Angiotensin II type 2 receptor promotes adipocyte differentiation and restores adipocyte size in high-fat/high-fructose diet-induced insulin resistance in rats

Michaël Shum, Sandra Pinard, Marie-Odile Guimond, Sébastien M. Labbé, Claude Roberge, Jean-Patrice Baillargeon, Marie-France Langlois, Mathias Alterman, Charlotte Wallinder, Anders Hallberg, André C. Carpenter, and Nicole Gallo-Payet

1 Division of Endocrinology, Department of Medicine, Faculty of medicine and sciences of the santé, Université de Sherbrooke, Sherbrooke, Québec, Canada; 2 Department of Medicinal Chemistry, Biomedicinska Centrum, Uppsala University, Uppsala, Sweden

Submitted 22 March 2012; accepted in final form 7 November 2012

High-fat/high-fructose (HFHF) diet induces insulin resistance in both adipocytes and nonadipose tissues (7, 16, 36). Therefore, an understanding of the specific properties and regulation of visceral (dysfunctional adipose tissue) depots in obesity and type 2 diabetes, and cardiovascular diseases (7, 25, 36, 50). In contrast, AT2R is highly expressed in fetal life, while in the adult its expression is much more variable, according to tissues and pathophysiologically situations (for recent reviews see Refs. 25, 45, 46, 51, 56). In conditions of insulin resistance, several studies, although not all (38), suggest that AT1R blockade provides end-organ protection independently of its antihypertensive effect by decreasing the size of adipocytes in visceral adipose tissue and by reversing the profile of adipokine secretion associated with obesity and type 2 diabetes (13, 17, 34, 48, 61) (for review see Refs. 20, 25). Therapeutic responses to angiotensin receptor blockers (ARBs) may result from enhanced AT2R activation in addition to inhibition of AT1R. Indeed, when AT1R is blocked, circulating ANG II binds to and activates AT2R only (45). However, most of the effects reported for AT2R have been inferred from studies using AT1R inhibition with ARBs (13, 49) or in AT2R-deficient mice (24, 57). Even in these conditions, it has proved difficult to clearly establish whether AT2R may play a role in adipocyte differentiation and adipose tissue physiology. Indeed, in the study of Iwai et al. (24) using atherosclerotic apoE-KO mice with an AT2R deficiency (AT2R/apoE double-knockout mice), the lack of AT2R decreased the expression of adipocyte differentiation factors, whereas adipocyte size, plasma cholesterol, and free fatty acid levels were increased. Conversely, Yvan-Charvet et al. (57) documented an increased number of small adipocytes in AT2R-deficient mice, and those mice were protected against high-fat diet-induced obesity.

In humans, it is well accepted that dysfunctional visceral adipose tissue is associated with an increased risk of insulin resistance and cardiovascular diseases (8, 15, 53). In contrast, increasing lipid storage in subcutaneous (SC) adipose tissue may be protective (1, 2, 15, 23, 27, 52, 53). This safe storage of lipids in adipose tissue is key to preventing lipotoxicity in nonadipose tissues (7, 16, 36). Therefore, an understanding of the specific properties and regulation of visceral (dysfunctional TG storage) as opposed to SC (functional TG storage) adipose tissues is of paramount importance (8, 15, 23).

Until recently, it was difficult to directly address the role of AT2R due to the absence of high affinity and selective ligands. Indeed, in addition to CGP42112A, only two AT2R agonists were available (35, 43), but all are peptides and not readily used in in vivo studies. In this context, we developed a stable, nonpeptide, and highly selective AT2R agonist, C21, now renamed M24 (14, 54). Using this compound, recent publications have argued in favor of a protective role of AT2R in...
Table 1. Primer sequences used for PCR

<table>
<thead>
<tr>
<th>Genes, Rats</th>
<th>Primer Sequences Forward, 5‘-3‘</th>
<th>Reverse, 5‘-3‘</th>
<th>Amplicon Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATR2</td>
<td>(F) GGTCTGCTGAGATGCTTTAAGT</td>
<td>(R) AGGAATGGTCTGACTGTTTCT</td>
<td>142</td>
</tr>
<tr>
<td>ATR1A</td>
<td>(F) GGTGGTACCTCGGATCTTCTTCT</td>
<td>(R) CTGTTGATTCGAGGAGA</td>
<td>121</td>
</tr>
<tr>
<td>ACTB</td>
<td>Primer pair from Qiagen</td>
<td></td>
<td>145</td>
</tr>
<tr>
<td>GAPDH</td>
<td>(F) TGGTGGAAAAGGTTCATC</td>
<td>(R) TGGTGCGATGGCGAAGTGGTTG</td>
<td>176</td>
</tr>
</tbody>
</table>

ATR2, angiotensin II receptor, type 2; ATR1A, angiotensin II receptor, type 1A; ACTB, β-actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Sequence of synthesized oligonucleotides of shAT2R

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>shAT2R. A</td>
<td>5’-CGGGGATTGACATCATGGTTTGCTGAGATGCTTTAAGCTGATGGAAGCCAGAAATGATGAAATGTTTT-3’</td>
</tr>
<tr>
<td>shAT2R. B</td>
<td>5’-CGGGGATTGACATCATGGTTTGCTGAGATGCTTTAAGCTGATGGAAGCCAGAAATGATGAAATGTTTT-3’</td>
</tr>
</tbody>
</table>

shRNA sequences used to downregulate mRNA level of AT2R. Boldface letters correspond to the loop of the shRNA sequences.
Fig. 1. Time-course of preadipocyte differentiation from rat retroperitoneal (RET) and subcutaneous (SC) adipose tissue in primary culture. Preadipocytes from RET (A) and SC (B) adipose tissues were plated in 35-mm Petri dishes at a density of 1.5 × 10^4/cm^2. To initiate the process of differentiation, cells were cultured in DMEM-F12 medium-enriched differentiation cocktail as described in RESEARCH DESIGN AND METHODS. A and B: phase-contrast microscopy of SC and RET preadipocytes. Images were acquired at days 2, 4, 6, and 12 using a Leica microscope equipped with a ×20 objective. Squares in top represent areas selected for magnification shown in bottom. Undifferentiated cells, elongated fibroblast-like cells or more polygonal cells, still persisted in SC cultures. Scale bars, 60 μm for full-size images, 20 μm for magnifications. C: proteins were extracted before differentiation (day 0, D0) and after 2, 4, 6, 8, and 12 days in differentiation medium and processed for Western blotting using appropriate primary antibodies against PPARγ (1:1,000), aP2/FABP4 (1:2,000), hormone-sensitive lipase (HSL; 1:2,000), fatty acid synthase (FAS; 1:2,000), perilipin A, and β-actin antibody (1:8,000, 1:2,000). Data shown are representative results from 3 independent experiments.
expression in primary cultures of preadipocytes was achieved using lentiviral constructs. Two AT2R shRNA sequences (shRNA A: TRCN0000027316 NM_007429.2–956s1c1; (shRNA B: TRCN0000027390 NM_007429.2–1229s1c1) subcloned into the pLKO.1-Puro vector and one control shRNA (empty vector) obtained from Sigma-Aldrich were used, as described in Table 2. The shRNA A was more potent in abolishing AT2R expression and was thus selected for subsequent experiments.

In vivo experiments. For in vivo experiments, Wistar rats (8 wk old) were randomly distributed in control or treated groups. Experimental animals received a high-fat/high-fructose (HFHF) diet [consisting of Teklad rat chows containing 46.5% fructose and 25.7% lard (TD no. 05482 rat chow), Teklad, Harlan Laboratories, Mississauga, ON, Canada] for 6 wk, whereas the control groups were fed a standard laboratory rodent diet (Rodent laboratory chow 5001 from Purina), as previously described (32). During the experiments, rats had free access to food and water. Weight, energy intake, and water consumption were measured three times a week. Both diet groups were subsequently subdivided into treatment groups: the standard chow diet-fed control group was treated with saline or C21/M24 (0.3 mg·kg⁻¹·day⁻¹), while the HFHF group was treated with saline, C21/M24 (0.3 mg·kg⁻¹·day⁻¹) or losartan (1 mg·kg⁻¹·day⁻¹). Treatments were administered intraperitoneally by constant infusion via Alzet osmotic minipumps (model 2006, Durect, Cupertino, CA). After 6 wk of diet and treatments, rats were fasted overnight and submitted to 2 h of euglycemic-hyperinsulinemic clamps to determine their insulin sensitivity, as previously described (32). At the end of experimentation, rats were euthanized by decapitation. Blood samples were collected for metabolic measurements [glucose, triglycerides (TG), nonesterified-fatty acids (NEFA)] as well as insulin assay. Adipose tissues (SC, RET), liver, heart, and muscles (gastrocnecmius, soleus) were also collected. Adipose tissues were excised, washed with isotonic saline solution, and subsequently fixed in phosphate-buffered 4% formaldehyde and embedded in paraffin blocks; 5 μm paraffin sections were prepared, mounted on slides, and stained with hematoxylin-eosin (H&E). Images were acquired with a Nikon Eclipse 300 microscope (Mississauga, ON, Canada) equipped with a CoolSnap fx digital camera (Roper Scientific, Tucson, AZ). Images were acquired using a ×20 objective. Measurement of adipocyte size (as area in μm²) was performed as described above with a custom-designed software program written using MATLAB.

Laboratory assays. Blood glucose levels were measured with an Accu-Chek Aviva nanoglucometer (Roche, Mississauga, ON, Canada). Plasma insulin was determined by radioimmunoassay (Linco Research, St. Charles, MO). Triglycerides (TG) and nonesterified fatty acids (NEFA) were measured by enzymatic methods according to the manufacturer’s instructions (Wako Chemicals, Hayward, CA). Liver lipids were extracted with chloroform-methanol 2:1 as described by Folch et al. (10). The concentration of hepatic TG was measured with enzymatic reagent kits (Roche, Indianapolis, IN).

Data analysis. The data are presented as means ± SE of the number of experiments indicated in the text. Statistical analyses of the
RESULTS

SC and RET preadipocytes exhibit different differentiation profiles. Preadipocytes were cultured in the presence of a well-established cocktail of differentiating factors. Both were characterized by a progressive accumulation of lipid droplets, which began after 3–4 days and progressed until day 12 onward. At that time, RET adipocytes contained two to five large lipid droplets (Fig. 1A), whereas SC adipocytes were more heterogeneous, with cells exhibiting a few large lipid droplets, often surrounded by small lipid droplets (Fig. 1B). These morphological changes were accompanied by a time-dependent increase in the expression of selective markers of differentiation (PPARγ and aP2/FAPB4) and of adipogenesis (HSL, FAS, and perilipin A; Fig. 1C).

SC and RET adipocytes express AT1R and AT2R. Time course analyses indicated that in SC preadipocytes the level of AT2R mRNA increased at the onset of the differentiation process, remained elevated throughout differentiation, and decreased after day 8. In contrast, in RET cells, AT2R mRNA was already present at the beginning of differentiation and decreased rapidly after 4 days of differentiation (Fig. 2A). AT1R mRNA levels, on the other hand, did not change significantly throughout the time course of differentiation in either SC or RET cultures (Fig. 2B). Western blotting confirmed the presence of the receptors in both cell types. Although there was a marked increase in AT2R expression at day 2 of differentiation, AT1R and AT2R expression levels remained thereafter relatively stable throughout the experimental period (Fig. 2, C–F).

![Fig. 3. Effect of selective ligands of type 1 and type 2 receptors on morphology of preadipocytes from SC rat adipose tissue in primary culture. Preadipocytes were plated in 35-mm Petri dishes at a density of 1.5 × 10^4/cm^2 and cultured for 7 days as described in RESEARCH DESIGN AND METHODS in the absence (control) or presence of ANG II (100 nM), or C21/M24 (10 nM) alone or in combination with AT1R antagonist losartan (LOS; 1 µM) or AT2R antagonist PD123,319 (PD; 1 µM). A: histograms illustrating percentage of cell types exhibiting small lipid droplets (LD). B: analyses of mean diameter of small LD. Values were obtained from data analyses (histograms and fitting curves) constructed as described in RESEARCH DESIGN AND METHODS and in Supplemental Data. Data are means ± SE of LD from 36 images taken from 3 different Petri dishes from 2 different experiments. Results include mean values obtained at day 7 in culture, thus n = 24. Statistical analyses were performed using two-way ANOVA followed by Bonferroni multiple comparisons. Statistical significance vs. respective control: ***P < 0.001. C: preadipocytes were plated in 35-mm Petri dishes at a density of 1.5 × 10^4/cm^2 and cultured for 7 days in the absence (control) or presence of ANG II (100 nM), or C21/M24 (10 nM) alone or in combination with losartan (1 µM) or PD123,319 (1 µM). Scale bars, 40 µm.](http://ajpendo.physiology.org/doi/abs/10.1152/ajpendo.00149.2012)
Selective pharmacological activation of AT2R has opposite effects on cell morphology in SC adipocytes. SC and RET preadipocytes were cultured in the absence or presence of ANG II (100 nM) or the AT2R agonist C21/M24 (10 nM), alone or in the presence of the respective AT1R (losartan, 1 μM) or AT2R (PD123,319, 1 μM) antagonists. Although it was difficult to assess changes in the size of adipocytes themselves, selective effects were clearly observed on both the number and size of lipid droplets. During the process of differentiation (between days 2 and 7 of culture), C21/M24 treatment increased the proportion of cells exhibiting small lipid droplets (≤5.5 μm), an effect not modified by cotreat-
ment with losartan, but abolished with PD123,319 (Fig. 3, A and C). ANG II alone also increased the proportion of cells accumulating lipid droplets. However, the various treatments did not affect the size of these small LD (Fig. 3, B and C). In contrast, such effects were not observed in RET preadipocytes (not shown).

After 11 days of culture and onward, stimulation of SC adipocytes with C21/M24 induced a significant reduction in the number (Fig. 4A) and size (Fig. 4B) of large lipid droplets (19–25 μm) while increasing the proportion of cells exhibiting medium LD (13–15 μm; Fig. 4C), without altering that of small LD (≤5.5 μm; Fig. 4D). These effects were not modified by treatment with losartan, whereas incubation with PD123,319 abolished the effect of C21/M24 and significantly blunted the effect of ANG II on the size of large LD (Fig. 4B). Of note, the percentage of cells exhibiting medium and small LD was not modified by ANG II (Fig. 4, C and D). Representative images are illustrated in Fig. 4E. These same treatments, on the other hand, did not affect the appearance of RET adipocyte morphology, nor their size, which were all very large, even in control conditions (data not shown).

Selective AT2R stimulation increases lipid accumulation and PPARγ during adipogenesis in SC adipocytes. Stimulation of cells for 11 days with various doses of C21/M24 significantly increased lipid accumulation in SC but not in RET preadipocytes (Fig. 5, A and B). When stimulated with C21/M24 (1 nM), this increase was mainly due to AT2R activation, since this effect was abolished by PD123,319 while not being affected by losartan (Fig. 5C). In contrast, adipocytes stimulated with ANG II showed less lipid accumulation both in SC and RET preadipocytes, an effect decreased by losartan in SC and abolished in RET (Fig. 5, C and D). Furthermore, losartan

![Figure 5](https://via.placeholder.com/150)

**Fig. 5.** Effect of AT2R activation on lipid accumulation (A–D) and on on PPARγ expression (E and F) in rat adipocytes from SC and RET adipose tissue. SC and RET preadipocytes were plated in 35-mm Petri dishes at a density of 1.5 × 10^4/cm² and cultured for 12 days as described in RESEARCH DESIGN AND METHODS. Treatments were initiated at day 0 and continued every 2 days. SC (A) and RET (B) preadipocytes were treated with C21/M24 from 0.01 to 100 nM. Preadipocytes from SC (C) and RET (D) adipose tissues were treated with ANG II (100 nM) or C21/M24 (1 nM) alone or in combination with AT1R antagonist losartan (1 μM), or AT2R antagonist PD123,319 (PD; 1 μM). Lipid accumulation was assessed with an AdipoRed kit on day 11. Results are expressed as means ± SE of ≥6 independent experiments, each condition conducted in quadruplicate. Results were corrected for protein content. Statistical analyses were performed using one-way ANOVA followed by Newman-Keuls post hoc test. Statistical significance: * P < 0.05, *** P < 0.001; vertical asterisks represent comparison with respective controls; horizontal lines indicate comparison between groups. SC (E) and RET (F) preadipocytes were plated in 35-mm Petri dishes at a density of 1.5 × 10^4/cm² and cultured until day 12 as described in RESEARCH DESIGN AND METHODS. Proteins were extracted on days 0, 2, 4, 6, 8, and 12, and cell lysates containing equal amounts of protein (20 μg) were processed for Western blotting using a primary antibody against PPARγ (1:1,000). Expression levels of PPARγ were normalized to that of actin content with an anti-β-actin antibody (1:8,000). Statistical analyses were performed using one-way ANOVA followed by Tukey’s multiple comparison test. Statistical significance vs. respective control: * P < 0.05 (n = 4–5).
alone stimulated lipid accumulation in SC adipocytes (Fig. 5C). During the time course of differentiation, C21/M24 increased PPARγ expression in both SC and RET preadipocytes, with significant differences at days 4 and 6, respectively (Fig. 5, E and F).

Finally, primary adipocytes, infected with lentivirus expressing shRNA-mediated knockdown of AT2R (shAT2R) exhibited a spindle-like or polygonal morphology, without any lipid droplets, whereas cells infected with control shRNA (shCtl) differentiated normally (Fig. 6A). In shAT2R-infected cells, AT2R protein expression was abolished, and expression of the differentiation markers PPARγ and aP2 remained very low or absent (Fig. 6B). These results clearly indicate that AT2R is critical for initiating preadipocyte differentiation and lipid accumulation.

Six-week treatment with C21/M24 restores adipocyte morphology and improves insulin resistance in vivo. In an attempt to correlate the above in vitro observations with the in vivo situation, rats were fed an HFHF diet for 6 wk to induce insulin resistance. Analyses of adipocyte size distribution in several adipose tissue depots revealed that the HFHF diet resulted in a significant shift toward larger adipocytes, particularly in SC and RET adipose depots (Fig. 7, A–D, and Fig. 8C, C = vs. A, A =). Treatment of HFHF-fed rats with C21/M24 or with the AT1R antagonist losartan prevented this shift and restored the normal distribution profile of adipocyte size (Fig. 7, A and B, and Fig. 8D, D = vs. A, A =), and the overall mean diameter of LD decreased both in SC and RET adipocytes (Fig. 7, C and D). These results indicate that both blockade of AT1R and stimulation of AT2R induced the same effect on adipocyte size distribution in HFHF-fed rats. In addition, C21/M24 treatment decreased SC adipocyte size in the control group but not in RET adipose tissue (Fig. 7, A and C, and Fig. 8B, B = vs. A, A =). Western blot analyses indicated that, in SC adipose tissues, HFHF diet for 6 wk increased AT1R expression compared with control groups and that this increase was prevented by both C21/M24 and losartan. No significant differences between groups were observed among other measured protein expression levels, except for a tendency for C21/M24 to increase PPARγ in RET adipose tissues in control groups (Fig. 9, A–D).

Six weeks of HFHF diet had only a small, nonsignificant impact on caloric intake and body weight but led to an increase in total adipose tissue mass without change in lean body mass (Table 3). Rats under HFHF diet developed hyperinsulinemia and insulin resistance, as shown by the decreased glucose infusion rate (GIR) during euglycemic-hyperinsulinemic clamp. C21/M24 and losartan treatment decreased hyperinsulinemia and improved diet-induced insulin resistance, as indicated by the significant increase in GIR (Table 4). Under fasting conditions, C21/M24 treatment also reduced insulin and TG levels in the control diet, but had no significant effect on NEFA and TG in the HFHF groups. In addition, we observed that only losartan was able to reverse TG content increased by HFHF diet in liver (Table 4). Of note, although

![Fig. 6. Effect of inactivation of AT2R expression on adipocyte differentiation. SC and RET preadipocytes were noninfected (normal) or infected with control (shCtl) or shAT2R lentivirus, followed by initiation of differentiation by adding a standard differentiation medium for 8 days. A: illustrations of SC and RET differentiated preadipocytes untreated (without shRNA), with control shRNA (shCtl), and with AT2R shRNA (shAT2R) at day 8. Scale bars, 60 μm. B: cell lysates from conditions in A (20 μg) were analyzed by immunoblotting with antibodies against AT2R (1:500), PPARγ (1:1,000), or aP2/FABP4 (1:2,000) and normalized to anti-β-actin antibody (1:8,000) for protein content. Data shown are representative results from 3 independent experiments.](http://ajpendo.physiology.org/fig6.jpg)
there was an absence of significant changes in fasting NEFA and TG in the HFHF groups, C21/M24 improved the insulin-mediated lowering of blood NEFA and TG concentrations during the clamp studies compared with saline treatment in the HFHF group, an effect not observed with losartan treatment. Results also indicated a correlation between larger adipocytes with lower GIR in both SC and RET adipose tissues (Fig. 10, A and B).

DISCUSSION

The role of ANG II receptors in adipocytes and in insulin resistance has generated considerable interest in recent decades (59). The present data are the first to address the direct effects of AT2R stimulation in adipocytes from SC and visceral adipose tissues. Results show that selective AT2R activation acts preferentially on SC preadipocytes and exhibits two opposite effects according to the differentiation status of the cells. In primary cultures of preadipocytes, we demonstrate that AT2R is involved in the initial steps of differentiation by favoring PPARγ expression and early lipid accumulation. However, once adipocytes reach their full capacity to store lipids, AT2R stimulation decreases the size of large lipid droplets but increases lipid accumulation. On the other hand, in a model of diet-induced insulin resistance (HFHF rats), treat-
ment with the AT2R agonist C21/M24 or the AT1R antagonist losartan for 6 wk is sufficient to restore normal adipocyte size distribution and reduce HFHF diet-induced insulin resistance.

We found that both AT1R and AT2R were present in primary cell cultures of preadipocytes from rat SC and RET adipose tissue, thus corroborating previous results in primary cultures of human SC preadipocytes (40, 55). However, AT2R protein expression differs from its mRNA expression in RET preadipocytes. One hypothesis may be that the protein turnover of AT2R could be very slow compared with its mRNA level.

Table 3. Tissue weights after 6-wk treatment with C21/M24 or losartan in control- and HFHF-fed rats

<table>
<thead>
<tr>
<th>Diet</th>
<th>SC adipose tissue</th>
<th>RET adipose tissue</th>
<th>Gastronecimus</th>
<th>Soleus</th>
<th>Heart</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.60 ± 0.37</td>
<td>5.0 ± 0.8</td>
<td>5.36 ± 0.17</td>
<td>0.47 ± 0.01</td>
<td>1.27 ± 0.03</td>
<td>12.7 ± 0.4</td>
</tr>
<tr>
<td>C21/M24</td>
<td>3.49 ± 0.33</td>
<td>3.8 ± 0.4</td>
<td>5.52 ± 0.12</td>
<td>0.48 ± 0.01</td>
<td>1.22 ± 0.05</td>
<td>12.6 ± 1.1</td>
</tr>
<tr>
<td>HFHF</td>
<td>7.36 ± 1.07**</td>
<td>10.8 ± 1.2*</td>
<td>5.43 ± 0.12</td>
<td>0.52 ± 0.03</td>
<td>1.25 ± 0.06</td>
<td>12.0 ± 0.6</td>
</tr>
<tr>
<td>LOS</td>
<td>8.16 ± 1.03**</td>
<td>11.3 ± 2.4*</td>
<td>5.14 ± 0.24</td>
<td>0.49 ± 0.02</td>
<td>1.23 ± 0.06</td>
<td>12.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>6.92 ± 0.57*</td>
<td>11.2 ± 1.9*</td>
<td>5.25 ± 0.11</td>
<td>0.51 ± 0.03</td>
<td>1.27 ± 0.03</td>
<td>12.8 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE, in g, from 9-10 animals per group. Control, standard diet-fed rats; HFHF, high-fat/high-fructose-fed rats; SC, subcutaneous; RET, retroperitoneal; Sal, saline treatment; LOS, losartan treatment; Statistical analysis was performed using one-way ANOVA with Newman-Keuls post hoc test; *P < 0.05, **P < 0.01, difference between HFHF diet vs. saline and C21/M24 in control diet.
Indeed, the mechanism of AT2R degradation is still unknown and may vary according to experimental model (19, 33). Thus the protein turnover of AT2R could be very slow compared with its mRNA level.

Direct activation of AT2R with C21/M24 increased the expression of PPARγ, whereas AT2R downregulation by shRNA infection blunted PPARγ expression and prevented preadipocyte differentiation in both SC and RET preadipocytes. These results thus demonstrate that AT2R is involved in the first steps of preadipocyte differentiation and suggest that PPARγ activation could represent one mechanism by which AT2R initiates differentiation, as previously shown in neuronal cells (60). However, C21/M24 accelerated the appearance of LD and selectively increased lipid accumulation only in SC preadipocytes. These discrepancies between SC and RET preadipocytes are of particular interest, since knockdown of AT2R with shRNA had the same effect in both types of adipocytes, indicating that expression of AT2R is essential, but not necessarily its activation. Such a hypothesis may be explained by the constitutive activity of the receptor prior to differentiation (12).

In that condition, AT2R action may thus occur through interaction with some of its recently identified partner proteins such as promyelocytic leukemia zinc finger (PLZF) and AT2R-interacting protein (ATIP) (for recent review see Ref. 21). However, the role of these interactions is not yet fully understood.

In contrast to the C21/M24 results in which lipid storage was increased, cells stimulated solely with ANG II exhibited a decrease in lipid accumulation, suggesting that ANG II, through the AT1R, was unable to favor TG accumulation in both SC and RET adipocytes. Accordingly, Cabassi et al. (4) have shown that ANG II stimulates lipolysis in both SC and RET adipose tissue by AT1R activation in Sprague-Dawley rats. In addition, ANG II-treated rats exhibited lower adipose tissue mass and lower TG content in adipose tissue depots. These effects were inhibited by losartan. Therefore, ANG II-stimulated lipolysis by AT1R may explain the decrease in lipid accumulation observed in our in vitro model.

As largely documented (30, 31, 37, 42, 52), we observed enlarged adipose tissue in both visceral and SC adipose tissue depots in HFHF-treated rats. In this situation, C21/M24 and losartan decreased adipocyte size in SC and RET adipose tissue depots, restoring adipocytes of smaller size, even though these treatments did not change total adipose tissue mass in HFHF-fed rats. Decreasing the size of adipocytes under lipid overload may limit the damages associated with large adipocytes. Such a strategy may also increase the ability of adipocytes to further store TG. Indeed, a new hypothesis proposes that increased adipose tissue storage capacity, enabled by increasing the storage capacity of individual adipocytes as well as stimulating the differentiation of new preadipocytes, may limit adipocyte hypertrophy and improve adipose tissue function (47, 52). The inverse relationship between adipocyte size and insulin sensitivity observed herein has also been described previously (1).

In various mouse and rat models of type 2 diabetes, ARB administration increased the number of differentiated adipocytes, PPARγ expression, and improved insulin sensitivity (5, 9, 13, 29, 41, 49, 61). Supporting a link between AT2R-increased PPARγ expression and AT2R-induced differentiation, PPARγ-activating drugs (such as thiazolidinediones) have been described as the prototypical example of the potent antidiabetic effects of PPARγ-mediated adipose tissue expansion and improvement in metabolic functions (28). The present study also demonstrates that direct and selective AT2R stimulation can reproduce the effects of AT1R blockers and are in agreement with studies conducted in preadipocyte cell lines (6) and in AT2R-deficient mice (24, 49), thus suggesting a role of AT2R in adipogenesis. However, certain results obtained herein are in contrast with whole body AT2R-deficient mice studies from Yvan-Charvet et al. (57). The major difference is that we show that AT2R activation improves insulin sensitivity by decreasing the size of large adipocytes and by increasing new, small adipocytes without inducing obesity, whereas Yvan-Charvet and colleagues (57, 58) showed that AT2R deletion improved insulin sensitivity and prevented diet-induced obesity. In both models, adipocyte physiology was restored. However, adipose tissue from AT2R-deficient mice appears to be resistant to normal expansion upon physiological insulin such as a high-fat diet. It is known that adipose tissue is an important endocrine tissue and a sink for lipids to prevent lipotoxicity (47, 52). Thus, stimulating AT2R may be better than blocking it to restore normal adipocyte physiology. Our results are nevertheless in agreement with their studies on adipose tissue since we show that AT2R expression is essential.

Table 4. Metabolic parameters after 6-wk treatment with C21/M24 or losartan in control- and HFHF-fed rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>C21/M24</th>
<th>LOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total caloric intake, kcal/6 wk</td>
<td>100.8 ± 2.3</td>
<td>99.9 ± 2.6</td>
<td>102.3 ± 2.4</td>
</tr>
<tr>
<td>Water intake, ml/day</td>
<td>33.76 ± 1</td>
<td>39.22 ± 0.9</td>
<td>28.33 ± 2</td>
</tr>
<tr>
<td>Fasting glucose, mmol/l</td>
<td>8.4 ± 0.5</td>
<td>8.5 ± 0.4</td>
<td>10.0 ± 0.5</td>
</tr>
<tr>
<td>Fasting insulin, pmol/l</td>
<td>129.4 ± 60</td>
<td>77.4 ± 20</td>
<td>404.6 ± 125</td>
</tr>
<tr>
<td>Fasting NEFA, μmol/l</td>
<td>454 ± 50</td>
<td>414 ± 33</td>
<td>514.1 ± 41</td>
</tr>
<tr>
<td>Fasting TG, μmol/l</td>
<td>504.1 ± 52</td>
<td>369 ± 35</td>
<td>662 ± 91</td>
</tr>
<tr>
<td>Liver TG content, mg/g liver</td>
<td>2.15 ± 0.15</td>
<td>1.76 ± 0.20</td>
<td>3.07 ± 0.24</td>
</tr>
<tr>
<td>Clamp NEFA, μmol/l</td>
<td>0.041 ± 0.003</td>
<td>0.036 ± 0.003</td>
<td>0.051 ± 0.003</td>
</tr>
<tr>
<td>Clamp TG, μmol/l</td>
<td>0.222 ± 0.016</td>
<td>0.159 ± 0.020</td>
<td>0.264 ± 0.028</td>
</tr>
<tr>
<td>GIR, μmol/kg/min</td>
<td>133.2 ± 8.3</td>
<td>138.8 ± 7.5</td>
<td>77 ± 5.7</td>
</tr>
</tbody>
</table>

Values are means ± SE from 9-10 animals per group. NEFA, nonesterified fatty acids; TG, triglycerides; GIR, glucose infusion rate. Statistical analysis was performed using one way ANOVA followed by Newman-Keuls test. *P < 0.05, difference between saline in HFHF diet and C21/M24 in control diet; **P < 0.05, difference between C21/M24 vs. saline in control diet; †P < 0.05, difference between C21/M24 vs. saline in HFHF diet; ‡P < 0.05 difference between C21/M24 in control diet vs. saline in HFHF diet; §P < 0.05, difference between losartan vs. saline in HFHF diet; ¶P < 0.05, difference between losartan vs. saline in control diet; ‖P < 0.05, difference between losartan in control diet vs. saline and C21/M24 in HFHF diet.
For adipo genesis in vitro, an effect similar to a reduction in adipo genesis in AT2R-deficient mice. Moreover, our study also confirms that stimulating AT2R not only does not induce obesity itself, an effect that might be expected from the results from AT2R-deficient mice, but also improves insulin sensitivity.

We also observed some discrepancies between the effects of C21/M24 in vitro vs. in vivo. Indeed, C21/M24 improved adipocyte size in both SC and RET adipose tissue, but in vitro C21/M24 acted only in SC preadipocytes. Besides the fact that C21/M24 could act on other tissues or cells that could improve adipocyte size, lower AT1R expression in SC adipose tissue of C21/M24- and losartan-treated animals cannot explain the improvement of adipocyte size, since in RET adipose tissue AT1R expression is similar among all groups. Moreover, some distinctions should be made about the projection of our pharmacological in vitro results to in vivo, since we worked on differentiated preadipocytes (multilocular) and not fully mature (unilocular) adipocytes as in adipose tissue. Indeed, our in vitro model aims more at the differentiation process that may also affect adipocyte size in vivo than the effect of AT2R and AT1R on adipocyte size from mature adipocyte. On the basis of our results and others’ (24, 57), we think that the effect of C21/M24 and losartan on adipocytes size is more due to an increase in number of new adipocytes by increasing adipogenesis, which increases the lipid storage capacity of the adipose tissue rather than by a direct effect on reduction of adipocyte size.

In conditions of insulin resistance and after hyperinsulinemic-euglycemic clamp, Hsieh et al. (22) showed that AT1R blockade increased whole body glucose uptake, in part due to an unmasked AT2R activation by AT1R blockade. In our model of HFHF diet, both C21/M24 and losartan were able to improve insulin resistance. To date, our study is the first to document that selective AT2R activation can also improve insulin resistance as previously depicted with AT1R antagonists. The fact that we did not observe a significant improvement in fasting glucose, TG, or NEFA with C21/M24 or losartan may be due to our experimental approach, designed to induce a moderate state of insulin resistance without any excessive disturbances in AT1R-mediating effects. Indeed, losartan is well known to attenuate several of the deleterious effects associated with well-established insulin resistance, as observed in longer treatment durations in various diet-induced obesity or genetic models of obesity (11, 39, 61). Hence, we selected a moderate duration of HFHF diet to target adipose tissue storage capacity without inducing hypertension- or obesity-associated damage such as inflammation or oxidative stress.

Our results did identify certain differences between AT2R activation and AT1R blockade, especially on TG levels. C21/M24 decreased fasting TG levels in control diet and also had a tendency to reduce fasting TG levels in HFHF-fed animals. Whereas no changes in TG level were observed with losartan in fasting state, we did show a reduction in TG content in liver compared with the HFHF and C21/M24+HFHF groups. Similar results have also been reported by Ran et al. (39), which observed less TG content in liver of obese Zucker rats treated for 4 wk with olmesartan, albeit with no significant changes in fasting TG level. In addition, we observed an increase in insulin-mediated NEFA and TG suppression during clamp studies with C21/M24 but not with losartan. Thus, from these results it is our hypothesis that losartan is less potent than C21/M24 specifically with regard to increasing insulin sensitivity for systemic lipid metabolism, although this hypothesis remains to be explored. Nonetheless, even with high TG levels, losartan-treated rats were more insulin sensitive for glucose metabolism than C21/M24-treated rats. These results may be explained by a different regulation of insulin signaling, lipolysis, lipoprotein lipase activity, and NEFA uptake proteins by AT2R activation vs. AT1R blockade (26).

In conclusion, the present study demonstrates that stimulation of AT2R promotes functional differentiation of SC and RET preadipocytes and improves lipid storage in SC adipocytes but not in visceral adipocytes. Moreover, in vivo AT2R activation is able to restore the adipocyte cell size profile and to reduce the insulin resistance induced by HFHF diet. Therefore, since AT2R is expressed in a limited number of tissues compared with AT1R, C21/M24, in association with ARBs, could represent a new and attractive therapeutic tool for prevention and management of patients presenting a potential risk of developing metabolic complications of obesity.
ACKNOWLEDGMENTS

We thank our technicians Lucie Chouinard and Lucie Bouffard (both from Division of Endocrinology, Department of Medicine, Faculté de médecine et des sciences de la santé, Université de Sherbrooke; Sherbrooke, QC, Canada) for experimental assistance with cell cultures and metabolic measurements. We are also grateful to Dr. Marcel D. Payet (Department of Physiology and Biophysics, Faculté de médecine et des sciences de la santé, Université de Sherbrooke) for developing morphometric software and assistance with data analyses and to Pierre Poiher for critical reading of the manuscript and editorial assistance (Les Services PM-SYS Enr., Sherbrooke). We also thank Audrey Emond and Thomas Grenier-Laroche (students from our group) for stimulating discussions. Dr. Nicole Gallo-Payet is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

GRANTS

This work was supported by grants from the Canadian Diabetes Association to N. Gallo-Payet, A. C. Carpentier, M.-F. Langlois, and J.-P. Baillargeon and by the Canada Research Chairs Program to N. Gallo-Payet. J.-P. Baillargeon and M.-F. Langlois are recipients of a Chercheur-boursier senior scholarship by the Canada Research Chairs Program to N. Gallo-Payet. J.-P. Baillargeon to N. Gallo-Payet, A. C. Carpentier, M.-F. Langlois, and J.-P. Baillargeon and the Canada Research Chairs Program and the FRSQ-funded Centre de recherche clinique Étienne-Le Bel. A. C. Carpentier is the recipient of the CIHR-GSK Chair in Diabetes. All are members of the FRSQ-funded Centre de recherche clinique Étienne-Le Bel.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


