Peroxisome Proliferator-Activated Receptor-γ (PPARγ) Mutations Responsible for Lipodystrophy With Severe Hypertension Activate the Cellular Renin–Angiotensin System

Martine Auclair, Corinne Vigouroux, Franck Boccara, Emilie Capel, Catherine Vigeral, Bruno Guerci, Olivier Lascols, Jacqueline Capeau, Martine Caron-Debarle

Objective—Inactivating peroxisome proliferator-activated receptor-γ (PPARγ) mutations lead to a syndrome of familial partial lipodystrophy (FPLD3) associated with early-onset severe hypertension. PPARγ can repress the vascular renin–angiotensin system (RAS) and angiotensin II receptor 1 expression. We evaluated the relationships between PPARγ inactivation and cellular RAS using FPLD3 patients’ cells and human vascular smooth muscle cells expressing mutant or wild-type PPARγ.

Approach and Results—We identified 2 novel PPARγ mutations, R165T and L339X, located in the DNA and ligand-binding domains of PPARγ, respectively in 4 patients from 2 FPLD3 families. In cultured skin fibroblasts and peripheral blood mononuclear cells from the 4 patients and healthy controls, we compared markers of RAS activation, oxidative stress, and inflammation, and tested the effect of modulators of PPARγ and angiotensin II receptor 1. We studied the impact of the 2 mutations on the transcriptional activity of PPARγ and on the vascular RAS in transfected human vascular smooth muscle cells. Systemic RAS was not altered in patients. However, RAS markers were overexpressed in patients’ fibroblasts and peripheral blood mononuclear cells, as in vascular cells expressing mutant PPARγ. Angiotensin II–mediated mitogen-activated protein kinase activity increased in patients’ fibroblasts, consistent with RAS constitutive activation. Patients’ cells also displayed oxidative stress and inflammation. PPARγ activation and angiotensin II receptor 1 mRNA silencing reversed RAS overactivation, oxidative stress, and inflammation, arguing for a role of angiotensin II receptor 1 in these processes.

Conclusions—Two novel FPLD3-linked PPARγ mutations are associated with a defective transrepression of cellular RAS leading to cellular dysfunction, which might contribute to the specific FPLD3-linked severe hypertension. (Arterioscler Thromb Vasc Biol. 2013;33:829-838.)

Key Words: angiotensin ■ hypertension ■ lipodystrophy ■ mutation ■ peroxisome proliferator-activated receptor-γ

Peroxisome proliferator-activated receptor-γ (PPARγ) is a ligand-activated transcription factor of the nuclear hormone receptor superfamily expressed in a variety of cell types that plays a prominent role in adipogenesis and insulin sensitivity.1,2 PPARγ is also involved in the regulation of vascular tone and blood pressure3 and is expressed in many components of the vascular system (endothelial and smooth muscle cells, monocytes/macrophages),4,5 where it exerts anti-inflammatory and antioxidant effects6,7 through gene transrepression.4 Thus, signaling pathways activated by PPARγ in the vasculature contribute to prevent endothelial dysfunction and atherosclerosis.8,9

Humans with germline-inactivating mutations in PPARγ develop a syndrome of familial partial lipodystrophy of the Dunnigan type 3 (FPLD3) characterized by limb and gluteal lipatrophy, severe hypertension together with diabetes mellitus, insulin resistance, dyslipidemia, and liver steatosis, leading to an increased cardiovascular risk.10-14 These clinical manifestations result from loss-of-function and dominant-negative effects of the mutated PPARγ proteins, modified in their DNA (DBD) or ligand (LBD)-binding domains.15 Compared with patients with other forms of lipodystrophies, patients with FPLD3 have an earlier and more severe hypertension.16 Hypertension is also associated with PPARγ dysfunction in lipodystrophic human immunodeficiency virus–infected patients under antiretroviral treatment17 and
is observed in animal models of PPARγ dysfunction, such as the knock-in PPARγ P465L mice, and to a lesser extent, the L466A female mice, with vascular remodeling in the P465L model. Lowering PPARγ expression moderately increased blood pressure in mice and induced vascular dysfunction and phenotypic modulation of vascular smooth muscle cells (VSMCs) during hypertension in rats, which were attenuated by PPARγ ligands.

The mechanism whereby PPARγ modulates blood pressure is poorly understood. In rat VSMCs, PPARγ modulates the renin–angiotensin system (RAS), a major regulator of systemic blood pressure and interstitial fluid volume, by transcriptional repression of angiotensin II receptor 1 (AT1R) expression. Overexpression of AT1R in adipose tissue was proposed to contribute to hypertension observed in obese subjects or in lipoatrophic mice. Otherwise, we previously proposed to contribute to hypertension observed in obese subjects or in lipoatrophic mice.26–28

Patients and Methods are available in the online-only Supplement.

Results

Identification of 2 Novel PPARγ Mutations

We identified 2 previously unreported heterozygous PPARγ substitutions, R165T (44-year-old mother and 22-year-old daughter) and L339X (70-year-old father and 40-year-old son), in 4 patients from 2 families with FPLD3. Clinical presentation was typical of FPLD3 in all patients, with peripheral lipodystrophy, muscular hypertrophy, insulin resistance (with diabetes mellitus in 3 patients), hypertriglyceridemia, liver steatosis, and severe hypertension (Table). The 2 patients with the L339X mutation also presented with extensive psoriasis lesions. Treatment with thiazolidinediones strikingly improved hyperglycemia in the 3 diabetic patients. In the 2 patients previously treated with insulin, thiazolidinediones allowed to markedly reduce or even stop high-dose insulin therapy (2 U/kg per d at the time of diagnosis). Regarding systemic renin–angiotensin regulation, none of the patients had low-potassium levels. However, because of the severe hypertension, which requires the use of several medications, measurements of circulating renin and angiotensin were difficult and were reliably investigated in 2 patients only. The 22-year-old woman with the PPARγ R165T mutation and the 40-year-old man harboring the L339X mutation had normal values of plasma renin and aldosterone, both in supine and upright position, as well as normal levels of urine aldosterone. The addition of AT1R blockers to their antihypertensive regimen significantly improved hypertension in all patients. Clinical and biological data are detailed in the Table.

Arginine 165 (according to the PPARγ2 nomenclature) is a residue preserved among PPAR species and nuclear receptors (Figure 1A). In this highly conserved region of the DBD (residues 139–192), 5 missense mutations (C142R, Y151C, C159Y, C190S, and C190W) have been associated with FPLD3 with severe hypertension.

Leucine 339 is conserved among PPARγ species and in PPARα and δ (Figure 1A). The nonsense L339X mutation predicted a protein truncation within the central part of the LBD, as described in other natural FPLD3-linked PPAR mutations, that is, the dominant-negative Fs343X and R385X mutations that alter PPAR/RXR dimerization and the Y355X alteration, which acts via a haploinsufficiency mechanism.

Several somatic PPARγ amino acid substitutions close to residue 339 have been found in sporadic colorectal cancers, and the germline dominant-negative PPARγ S317C substitution was associated with colorectal cancer and dyslipidemia in a family. Interestingly, a screening colonoscopy revealed several polyps, without dysplasia, in the 40-year-old patient with the L339X mutation.

PPARγ was similarly expressed in control and patients’ fibroblasts at both the protein (Figure 1B) and mRNA levels (data not shown). As shown by cDNA sequencing, both the normal and R165T-mutated alleles were expressed in patients’ peripheral blood mononuclear cells (PBMCs; data not shown). In cell lysates from L339X-mutated fibroblasts and PBMCs, 2 bands of 55 and 40 kDa were revealed by N-terminal PPARγ antibodies, corresponding to the WT and truncated forms of the protein, respectively. The latter form was not detected with C-terminal PPARγ antibodies (Figure 1B). Similar patterns of PPARγ protein expression were observed in human VSMCs (Figure 1B) or human embryonic kidney (HEK)-293 cells (not shown) transfected with WT, R165T-, or L339X-mutant PPARγ.

In HEK-293 cells transfected with R165T- or L339X-mutant PPARγ, the basal PPARγ transcriptional activity was markedly decreased (by 70%–80%) as compared with that measured in cells transfected with WT PPARγ (Figure 1C). As expected, rosiglitazone increased PPARγ basal transcriptional activity of cells transfected with WT PPARγ (by 2-fold), whereas in mutant cells the effect of rosiglitazone only reached 30% to 40% of that observed in cells transfected with WT PPARγ. However, R165T- or L339X-mutant PPARγ failed to significantly suppress WT-driven transcription indicating that the 2 mutations lacked dominant-negative activity and probably acted through a haploinsufficiency mechanism (Figure 1C).
The basal protein expression of AT1R, renin, and angiotensinogen (AGT) was barely detectable in control cells but was markedly increased in R165T- and L339X-mutated fibroblasts (Figure 2A; 5- to 7-fold, 9- to 10-fold, and 13- to 16-fold increase, respectively). AT1R, renin, and AGT overexpression was also observed in PBMCs from the patients bearing the PPARG mutations (5- to 7-fold increase) and in human VSMCs transfected with R165T- or L339X-mutant PPARγ (Figure 2A; 9-, 4.5-, and 4.1-fold increase versus WT PPARγ, respectively), but not in human VSMCs transfected with WT-PPARγ. A short-term treatment with angiotensin II (100 nmol/L, 10 minutes) activated extracellular-signal-regulated kinase1/2 in patients’ fibroblasts to a higher level than in control fibroblasts (Figure 2B; 4.4- to 6.2-fold increase). A similar alteration of the RAS markers was observed in HEK-293 cells transfected with mutant PPARγ (data not shown). These studies indicated that the 2 PPARγ mutations deregulate the cellular RAS in all cell types studied, including vascular cells.

Table. Main Clinical and Biological Features of Patients

<table>
<thead>
<tr>
<th></th>
<th>Mother</th>
<th>Daughter</th>
<th>Father</th>
<th>Son</th>
</tr>
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<td>Age, y</td>
<td>44</td>
<td>22</td>
<td>70</td>
<td>40</td>
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<td>73/1.66</td>
<td>70/1.71</td>
<td>78/1.65</td>
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<tr>
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<td>26.5</td>
<td>23.9</td>
<td>28.6</td>
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<td>Yes, axillary</td>
<td>No</td>
<td>No</td>
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<td>4 limbs</td>
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<td>Face, neck</td>
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<td>Face, neck</td>
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<tr>
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<td>Generalized, moderate</td>
<td>Generalized, moderate</td>
<td>Calves</td>
<td>Generalized</td>
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<td>37/45/22.5/25.2</td>
<td>…</td>
<td>22.6/29.2/12.6/16.9</td>
</tr>
<tr>
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<td>135/75</td>
<td>140/70</td>
<td>140/90</td>
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<td>Diabetes mellitus</td>
<td>Diabetes mellitus</td>
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<td>Diabetes mellitus</td>
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<td>Hydrochlorothiazide, Verapamil, Indapamide, Telmisartan</td>
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<td>Lipid-lowering medications</td>
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<td>Bezafibrate</td>
<td>Rosuvastatin</td>
<td>Fenofibrate</td>
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<td>Vascular and cardiac examination</td>
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<td>Normal</td>
<td>Diffuse peripheral atherosclerosis</td>
<td>Normal, but left-ventricular hypertrophy</td>
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<td>Liver steatosis</td>
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<td>Yes (US imaging) with elevated liver enzymes and moderate fibrosis at elastography</td>
<td>Yes (US imaging) with mildly elevated liver enzymes</td>
<td>Yes (US imaging) with mildly elevated liver enzymes</td>
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<td>Fasting glycemia, mmol/L</td>
<td>7.9</td>
<td>7.7</td>
<td>4.8</td>
<td>7.3</td>
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<td>Fasting insulinemia, mU/L (n&lt;10)</td>
<td>…</td>
<td>23 (before insulin therapy)</td>
<td>17</td>
<td>14.3 (without insulin therapy)</td>
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<tr>
<td>HbA1c, %</td>
<td>7.2</td>
<td>8.2</td>
<td>5.5</td>
<td>8.2</td>
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<td>Triglycerides, mmol/L</td>
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<td>6.6</td>
<td>2.7</td>
<td>8.3</td>
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<td>Total cholesterol, mmol/L</td>
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<td>4.0</td>
<td>4.7</td>
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<td>0.8</td>
<td>0.6</td>
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<td>LDL-cholesterol, mmol/L</td>
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<td>3.3</td>
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<td>2.2</td>
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<td>36/38</td>
<td>…</td>
<td>20.1/21.5</td>
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<tr>
<td>Plasma aldosterone, pmol/L:supine/upright position (n: 83–414 in supine position)</td>
<td>…</td>
<td>86/108</td>
<td>…</td>
<td>351/789</td>
</tr>
<tr>
<td>Urine aldosterone, nmol/24-h (n: 14–55)</td>
<td>…</td>
<td>20</td>
<td>…</td>
<td>51</td>
</tr>
</tbody>
</table>

CT indicates computed tomography; DEXA, dual energy X-ray absorptiometry; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PPARγ, peroxisome proliferator-activated receptor-γ; and US, ultrasound.
Figure 1. Identification and characterization of peroxisome proliferator-activated receptor-γ (PPARγ) mutations. A, Schematic representation of the major domains of PPARγ showing the locations of the 2 novel mutations R165T and L339X (according to the PPARγ2 nomenclature). The protein sequence surrounding the R165T mutation in the PPARγ2 DNA-binding domain is shown, as well as the corresponding region of all PPAR species and nuclear receptors. The regions of the PPARγ2 DNA- (DBD) and ligand-binding domains (LBD), including the mutations, are conserved among the PPAR species. *Previously identified germline PPARG mutations in these 2 regions are indicated.

B, Fibroblast, peripheral blood mononuclear cell (PBMC), or human vascular smooth muscle cell (hVSMC) lysates were subjected to Western blotting and revealed with antibodies directed against the N- or C-terminal regions of PPARγ. To note, L339X-mutated PPARγ are recognized by the N-ter, but not the C-ter antibodies. β-actin was used as an index of the cellular protein level. Representative blots (performed in triplicate) are shown.

C, R165T- and L339X-mutated PPARγ show impaired transactivation activity. PPARγ transcriptional activity was evaluated in human embryonic kidney (HEK)-293 cells using a PPAR Cignal Reporter Assay. The cells were transfected with wild-type (WT) mutant (R165T or L339X) or empty expression vectors together with the reporter gene. Rosiglitazone (1 µmol/L) was added (white bars) or not added (dark bars) 24-h post transfection. Negative and positive controls were added to each transfection to determine background reporter activity, normalize transfection efficiencies, and visually control the transfection. The transcriptional activity of WT PPARγ was also tested in the presence of mutant PPARγ. Results are expressed as a percentage of the maximum activation obtained with WT PPARγ. Results are the mean±SEM of 4 independent experiments, each performed in triplicate. *P<0.05 vs cells transfected with WT PPARγ. #P<0.05 vs cells transfected with WT PPARγ and treated with rosiglitazone.
**PPARγ Mutations Induce Oxidative Stress and Inflammation**

The 2 *PPARG* mutations increased reactive oxygen species production in patients' fibroblasts, as demonstrated by the 5- to 6-fold and the 2.5- to 3-fold increase of CM-H$_2$DCFDA oxidation and nitro blue tetrazolium reduction, respectively (Figure 3A). The secretion of the inflammation markers interleukin-6 and MCP-1 also increased by 3- to 3.7-fold and 2.2- to 2.8-fold, in *PPARG*-mutated versus control fibroblasts, respectively (Figure 3B), in the same range whatever the mutation and the patient (not shown). Activation of the inflammation signaling was further demonstrated by the increased expression of the phosphorylated form (serine 536) of the p65/RelA subunit of nuclear factor κ-B in patients' fibroblasts (15-fold increase) and PBMCs (3- to 4-fold increase), as compared with control cells (Figure 3C).

**Effects of the Modulators of PPARγ, Rosiglitazone, and GW9662**

The strong pharmacological activator of PPARγ, rosiglitazone, markedly improved cell dysfunctions induced by heterozygous *PPARG* mutations. Incubation of cells with rosiglitazone decreased AT1R, renin, and AGT overexpression observed in patients' fibroblasts by 2- to 4-fold (Figure 4A). Rosiglitazone treatment also decreased oxidative stress, evaluated by nitro blue tetrazolium reduction (by 2.4- to 3.3-fold), and inflammation, assessed by interleukin-6 secretion (by 3.1- to 3.8-fold; Figure 4B), and p65 nuclear factor κ-B phosphorylation (by 4.2- to 6.8-fold; Figure 4A) in R165T- and L339X-mutated fibroblasts without modifying the basal level in control cells. Thus, forced activation of PPARγ by rosiglitazone in cells bearing heterozygous mutations in the *PPARG* gene could partly reverse RAS overactivation and subsequent oxidative stress and inflammation, consistent with the results of the transactivation studies.

Conversely, the irreversible inhibition of WT PPARγ by GW9662 in control fibroblasts (Figure 4C) markedly enhanced AT1R, renin, and AGT expression, and triggered oxidative stress, evaluated by nitro blue tetrazolium reduction (3.5-fold increase), and inflammation, assessed by nuclear factor κB activation (3.2-fold increase). These data further argue for the ability of PPARγ to repress RAS activation and indicate that RAS overactivation is associated with oxidative stress and inflammation.

**AT1R mRNA Silencing Decreases Oxidative Stress and Inflammation Resulting From PPARγ Mutations**

To directly evaluate the consequences of RAS inhibition, we silenced AT1R mRNA by using siRNA. As expected, AT1R siRNAs blocked AT1R overexpression in R165T- and L339X-mutated fibroblasts (5.9- to 7.4-fold decrease, Figure 5A) without affecting control cells (data not shown), in which AT1R expression was minimal (Figures 2A and 4A). Renin and AGT overexpression was also partly decreased by AT1R mRNA silencing (by 2.3- to 4.6-fold; Figure 5A). Scrambled siRNAs,
used as controls, had no effect on AT1R, renin, and AGT overexpression (data not shown). Interestingly, AT1R mRNA silencing reduced oxidative stress (evaluated by nitro blue tetrazolium reduction, by 2.5-fold) and inflammation (measured by interleukin-6 secretion, by 5-fold) resulting from PPARγ mutations (Figure 5B), arguing for the role of AT1R in these cell dysfunctions. Scrambled siRNA had no effect.

As well, the constitutive activation of nuclear factor-κB observed in R165T- and L339X-mutated fibroblasts was completely reversed by AT1R mRNA silencing (Figure 5C), further stressing the importance of AT1R overactivation in cellular inflammation resulting from PPARγ mutations.

Discussion

We report here that components of the cellular RAS were markedly overexpressed and activated in fibroblasts and PBMCs issued from 4 patients with FPLD3 attributable to novel PPARγ mutations. PPARγ mutations also induced oxidative stress and inflammation in patients’ fibroblasts. The adverse effects of the PPARγ mutations were reproduced in VSMCs overexpressing the R165T- or L339X-mutant PPARγ. The ability of a pharmacological agonist of PPARγ (rosiglitazone), and, conversely, were induced either an arginine (R165) within the DBD, or a leucine (L339) within the LBD, which induced PPARγ truncation, as confirmed by the protein expression analysis. The pathogenicity of the R165T variant could result from the loss of hydrogen bond formation by the positively charged arginine. The substituted threonine is, however, not predicted to turn out to be a new site of phosphorylation (http://www.cbs.dtu.dk/services/NetPhos). In the same region of the DBD, several other human natural PPARγ amino acid substitutions, leading to loss-of-function or dominant-negative effects, have been reported resulting in FPLD3 with severe hypertension. We demonstrated using a reporter gene assay in HEK-293 cells that these 2 new mutations, R165T and L339X, repressed basal and ligand-induced PPARγ transcriptional activity and acted via a loss-of-function mechanism.

We studied the fibroblasts and PBMCs from these 4 severely hypertensive patients. Whatever the mutation, R165T or L339X, patients’ cells exhibited increased levels of tissue RAS components, including AT1R, renin, and AGT, with overactivation of angiotensin II signaling together with oxidative stress and inflammation. Several arguments indicate that RAS overactivation might play a role in the cellular consequences of PPARγ inactivation as follows: (1) RAS overactivation and cellular dysfunctions observed in PPARγ-mutated fibroblasts were reversed, at least partially, by the PPARγ agonist rosiglitazone, and, conversely, were induced...
in control cells by the strong PPARγ antagonist GW9662 and (2) AT1R mRNA silencing, which decreased RAS markers as expected, reversed oxidative stress and inflammation in PPARγ-mutated fibroblasts. These data indicated that the patients’ cell dysfunctions may result from AT1R overactivation induced by PPARγ mutations. AT1R overactivation has been reported to have damaging consequences not only on cell cultures but also in animal studies and clinical intervention trials.41 In patients with essential hypertension, blockade of AT1R reduced the expression of markers of oxidative stress and inflammation and improved endothelial function and arterial stiffness.42 Otherwise, several studies have reported that PPARγ can directly repress AT1R by acting at the Sp1 site level.23,47,48 The mechanism whereby these new PPARγ mutations prevented PPARγ-induced repression of AT1R expression and activity deserves further studies.

Whether AT1R overactivation measured on patients’ fibroblasts or PBMCs had an influence on the patients’ vascular system is not obvious, and we did not bring evidence of systemic RAS overactivation in these patients. However, PPARγ is expressed in the vascular system where it locally exerts a protective role10,16 by the control of endothelial cell function, smooth muscle cell proliferation, and migration,45 and of the phenotype of arterial wall macrophage foam cells.5,46 In VSMCs, PPARγ constitutively represses several genes involved in atherogenesis and angiogenesis,23,47,48 including components of the tissue RAS, stressing the importance of this system in the pathophysiology of hypertension.

When we tested the effect of these 2 PPARγ mutations on human VSMCs, we demonstrated that RAS components were overexpressed in human VSMCs transfected with mutant, but not WT PPARγ. These data reinforce the hypothesis that these PPARγ mutations may deregulate the patients’ vascular RAS, thus playing a role in their hypertension. The relative importance of the vascular versus the systemic RAS has recently emerged. In certain pathological conditions and cell types, particularly hypertension, atherosclerosis, and diabetes mellitus, the intracrine pathways may be the dominant mechanism of angiotensin II effect,32 which may function independently
of the circulating RAS. Thus, our results strongly suggest that AT1R activation resulting from PPARγ mutations might contribute to severe hypertension associated with FPLD3.

Our results also indicate that PPARγ agonists, but also AT1R blockers, might be beneficial on the long term for the treatment of patients with FPLD3. Because the use of thiazolidinediones is at present restricted or even forbidden in some countries, it might be relevant for the patients to use therapeutic molecules that both inhibit the RAS and have a PPARγ agonist activity, as AT1R blockers, which were efficient to control blood pressure in these patients. Other PPARγ modulators targeted against the complex post-transcriptional modifications of PPARγ have become a topic of therapeutic interest. Their capacity to restore a normal PPARγ transrepression mechanism of the RAS together with improved insulin signaling in the patients’ cells should be evaluated.

In conclusion, our data suggest that severe hypertension, which is a peculiar feature of patients with FPLD3 versus other lipodystrophic syndromes, might be linked to tissular RAS overactivation resulting from PPARγ dysfunction. In addition, overactivation of AT1R signaling could participate in cellular oxidative stress and inflammation observed in PPARγ-mutated cells, stressing the important role of the tissular RAS in these cell functions.

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Disclosures
None.

References


46. Auclair et al. PPARγ Mutations, Severe Hypertension, and RAS. 2008;837:837.
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Materials & Methods

Patients, cell cultures and treatment

Primary skin fibroblast cultures were established after punch biopsy in two families harboring PPARG mutations (R165T or L339X, each in two patients). Cells were removed from their in vivo environment and cultured during several passages which discarded the possibility of a residual effect of any medication, and allowed us to study their intrinsic characteristics. PPARG mutations were identified after amplification of genomic DNA using specific oligonucleotides and direct sequencing. Fibroblasts from two non-obese, non-diabetic, normotensive 20 and 33 yr-old women without any cardiovascular disease undergoing plastic surgery were used as controls. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood from the four patients and three healthy controls without diabetes and hypertension. All the subjects gave their written informed consent for these studies which have been approved by an institutional review committee.

Control and/or PPARG-mutated fibroblasts were cultured and incubated or not for 5 days with rosiglitazone (1 µmol/L, Alexis Corporation, Lausen, Switzerland), or for 24 hours with the PPARγ antagonist 2-chloro-5-nitro-N-phenylbenzamide (GW9662, 1µmol/L). Stock solutions of rosiglitazone or GW9662 were prepared in dimethylsulfoxide (DMSO, final concentration ≤ 0.05%). Commercially obtained human VSMCs from coronary artery (HCASMC, C-12512, PromoCell GmbH, Heidelberg, Germany) were cultured in smooth muscle growth medium 2 and transfected as described below.

Constructions of PPARG mutants and transfection assays

Full length wild-type (WT) and R165T- and L339X-mutated PPARγ2 cDNA were cloned in pcDNA3.1(+) to yield wild-type or mutant PPARγ expression vectors, respectively (GeneCust, Dudelange, Luxembourg). Human VSMCs were transfected with TurboFect™ (Fisher Scientific SAS, Illkirch, France) according to the instructions of the manufacturer. The resulting transfection efficiencies with wild-type and/or mutant PPARγ expression vectors were more than 70 %.

PPARγ transactivation assays

Transactivation assays were carried out with the PPAR Cignal Reporter Assay kit (Qiagen S.A.S., Courtaboeuf, France) in HEK-293 cells according to the instructions of the manufacturer. The PPAR reporter system is a mixture of a PPAR-responsive luciferase construct and a constitutively expressing Renilla luciferase construct (40:1) under the
transcriptional control of a minimal CMV promoter and tandem repeats of a PPAR transcriptional response element. HEK-293 cells were transfected with expression vectors containing WT or mutant PPARγ (100 ng) alone or in combination (ratio 1:1). When indicated, HEK-293 cells were treated with 1 µmol/L rosiglitazone for 24-h post transfection. The PPARγ activity was monitored by a dual luciferase assay (Promega Corporation, Charbonnieres, France).

**Western blotting**

Cell extracts were subjected to SDS/PAGE and western blotting. Antibodies against N-terminal (SC-7196) or C-terminal (γ1 and γ2, SC-6284) PPARγ, angiotensinogen (AGT) (SC-20717), AT1R (SC-1173), renin (SC-27318), and total or activated extracellular signal-regulated kinase (ERK) (ERK 1/2, SC-154 and p-ERK, SC-7383) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Beta-actin (A-5441, Sigma-Aldrich, Saint Louis, MO, USA) was used as an index of the cellular protein content. Antibodies against NF-κB p65/RelA (#3987) and phospho-NF-κB p65/RelA (ser 536) (#3033) were from Cell Signaling Technology, Inc. (Danvers, MA, USA).

**Oxidative stress and inflammation**

The production of reactive oxygen species (ROS) was indirectly measured by the oxidation of CM-H₂DCFDA derivatives (5- (and 6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate, acetyl ester) and the reduction of NBT as described. Secretion of interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) was analyzed into 24-h culture supernatants. Inflammation was also determined by the protein expression of the phosphorylated form (serine 536) of the p65/RelA subunit of NF-κB.

**AT1R mRNA silencing**

We used small interfering RNA (siRNA) to selectively inhibit the mRNA expression of AT1R. Fibroblasts were incubated for 6 hours with 100 pmoles of a pool of three target-specific AT1R siRNA (SC-29750, Santa Cruz Biotechnology) in the transfection reagent (SC-29528). The effect of AT1R siRNA was evaluated 3-days later. The appropriate controls with scrambled siRNA (SC-37007) were performed in parallel.

**Statistical analysis**

All experiments were performed at least three times on triplicate samples. All quantitative results were expressed as mean ± SEM. Statistical significance was determined using
ANOVA and the Kruskal-Wallis nonparametric test, followed by a Fisher protected least significant difference test for pair-wise differences. \( P \) values of less than 0.05 were considered significant.

References

