cytochrome-P450 degradation into EXP3174, serving as the AT1R-blocking metabolite, and into EXP3179 with no AT1R-blocking properties. The latter exhibits molecule homology to indomethacin, a cyclooxygenase inhibitor with antiinflammatory and antiaggregatory

Received March 20, 2009; first decision April 11, 2009; revision accepted July 29, 2009.

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DOI: 10.1161/HYPERTENSIONAHA.109.132886

properties.³ Consistently, this metabolite was capable of blocking cyclooxygenase-2 and ICAM-1 mRNA upregulation, and cyclooxygenase-dependent thromboxane A2 and prostaglandin F₂ α generation in vitro. Moreover, EXP3179 stimulated the phosphorylation of the endothelial nitric oxide synthase through a PI3-kinase/Akt-pathway downstream of the VEGF-receptor 2 in cultured endothelial cells.⁴

We and others demonstrated that a subset of ARBs, including losartan, induces the activity of a nuclear hormone receptor named peroxisome proliferator-activated receptor γ (PPAR γ) by partial agonism.^{5,6} Importantly, this activation was shown to be independent of AT1R expression, and therefore not related to the AT1R-blocking properties.⁶ PPAR γ functions as a transcriptional regulator in adipose tissue where it regulates multiple genes involved in lipid and glucose metabolism.⁷ PPAR γ is abundantly expressed in adipose tissue, but also in vascular and nonvascular cells in the vessel wall (monocytes/macrophages, endothelial cells, vascular smooth muscle cells).⁸ PPAR γ activated by synthetic agonists like glitazones improves whole-body insulin sensitivity resulting in decreased levels of fasting plasma glucose, insulin, and triglycerides.⁹ In addition, ligand-activated PPAR γ mediates direct antiatherosclerotic actions resulting in marked reduction of cardiovascular morbidity and mortality.^{10–12} We could demonstrate that EXP3179, but not the losartan metabolite EXP3174, markedly stimulated PPAR γ activity and target gene expression of adipose protein 2 in vitro.¹³ Whether EXP3179-induced PPAR γ -activity plays a significant role in the observed beneficial actions of losartan in patients is currently unknown. Therefore, we analyzed (1) levels of losartan and losartan metabolites in hypertensive patients chronically treated with losartan, and (2) PPAR γ activation in isolated monocytes.

Materials and Methods

The extended material and methods used are described in the online Data Supplement (please see <http://hyper.ahajournals.org>).

Patients' Characteristics

Hypertensive patients who had been treated with losartan 100 mg/daily for at least 2 months (n=15) and hypertensive volunteers without losartan-treatment (n=7) were recruited in the Department of Internal Medicine/Cardiology at the Deutsche Herzzentrum Berlin, Germany. Patients' characteristics are summarized in Table 1. No patient was taking indomethacin or any other antiphlogistic drug (NSAIDs), except for antiplatelet acetyl salicylic acid, or other ARBs or glitazones for the past 2 months. The study was approved by the ethics committee of the Charité-Universitätsmedizin Berlin, Germany. All patients provided written informed consent. The study was registered as ClinicalTrials.gov-ID NCT00561327.

Results

Losartan and Losartan Metabolites Serum Levels

Levels of losartan and metabolites were clearly detectable in serum from losartan-treated patients at t=0 hour, demonstrating that chronic administration of losartan leads to steady-state levels that can be discriminated to levels of untreated control patients, which were negative in our experimental set-up, providing evidence for yielding valid serum levels (no biological noise; Table 2).

Table 1. Characteristics of the Study Population

Patients' Characteristics	Control, n=7 (%)	Losartan, n=15 (%)	P for Difference
Sex, male/female	7/0	11/4	n.s.
Age, y	59 \pm 9	68 \pm 6	<0.05
Body mass index, kg/m ²	28.3 \pm 2.0	31.4 \pm 3.1	<0.05
Mean systolic blood pressure, mm Hg	145.0 \pm 17.3	147.8 \pm 14.3	n.s.
Mean diastolic blood pressure, mm Hg	78.9 \pm 10.9	79.4 \pm 8.5	n.s.
Diagnosis			
Hypertension	7 (100%)	15 (100%)	n.s.
Diabetes	2 (29%)	7 (47%)	n.s.
Coronary artery disease	5 (71%)	12 (80%)	n.s.
Comedication			
Antiplatelets	5 (71%)	13 (87%)	n.s.
Statins	5 (71%)	12 (80%)	n.s.
ACE-inhibitors	4 (57%)	2 (13%)	n.s.
Beta-blockers	4 (57%)	12 (80%)	n.s.
Calcium channel blockers	3 (43%)	9 (60%)	n.s.
Serum parameters			
Creatinine, mg/dL	1.14 \pm 0.24	1.06 \pm 0.17	n.s.
Sodium, mmol/L	140.2 \pm 3.7	140.5 \pm 2.5	n.s.
Potassium, mmol/L	3.78 \pm 0.55	3.81 \pm 0.35	n.s.

Consistent with previous reports, mean losartan baseline serum levels were 348.3 ng/mL corresponding to 0.82 μ mol/L (Table 2). The AT1R-blocking metabolite EXP3174 was characterized by low baseline levels, reached a maximum after 2 hours with 1706.0 ng/mL (=3.91 μ mol/L), and showed a second peak after 6 hours (1222.7 ng/mL=2.80 μ mol/L; Table 2). The PPAR γ -activating EXP3179 could be clearly detected in serum samples from losartan-treated patients. Serum levels peaked after 2 hours with 808.9 ng/mL, which represents 1.92 μ mol/L followed by a decrease over time (Table 2).

PPAR γ Target Gene Expression in Monocytes

As a model for in vivo target gene expression, monocytes were isolated from hypertensive losartan-treated patients and

Table 2. HPLC-Based Analyses of Losartan and Losartan Metabolites

	t=0	t=2 Hours	t=4 Hours	t=6 Hours
Losartan				
ng/mL	348.3 \pm 101.8	Not determined	799.9 \pm 580.5	Not determined
μ mol/L	0.82 \pm 0.24	Not determined	1.89 \pm 1.37	Not determined
EXP3174				
ng/mL	115.3 \pm 56.1	1706.0 \pm 760.1	857.7 \pm 373.3	1222.7 \pm 391.0
μ mol/L	0.26 \pm 0.13	3.91 \pm 1.74	1.96 \pm 0.86	2.80 \pm 0.90
EXP3179				
ng/mL	176.2 \pm 143.4	808.9 \pm 618.2	127.7 \pm 58.7	70.1 \pm 43.6
μ mol/L	0.42 \pm 0.35	1.92 \pm 1.47	0.30 \pm 0.14	0.17 \pm 0.10

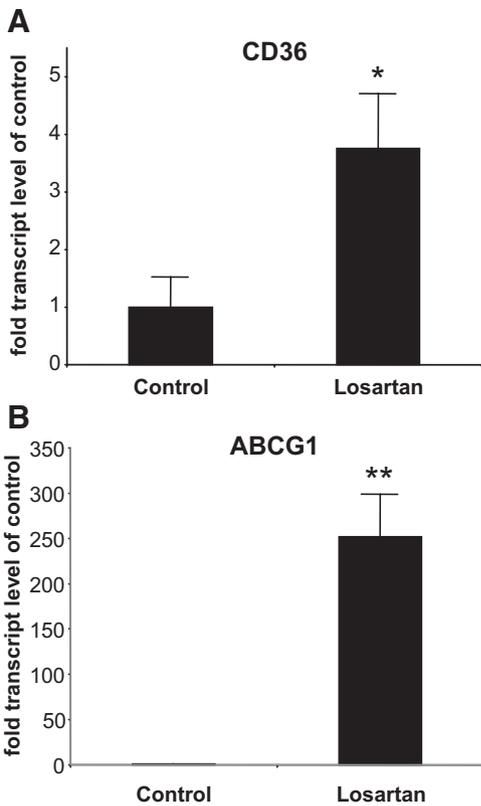


Figure 1. PPAR γ target gene expression in control and losartan-treated patients. Quantitative real-time PCR of the PPAR γ target genes (A) CD36 and (B) ABCG1 in isolated monocytes from hypertensive control patients and patients chronically treated with losartan. Levels of control patients were arbitrarily set to 1. Shown are analyses of transcript levels at t=0 hour, which equates to 24 hours after last oral ingestion of 100 mg losartan. Data are expressed as mean relative expression \pm SEM of CD36/18S, and ABCG1/18S mRNA ratios. * P <0.05, ** P <0.01 vs Control.

hypertensive control patients. Monocytes from losartan-treated and control patients were prepared by negative isolation from blood that was withdrawn at t=0 hour (before losartan intake in the treated group) and at t=6 hours (6 hours after oral losartan ingestion). RNA was extracted from isolated monocytes, reverse transcribed to cDNA, and used as a template for quantitative RT-PCR analyses on PPAR γ target gene expression (Figure 1). PPAR γ activators have

previously been described to enhance the expression of the scavenger receptor CD36 and the cholesterol efflux transporter ABCG1, implicated in the elimination of the excess of free cholesterol.¹⁴ Transcript levels were normalized to transcript expression of the house-keeping gene human 18S, and levels in control-patients were arbitrarily set to 1. PPAR γ target gene expression was significantly increased in patients chronically treated with losartan. CD36 was upregulated by 3.75 \pm 0.95-fold (P =0.043 versus control patients; Figure 1). ABCG1 levels were increased to 252.02 \pm 46.86-fold (P =0.0045 versus control patients) compared to expression of untreated control patients (Figure 1), in consonance with the concept of sufficient levels of losartan and losartan-metabolites to induce PPAR γ target gene expression in vivo under chronic treatment with losartan. Interestingly, gene transcript expression from monocytes isolated 6 hours after losartan ingestion (data not shown) did not differ significantly from values detected at t=0 hour, underlining the assumption of chronic losartan and losartan metabolite levels particularly impacting on target gene expression.

Functional Impact of PPAR γ Agonism on Monocyte Migration

Exploring the actual biological effects of PPAR γ agonism by losartan and its metabolites, we analyzed cellular responses of THP-1 human monocytes. Monocytes were characterized with regard to monocyte chemoattractant protein-1 (MCP-1)-directed chemotaxis in a transwell migration system in the absence or presence of ARBs known to activate PPAR γ (telmisartan, irbesartan), or valsartan, an ARB without PPAR γ agonistic properties. In addition, chemotaxis was analyzed in the presence of losartan, and the losartan metabolites EXP3174 and EXP3179, as well as pioglitazone as a PPAR γ full agonist. As shown in Figure 2, MCP-1-directed migration was significantly and concentration-dependently impaired by EXP3179, telmisartan, irbesartan, and pioglitazone. In contrast, losartan, EXP3174, and valsartan failed to alter migration. Importantly, monocytes being subjected to EXP3179 at levels that were actually achieved in hypertensive patients (approximately 2 μ mol/L) were characterized by significantly reduced migration. These data support that the

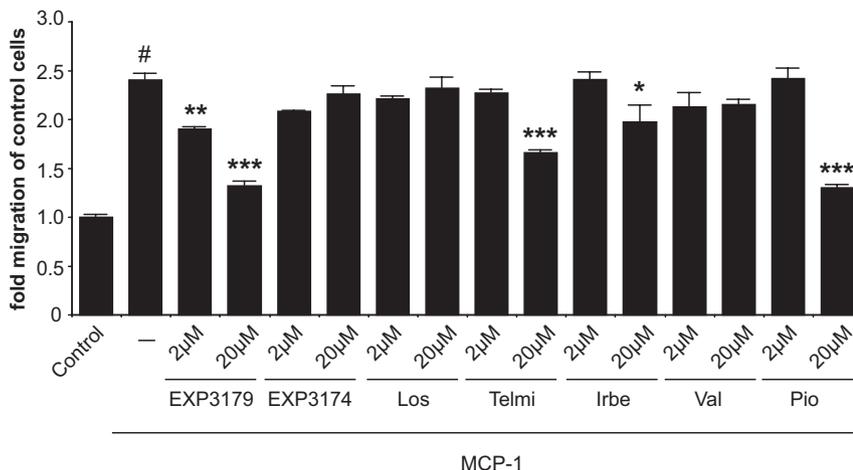


Figure 2. Migration of human monocytes. Human monocytic THP-1 cells were pre-treated for 24 hours with vehicle (control) or compounds at concentrations indicated. Cells were then subjected to chemotaxis assays using 0.2% gelatin-coated transwell chambers. Cells were placed in the upper chamber and were allowed to migrate for 2.5 hours toward a monocyte-chemoattractant protein-1 (MCP-1, 10 ng/mL) gradient, either in the presence of vehicle (control) or compounds as indicated. Each experiment was performed with n=3 wells per condition. Data are expressed as fold migration of control cells without MCP-1 chemotaxis-induction. Shown is the mean \pm SEM from 3 independent experiments. # P <0.001 vs Control; * P <0.05 vs MCP-1; ** P <0.01 vs MCP-1; *** P <0.001 vs MCP-1.

plasma levels of the PPAR γ agonist EXP3179 are capable of reducing monocytic cellular responses.

EXP3179 Induces PPAR γ Target Gene Expression in Monocytes Ex Vivo

PPAR γ target genes were significantly upregulated in hypertensive patients chronically treated with losartan. Thus, we next analyzed whether the PPAR γ agonistic losartan metabolite EXP3179 induces gene expression at concentrations achieved in vivo in primary human monocytes ex vivo. Monocytes were isolated from drug naïve healthy volunteers and treated with various ARBs (losartan, telmisartan, irbesartan), the losartan metabolites EXP3174 and EXP3179, and pioglitazone as a full PPAR γ agonist for 24 hours. In accordance with previous reports,¹³ EXP3179 treatment resulted in induction of CD36 (Figure 3A). Importantly, both losartan and the losartan metabolite EXP3174 failed to significantly induce CD36 in human monocytes. Moreover, the observed effect attributable to EXP3179 treatment was achieved after incubation at concentrations that were indeed detectable in hypertensive patients chronically treated with losartan. Confirming previous data,^{5,6} telmisartan and irbesartan treatment resulted also in PPAR γ target gene expression, underlining the dual molecular targets of these ARBs. Furthermore, repetitive incubation of monocytic THP-1 cells with the losartan metabolite EXP3179 (2 μ mol/L for 2 hours every 24 hours) produced a larger induction of PPAR γ target gene expression than one single dose (Figure 3B), thus substantiating the assumption of repeated peaks being capable of inducing higher and long-standing effects on PPAR γ responsive genes.

Discussion

Here we provide evidence for significant monocytic PPAR γ -target gene regulation by chronic treatment with losartan, which likely is mediated by its metabolite EXP3179. Our data show that serum levels of EXP3179, which have recently been acknowledged to induce PPAR γ target gene expression in vitro, are present under chronic losartan treatment of patients. These observations are consistent with the idea of losartan metabolites exhibiting advantageous effects in hypertensive patients beyond blood pressure reduction. This is also supported by impaired migration of EXP3179 treated monocytes. In conclusion, the observed PPAR γ activation by the ARB losartan may translate into synergistic beneficial actions (AT1-receptor blockade + PPAR γ activation) in monocytes.

Pleiotropic Actions of Losartan

The blockade of the renin-angiotensin system (RAS) by both ACE-inhibitors and ARBs provides a well established pharmacological strategy for treatment of patients with heart failure, chronic renal failure, atherosclerotic disease, and hypertension.¹⁵⁻¹⁷ In particular, ARBs provide effective inhibition of the RAS interfering with angiotensin II at the receptor level. Early findings indicated that the action of the ARB losartan could not solely be explained by the antagonistic effects of losartan and the metabolite EXP3174 at the AT1-receptor. Additional studies suggest blood pressure-independent pleiotropic effects of losartan and other ARBs on certain end points such as new-onset of diabetes. Along this

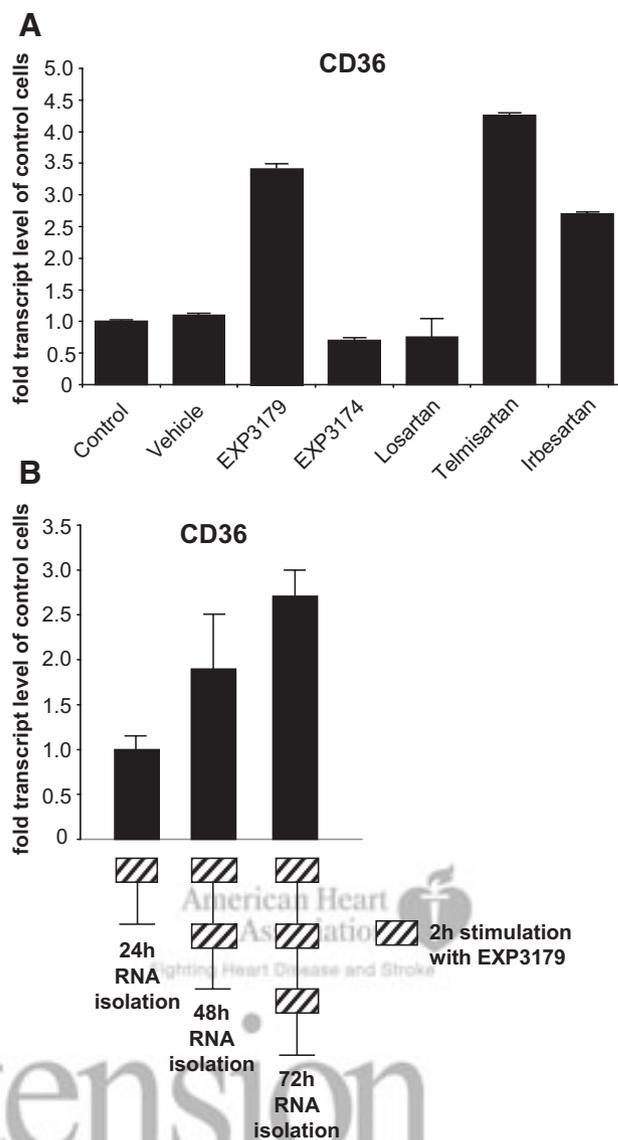


Figure 3. A, PPAR γ target gene expression in primary monocytes ex vivo. Human primary monocytes were isolated from drug naïve nonhypertensive individuals. Cells were left resting (control) or were cultured in the presence of vehicle, losartan metabolites EXP3174 and EXP3179, losartan, telmisartan, or irbesartan at 2 μ mol/L for 24 hours. Isolated RNA was transcribed to cDNA, and quantitative real-time PCR of the PPAR γ target gene CD36 was performed. Data are expressed as mean relative expression \pm SD of CD36/18S ratios. Shown are results from 1 representative experiment of n=4 individual experiments. B, PPAR γ target gene expression in human monocytic cells. Human monocytic THP-1 cells were cultured in the presence of EXP3179 (2 μ mol/L) for 2 hours every 24 hours and lysed at indicated times. Isolated RNA was transcribed to cDNA, and quantitative real-time PCR of the PPAR γ target gene CD36 was performed. Data are expressed as mean relative expression \pm SD of CD36/18S ratios (n=3).

line, the LIFE and VALUE trial have demonstrated antidiabetic actions of ARBs which cannot be directly attributed to blood pressure differences.^{1,2} Indeed, the losartan metabolite EXP3179 exhibits a variety of beneficial pleiotropic effects in vitro that may also account for antiinflammatory, antiaggregatory, and antidiabetic actions of losartan observed in clinical trials.^{1,18,19} The underlying molecular mechanism of

these actions is unknown. PPAR γ activation by EXP3179 demonstrated in the present study may provide a potential molecular explanation for these clinical findings.

Pharmacokinetics of EXP3174 and EXP3179

The kinetics of the serum concentration of EXP3174 and EXP3179 has to be interpreted with caution and requires future additional studies. EXP3179 is supposed to be a short-lived intermediate of losartan, which may explain its fast serum appearance and its marked tail-off after 2 hours. Serum concentration of EXP3174 peaks at 2 hours with an additional second peak after 6 hours showing a diversion of the EXP3174 – EXP3179 curves. The most probable explanation for this phenomenon might be that EXP3174 undergoes substantial enterohepatic circulation, which then results in a second peak of the serum concentration time curve. Previous reports on the pharmacokinetics of losartan and EXP3174 demonstrated a peak plasma concentration of EXP3174 between 3 to 6 hours.²⁰ However, in most cases pharmacokinetic data are based on results from early clinical development phases tested in untreated healthy volunteers with short-term treatment. In the present study, hypertensive patients chronically treated with losartan and concomitant medication were investigated. Thus, drug interactions on different levels of drug metabolism (eg, enteral transport, hepatic metabolism, etc) or potential saturation of metabolizing enzymes under chronic conditions may have affected our results.

EXP3179: A PPAR γ -Activator in Hypertensive Patients

It has been previously demonstrated that a single oral dose of losartan (100 mg) reached maximal serum levels of EXP3179 between 0.1 and 1 $\mu\text{mol/L}$.³ Our group showed PPAR γ -mediated adipocyte differentiation at concentrations between 1 and 10 $\mu\text{mol/L}$.¹³ The high lipophilicity of EXP3179 with possible accumulation of this metabolite in target cells (eg, monocytes), and the characteristics of repetitive elevated levels under chronic losartan treatment, may produce sufficient EXP3179 intracellular concentrations in vivo to activate PPAR γ . This is substantiated by our findings that repetitive incubation of monocytic cells with EXP3179 resulted in larger induction of PPAR γ target gene expression than 1 single dose (Figure 3B). Rapid hepatic metabolization of EXP3179, however, might not be negligible. In agreement with the data from Kramer and colleagues,³ we could now demonstrate maximal EXP3179 serum levels of 1.92 $\mu\text{mol/L}$ under chronic losartan (100 mg daily) treatment (Table 2). This serum concentration is in a range which is compatible with previous PPAR γ inducing concentrations in in vitro settings. In line with this, we could demonstrate increased PPAR γ target gene expression in monocytes treated with EXP3179 ex vivo at concentrations detectable in hypertensive patients treated with losartan (Figure 3).

CD36 and ABCG1, 2 previously recognized PPAR γ target genes, were significantly increased in patients chronically treated with losartan (Figure 1). Both genes have been implicated in cholesterol uptake and removal in monocytes/macrophages, suggesting a significant role in foam cell forma-

tion and, thus, atherosclerotic disease.^{21–23} In this context, PPAR γ -mediated cholesterol efflux via ABCA1 and ABCG1 appears to be more prominent than cholesterol influx resulting in a net antiatherosclerotic action of PPAR γ activation.^{24,25} In the present study we detected serum levels of EXP3179 in the range of PPAR γ -activating concentrations. Thus, it is likely that the differences in monocytic CD36 and ABCG1 expression levels between losartan-treated and -untreated patients result from the presence/absence of the PPAR γ -activating EXP3179 metabolite. These data are consistent with previous reports demonstrating that telmisartan, another PPAR γ -activating ARB, induces ABCG1 expression in human THP-1 macrophages.²⁶ Notably, downregulation of PPAR γ by siRNA abolished the telmisartan-induced expression, underlining the importance of PPAR γ for the induction of ABCG1 by this distinct group of ARBs.²⁶ However, the role of AT1R blockade by losartan for monocytic gene expression and cholesterol efflux has to be taken into consideration. Angiotensin II has been consistently shown to reduce cholesterol efflux from monocytes/macrophages.^{27,28} Takata and colleagues demonstrated that angiotensin II-induced cholesterol efflux is mediated via downregulation of the ABCA1 transporter in macrophages, whereas ABCG1 expression was not affected.²⁷ Moreover, treatment with the ARB valsartan resulted in an induction of ABCA1 gene expression but had no effect on ABCG1.²⁷ These studies suggest that the induction of ABCG1 observed in our study may mainly result from losartan-metabolite (EXP3179)-mediated PPAR γ activation, because AT1-blockade has not been described to regulate this gene. The bimodal mechanism of action of losartan and other PPAR γ -activating ARBs may provide synergistic benefits with regard to cholesterol efflux comprising AT1R-dependent ABCA1 regulation and PPAR γ -dependent ABCG1 regulation. Furthermore, proliferation and accumulation of monocytes account for inflammation in both obesity/insulin-resistance and atherosclerosis. In line with previous reports²⁹ we demonstrate impaired monocyte migration attributable to PPAR γ activation (Figure 2). In particular, EXP3179 at concentrations detectable in vivo in losartan-treated hypertensive patients significantly inhibited MCP-1-directed transwell migration. These results may provide an additional mechanism of the cardiovascular protective actions of losartan observed in clinical end point trials.

Taken together, chronic losartan treatment in hypertensive patients leads to serum levels of losartan and losartan metabolites, which seem sufficient for induction of PPAR γ target gene induction. Whereas earlier findings demonstrated significant reduction of atherosclerotic disease by losartan,^{30,31} our data suggest PPAR γ activation as a potential mechanism by which beneficial clinical impact is induced under chronic treatment with the ARB losartan.

Clinical Perspective

AT1 receptor blockade and PPAR γ activation have been shown to exert beneficial actions on cardiovascular morbidity and mortality. Combination of the 2 pharmacological principals in 1 compound may result in synergistic actions in patients with cardiovascular risk. In this regard, the present study provides a potential cardiovascular protective mecha-

nism for losartan. In addition, these data demonstrate the possibility to activate monocyte PPAR γ in patients treated with an AT1 receptor blocker serving as a clinical model for the future development of bimodal drugs.

Sources of Funding

This work was supported by MSD SHARP & DOHME GMBH, Haar, Germany. K.K. was supported by a research scholarship from the Charité-Universitätsmedizin Berlin, Germany, and is supported by the Deutsche Forschungsgemeinschaft (KA 1820/4-1). R.G. is supported by the Deutsche Forschungsgemeinschaft (GU-285/7-1). T.U. is supported by the Deutsche Forschungsgemeinschaft (GK 754-III, GK 865-II). U.K. is supported by the Deutsche Forschungsgemeinschaft (GK 754-III, KI 712/3-1, FG 1054 [KI 712/5-1]).

Disclosures

T.U. has received research support and speaker fees from Sanofi-Aventis, Bayer-Schering Pharma, Boehringer Ingelheim, Berlin Chemie, and MSD. U.K. has received research support and speaker fees from Sanofi-Aventis, Bayer-Schering Pharma, Boehringer Ingelheim, Berlin Chemie, and MSD.

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