Angiotensin AT$_1$ receptor antagonism by losartan stimulates adipocyte browning via induction of apelin

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Adipocyte browning appears to be a potential therapeutic strategy to combat obesity and related metabolic disorders. Recent studies have shown that apelin, an adipokine, stimulates adipocyte browning and has negative cross-talk with angiotensin II receptor type 1 (AT$_1$ receptor) signaling. Here, we report that losartan, a selective AT$_1$ receptor antagonist, induces browning, as evidenced by an increase in browning marker expression, mitochondrial biogenesis, and oxygen consumption in murine adipocytes. In parallel, losartan up-regulated apelin expression, concomitant with increased phosphorylation of protein kinase B and AMP-activated protein kinase. However, the siRNA-mediated knockdown of apelin expression attenuated losartan-induced browning. Angiotensin II cotreatment also inhibited losartan-induced browning, suggesting that AT$_1$ receptor antagonism-induced activation of apelin signaling may be responsible for adipocyte browning induced by losartan. The in vivo browning effects of losartan were confirmed using both C57BL/6J and ob/ob mice. Furthermore, in vivo apelin knockdown by adenoad-associated virus carrying–apelin shRNA significantly inhibited losartan-induced adipocyte browning. In summary, these data suggested that AT$_1$ receptor antagonism by losartan promotes the browning of white adipocytes via the induction of apelin expression. Therefore, apelin modulation may be an effective strategy for the treatment of obesity and its related metabolic disorders.

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Obesity is a disease that occurs when the balance between energy uptake and consumption is disrupted, leading to excess fat accumulation in adipocytes (1). Although many therapies have been developed for the treatment of obesity, the most commonly used appetite suppressants may have some adverse side effects. Therefore, there is a growing need for novel therapeutics to prevent or treat obesity, without or with fewer side effects (2).

Generally, adipocytes are classified into two types: white and brown. White adipocytes are the main deposits of fat, and they are increased in obesity, whereas brown adipocytes specialize in thermogenic functions (generation of heat from fat) (1). Brown adipocytes are abundant in newborns of nearly all mammalian species, but their numbers gradually decrease in adults (3). It has recently been observed that white adipocytes can be converted to a brown-like phenotype (so-called beige adipocytes), characterized by multilocular lipid droplets, a high number of mitochondria, and uncoupling protein 1 (UCP1) expression (4, 5). These beige adipocytes also uncouple the oxidative metabolism of energy substrates from ATP synthesis, consequently enhancing heat generation (6). Therefore, the suppression of white adipocyte expansion, the activation of brown adipocytes, and the browning of white adipocytes (converting white adipocytes to beige adipocytes) are considered as potential new therapeutic strategies to treat obesity and its related metabolic diseases (7, 8), with potentially fewer side effects compared with existing therapeutics.

Apelin is a recently identified member of the adipokine family and is widely expressed (9). It has various functions, including effects on energy metabolism via binding to angiotensin 1 receptor-related protein (API) receptors (10). These metabolism-related effects include the promotion of glucose uptake in adipocytes and adipose tissue (11, 12), inhibition of lipolysis in 3T3-L1 adipocytes (13), promotion of brown adipogenesis (14), and a decrease in insulin secretion in the pancreas (15). Correspondingly, apelin-transgenic mice are protected from diet-induced obesity, with increased energy expenditure (16), whereas apelin KO mice have increased white adipose tissue mass free fatty acid and leptin levels, and body adiposity (17). Moreover, the injection of apelin into apelin KO mice reverses these changes (17). Recent studies have shown that apelin increases the activation of Akt and AMPK in white adipocytes and brown preadipocytes (14). As a result, UCP1 and PRDM16 expression levels are increased, triggering brown adipogenesis in brown adipose tissue and browning of white adipose tissue (14).

Recent studies have shown that the renin-angiotensin system plays a role in regulating energy homeostasis. White adipocytes are known to produce angiotensin II (AT) and express both the AT$_1$ and AT$_2$ receptors, with AT$_1$ receptors being the major subtype (18, 19). Compared with the beneficial effects of apelin on energy homeostasis, increased activity of the AT$_1$ receptor system is often associated with various metabolic disorders (20). For example, the plasma levels of AT$_1$ increase considerably in obesity (21). The AT$_1$ receptor interaction induces the production of various pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) (22, 23), in adipose tissue, which in turn inhibits the browning of white adipocytes and stimulates the apoptosis of brown adipocytes (24). However, blockade of the AT$_1$ receptor prevents excessive lipid accumulation and the generation of reactive oxygen species in differentiated 3T3-L1 adipocytes (25). This is accompanied by an
increase in the production of adiponectin and apelin but a decrease in the expression of TNFα, renin, retinol binding protein 4, and visfatin (25, 26). Similarly, knockdown of either the AT1 receptor or the AT1 receptor blockade attenuates adiposity and diet-induced weight gain (27, 28), with increased thermogenic gene expression and the appearance of multilocular lipid droplets in adipose tissues (29). On the other hand, the opposing actions of cross-talk between apelin and the AT-AT1 receptor exist during energy homeostasis, such that apelin leads to the negative allosteric regulation of the AT1 receptor function by triggering heterodimerization of APJ and AT1 receptors (30).

Losartan is an anti-hypertensive drug that acts as a selective and competitive AT1 receptor antagonist (31). To date, the effects of losartan on adipose browning and the associated mechanisms have not been clearly elucidated. In the present study, we investigated the effects of losartan on adipocyte browning, with a special focus on the modulation of apelin.

**Results**

**Losartan induced a browning effect in differentiated 3T3-L1 adipocytes**

To investigate the browning effects of losartan in fully differentiated 3T3-L1 adipocytes, cells were treated with various concentrations of losartan (10–100 μM) for 48 h, and the mRNA expression of browning markers was then determined by real-time PCR. Losartan concentration dependently promoted the mRNA expression of Ucp1, peroxisome proliferator-activated receptor-γ coactivator 1α (Pgc1α), cell death inducing DNA fragmentation factor subunit a like effector A (Cidea), transmembrane protein 26 (Tmem26), and PR domain containing 16 (Prdm16) (Fig. 1A). T3 (50 nM), a known browning inducer (32), was used as a positive control. Consistent with the increased mRNA expression of browning markers, the protein levels of UCP1, PGC1α, CideA, and TMEM26 were also increased after 6 days of losartan (10–100 μM) treatment (Fig. 1B). Moreover, increased UCP1-positive immunostaining was evident after losartan treatment (Fig. 1C).

Browning of white adipocytes affects various cellular phenotypes, such as decreasing the number of lipid droplets and increasing mitochondrial biogenesis. An increased multilocular appearance, with reduced lipid droplet sizes, was observed after 6 days of losartan treatment (Fig. 1D). As shown in Fig. 1E, the density of MitoTracker staining (indicated in red) increased in both T3- (50 nM) and losartan-treated cells when compared with control cells, indicative of increased mitochondrial biogenesis and further supported by increased levels of mitochondrial proteins (Fig. 1B). In parallel, the oxygen consumption rate (OCR), which reflects mitochondrial respiratory activity and metabolic rate, was also increased by more than 160% after treatment with losartan for 6 days, with a maximum OCR occurring at a concentration of 50 μM (Fig. 1F). Furthermore, the losartan-treated cells exhibited greater uncoupled OCR as determined in the presence of oligomycin A (Fig. 1F). Taken together, our results suggested that losartan was able to induce the browning of murine white adipocytes under basal condition.

**Losartan induced apelin expression in differentiated 3T3-L1 adipocytes**

Both apelin and APJ receptors are expressed in white adipocytes, and the exogenous addition of apelin induced brown characteristics in white adipocytes (33). Hence, we investigated the effects of losartan on apelin mRNA expression in fully differentiated 3T3-L1 adipocytes after treatment with various concentrations of losartan (10–100 μM) for 48 h. Losartan concentration dependently increased apelin mRNA expression (Fig. 2A, upper graph) and also induced apelin secretion (Fig. 2A, lower graph). Apelin-positive immunostaining further confirmed the inducing effects of losartan on apelin expression (Fig. 2B).

To identify the mechanisms by which losartan induced apelin expression, the phosphorylation status of mitogen-activated protein kinase (MAPK; c-Jun N terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK)), Akt, and AMPK was assessed following acute losartan treatment (5–30 min). Whereas MAPK phosphorylation remained unchanged after losartan treatment, elevated phosphorylation of Akt and AMPK was detected (Fig. 2C). Moreover, either AMPK or Akt knockdown by siRNA (50 nM) markedly attenuated losartan-induced increase in apelin mRNA expression (Fig. 2D), consistent with the previous reports (14). These results suggest that losartan induced apelin expression in 3T3-L1 adipocytes and that increased phosphorylation of Akt and AMPK may play an important role in this effect.

To determine whether the increase in apelin mRNA is due to active transcription, differentiated white adipocytes were pretreated with actinomycin D for 1 h followed by losartan treatment. Actinomycin D completely blocked the increase of apelin mRNA expression (Fig. 2E, left graph), showing that increased apelin mRNA by losartan requires active transcription. Furthermore, losartan increased the apelin promoter activity compared with the control treatment as measured by luciferase activity (Fig. 2E, right graph). On the other hand, cycloheximide treatment did not affect losartan-induced apelin expression (Fig. 2F), suggesting that de novo protein synthesis is not required for the increase in apelin mRNA expression and instead is likely due to activation of preexisting transcription factor(s). Among possible transcription factors involved in apelin expression, signal transducer and activator of transcription 3 (STAT3) activation is regulated by Akt/AMPK phosphorylation (34), and interestingly, losartan induced the phosphorylation of STAT3 (Fig. 2C). In support of the role of STAT3 in losartan-induced apelin expression, increased mRNA expression of apelin by losartan was abolished in the presence of STAT3 inhibitor C188-9 (Fig. 2G).

**Knockdown of apelin expression attenuated losartan-induced browning**

To further test the role of apelin in losartan-induced browning, apelin was silenced in fully differentiated 3T3-L1 adipocytes using an apelin-specific siRNA (50 nM). These cells were then treated with losartan (20, 100 μM) for 48 h. When apelin was knocked down by siRNA (~65%), the losartan-induced mRNA expression of browning markers (Ucp1, CideA, Tmem26, and Prdm16) was completely inhibited (Fig. 3A). Coinciding with
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the blunted expression of browning markers, the reduction in the number of lipid droplets by losartan treatment was largely reversed by apelin silencing (Fig. 3B). This finding was supported by the observation that the MitoTracker staining, the levels of mitochondrial proteins, and the OCR increased by losartan were all markedly attenuated by the siRNA-mediated knockdown of apelin expression (Fig. 3, C–E), indicating that apelin induction was a key mechanism contributing to losartan-induced browning.

Signaling involved in losartan-stimulated browning

Previous studies have reported that apelin increases Akt and AMPK phosphorylation in white adipocytes to up-regulate browning marker expression (14). Hence, we examined whether losartan-induced browning is also dependent on Akt and AMPK phosphorylation upon apelin induction. Indeed, losartan treatment for 48 h concentration-dependently increased the phosphorylation of Akt and AMPK (Fig. 4A). To determine whether increased phosphorylation of Akt and AMPK was a downstream consequence of apelin signaling, the effect of apelin knockdown was investigated. As shown in Fig. 4B, losartan-induced phosphorylation of Akt and AMPK was inhibited in apelin-knockdown cells, implying that the actions of apelin, stimulated by losartan, were also dependent on Akt and AMPK phosphorylation. Correspondingly, either an AMPK-specific siRNA or an Akt-specific siRNA (50 nM) abrogated the losartan-induced expression of browning markers (Fig. 4C). However, MAPK phosphorylation was unaltered by losartan treatment, regardless of apelin siRNA treatment (Fig. 4B). Collectively, these results suggested that the effect of losartan on adipocyte browning was attributable to apelin induction, leading to activation of the Akt and AMPK signaling pathways.

Browning effect of losartan in primary adipocytes

To confirm the browning effects of losartan at the cellular level, differentiated primary adipocytes isolated from C57BL/6J mice were treated with losartan (1–20 μM) for 2 days. In line with the results in 3T3-L1 adipocytes, losartan induced the mRNA and protein levels of UCP1 and CideA, concurrently with increased apelin mRNA expression in primary adipocytes at lower concentrations of losartan than in 3T3-L1 adipocytes (Fig. 5, A and B). After 6 days of losartan treatment, increased UCP1 staining (Fig. 5C) and increased mitochondrial biogenesis (Fig. 5B and D), with a reduction in the number of lipid droplets, were observed by microscopic observation (Fig. 5E). All of these results indicated that losartan promoted browning in primary adipocytes in a similar manner to its effects in 3T3-L1 adipocytes.

AT inhibited the losartan-induced expression of apelin and browning

Losartan acts as a selective AT1 receptor antagonist with high affinity (35), and chronic AT reduced apelin expression through AT1 receptor binding followed by inactivation of either p38MAPK or PI3K/Akt signaling (36). Hence, we
examined whether exogenous AT could reverse the losartan-induced expression of UCP1 and apelin via its competitive binding to the AT1 receptor. Whereas the addition of AT itself suppressed the mRNA expression of UCP1 and apelin (Fig. 6A), cotreatment with AT (0.1–100 nM) and losartan (100 μM) masked the browning effects of losartan in an AT concentration–dependent manner (Fig. 6, B–E), suggesting that losartan stimulated browning via AT1 receptor blockade, which was surmounted by high concentrations of AT.

Of note, browning effects of the AT2 receptor have recently been demonstrated by Than et al. (37), although these findings remain controversial. Thus, we examined the involvement of the AT2 receptor in the effects mediated by losartan. Under our experimental conditions, an AT2 receptor–specific siRNA (50 nM) partially reduced the effects of losartan on browning marker expression but had little effect on apelin mRNA levels (Fig. 6F). Together, these results suggested that losartan primarily induces adipocyte browning via AT1 receptor antagonism-induced apelin expression and that partial involvement of AT2 receptor activation may have an additive effect on the actions of losartan (Fig. 6G). Consistent with our results, AT1 receptor antagonism has been shown to restore the AT1-induced reduction of apelin and APJ expression in 3T3-L1 adipocytes (36).

C57BL/6j mice (6 weeks old, male) were orally administered losartan at 10 mg/kg or 30 mg/kg (once a day) for 2 weeks (n = 6 per group). No significant difference in body weight, blood glucose, or food intake was observed between the vehicle- and losartan-treated groups (Fig. 7A). However, glucose tolerance was significantly improved in the losartan-treated group (30 mg/kg), which was positively correlated with apelin mRNA expression (Fig. 7B) and UCP1 immunostaining in subcutaneous fat (SCF, Fig. 8C). Similar effects were also observed in the relatively browning-resistant visceral (VF) and retroperitoneal (RF) fats (Fig. 8, A–C). In parallel, H&E staining and MitoTracker staining also suggested that losartan induced browning in vivo, in association with apelin induction (Fig. 8, D and E).
Congruent with increased UCP1 immunostaining and mitochondrial biogenesis, losartan-treated mice were resistant to the reduction of body temperature upon cold exposure for 48 h (Fig. 8 F), in which ex vivo OCR and the levels of mitochondrial proteins and apelin were increased in various adipose depots after losartan treatment (Fig. 8, G and H). On the contrary, AT1 receptor positive immunostaining was almost completely absent in the losartan (30 mg/kg)-treated group (Fig. 8 G), further supporting that losartan induces adipose browning via AT1 receptor antagonism–apelin induction.

To further confirm the browning effects of losartan, ob/ob mice (8 weeks old, male) were treated with losartan (50 mg/kg, n = 8) for 4 weeks and the effects of losartan on adipose browning were then examined. Body weight and fat weight were reduced after 4 weeks of treatment with losartan, with little effect on food intake (Fig. 9, A and B). Improved glucose tolerance was also observed in the losartan-treated group (Fig. 9C). The expression of browning markers (Fig. 9D), the levels of UCP1, apelin, and mitochondrial proteins (Fig. 9, E–G), and increased MitoTracker staining (Fig. 9H) were confirmed in ob/ob mice, indicating that losartan induced adipose browning, resulting in reduced body weight.

**In vivo knockdown of apelin blunted losartan-induced browning**

Finally, an in vivo demonstration that losartan’s action was apelin dependent was performed using an adeno-associated virus (AAV) packaged with apelin shRNA. In vivo knockdown of apelin was achieved by tail vein injection of C57BL/6J mice (n = 8 each group) with AAV-apelin shRNA (1 × 1011 genome copies/ml, 100 µl/mice), and 6 weeks later, losartan (30 mg/kg) was orally administered once daily for 2 weeks (experimental scheme is shown in Fig. 10 A). In agreement with our previous in vitro and in vivo results, apelin levels increased in fat tissues of the AAV-control shRNA/losartan-treated group, of which the level remained suppressed in the AAV-apelin shRNA/losartan-treated group (Fig. 10B). Although the effects on body weight and fat weight were negligible, possibly because of the leanness of C57BL/6J mice (Fig. 10C), the increased expression of browning markers in fats by losartan was markedly attenuated with the suppression of apelin expression (Fig. 10, D, E, and G), as was mitochondrial biogenesis (Fig. 10, F and G) in the AAV-apelin shRNA/losartan-treated group. Plasma apelin and adiponectin levels increased, and conversely, TNFα levels decreased in the AAV-control shRNA/losartan-treated group, which was also reversed by AAV-apelin shRNA (Fig. 10H). These results supported the notion that losartan induces in vivo adipose browning primarily via apelin induction.

**Discussion**

The present study demonstrated that AT1 receptor antagonism by losartan induced browning effects via apelin induction, both in vitro and in vivo. Losartan induced browning in
Figure 4. Chronic losartan treatment increased phosphorylation of Akt and AMPK in differentiated 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with various concentrations (10, 20, 50, or 100 μM) of losartan (48 h), and the phosphorylated levels of Akt and AMPK were determined by Western blotting, which was repeated three times. The representative results are shown with band density analysis (A). Gene silencing of apelin in fully differentiated 3T3-L1 adipocytes was accomplished using an apelin siRNA (50 nM). Apelin-silenced cells were treated with losartan (20 or 100 μM) for 2 days. Phosphorylation of Akt, AMPK, and MAPK was measured by Western blotting (B). Each experiment was repeated three times, and the representative results are shown with band density analysis. AMPK and Akt were knocked down by siRNA transfection (50 nM) in differentiated 3T3-L1 adipocytes, and the expression of browning markers was determined by real-time PCR (C, repeated three times, each done in triplicate). *p < 0.05 versus vehicle; #p < 0.05 versus control siRNA groups. NS, not significant.

Figure 5. Browning effect of losartan in primary adipocytes. Primary adipocytes (1 × 10⁶ cells/well) isolated from C57BL/6J mice were treated with losartan (1–20 μM) for 2 days after differentiation. Expression of browning markers was determined by real-time PCR (A, repeated three times, each done in triplicate) and by Western blotting with band density analysis (B, repeated three times). Differentiated primary adipocytes were treated with different concentrations of losartan for 6 days and then underwent immunostaining of UCP1 (C, green labeling, scale bar = 50 μM, n = 6) and Mitotracker staining (D, red labeling, scale bar = 50 μM, n = 6). Cell morphology (with lipid droplets) is shown (E, scale bar = 20 μM, n = 6). *p < 0.05 versus vehicle.
differentiated 3T3-L1 adipocytes and primary white adipocytes, in association with increased Akt/AMPK phosphorylation and apelin expression, whereas apelin silencing diminished losartan-induced adipocyte browning. However, AT attenuated the losartan-induced expression of apelin and browning markers, suggesting that the effects of losartan are primarily mediated by AT₁ receptor antagonism. The browning effects of losartan were confirmed in vivo by showing that the expression levels of browning markers and apelin were increased in SCF, RF, and VF tissues isolated from losartan-treated mice. Furthermore, in vivo apelin knockdown by AAV-apelin shRNA blunted losartan-induced browning effects, providing evidence that losartan induced adipose browning via apelin induction in vivo and in vitro. Based on our experiments, the proposed mechanism underlying losartan-induced browning action is schematically represented in Fig. 6G. Losartan induced adipocyte browning primarily by blockade of AT binding to the AT₁ receptor following Akt/AMPK/STAT3 activation and subsequent apelin induction. As an indirect mechanism, AT₂ receptor activation by AT, resulting from the AT₁ receptor blockade in the presence of losartan, leads to adipocyte browning, independent of apelin induction.

Accumulating evidences suggest that apelin plays an important role in energy metabolism and that its actions are either in autocrine or in paracrine manners (10, 22). In the present study, we focused on the action of apelin on adipocytes, limiting its investigation to autocrine circumstances. However, the finding that glucose tolerance was improved by losartan treatment in vivo suggested potential effects of apelin in other tissues, such as skeletal muscle, which warrants further study. In fact, besides apelin-mediated browning effects, apelin stimulates glucose uptake in various target tissues, such as skeletal muscle and adipose tissue, producing glucose-lowering and insulin-sensitizing effects (38). Intriguingly, losartan also increased the expression of APJ in our study, which needs further investigation.

Besides its effects on energy metabolism, including obesity, losartan actions linked to apelin induction have also been reported in cardiovascular and renal ischemia/reperfusion injury. Losartan protects against early and late renal ischemia/reperfusion injury by the induction of renal apelin through endothelial isoform of nitric oxide synthase phosphorylation (39). Similarly, renal fibrosis is alleviated after induction of the apelin/APJ system by losartan (40). Olmesartan, another angiotensin receptor blocker, has been shown to improve cardiac dysfunction and vascular remodeling through apelin/APJ induction and the Akt/endothelial isoform of nitric oxide synthase pathway (41).

Akt and AMPK signaling pathways are involved in apelin synthesis and secretion in 3T3-L1 adipocytes (42). In the present study, losartan treatment increased the phosphorylation levels of Akt and AMPK in the acute phase (<30 min), which possibly leads to apelin expression and secretion, whereas the
phosphorylation levels of JNK, p38, and ERK were not changed. Similarly, chronic treatment of losartan (48 h at 100 μM) significantly increased the phosphorylation of Akt and AMPK, possibly resulting from increased interaction between apelin with APJ, an apelin receptor. Moreover, knockdown of either AMPK or Akt attenuated the expression of browning markers induced by 48 h of losartan treatment, with a moderate effect on apelin expression, suggesting that AMPK and Akt activation is critical for apelin-induced browning effects. Consistent with the current results, a previous study showed that apelin restores inflammation-impaired brown adipocytes through the Akt and AMPK signaling pathway (14). However, the AT-induced suppression of apelin expression is p38/ERK1/2-dependent (37).

In line with the role of Akt and AMPK activation in losartan-induced apelin expression, we demonstrate that active transcription is necessary for apelin induction in response to losartan. Furthermore, increased apelin transcription does not require new protein synthesis because there was no reduction in losartan-induced apelin mRNA levels in the presence of cycloheximide. To further elucidate the mechanisms of transcriptional activation of apelin by losartan, we analyzed the promoter regions of the apelin gene and found that there are three STAT3 consensus sequences. Based on the previous findings that STAT3 phosphorylation was regulated by Akt and AMPK phosphorylation (34), STAT3 was selected as a possible candidate of transcriptional factors involved in apelin mRNA expression (43). Coinciding with increased Akt and AMPK phosphorylation by losartan, STAT3 phosphorylation was also increased by losartan, and STAT3 inhibitor prevented losartan-induced apelin mRNA expression, indicating that STAT3 may play an important role in losartan-induced apelin transcription. Other transcription factors may also contribute to the apelin transcription upon losartan treatment.

Counter-regulatory effects of apelin and the renin-angiotensin system have been detected in various conditions. In metabolic homeostatic settings, apelin secretion is increased by the renin-angiotensin system blockade, including by losartan treatment (37). Furthermore, it has been observed that 3T3-L1 adipocytes increase the secretion of AT during differentiation (23). The current study also showed that apelin mRNA levels were increased by losartan. On the contrary, exogenous addition of AT, at 0.1–100 nM, decreased the expression of UCP1 and apelin in 3T3-L1 adipocytes, and the losartan-induced increase in UCP1 and apelin expression was normalized by AT cotreatment. Thus, losartan increased apelin expression and secretion, mainly by the direct effect of competitive inhibition of AT1 receptor–binding with AT. Consistent with these findings, increased UCP1 expression and oxygen consumption were observed in apelin-transgenic mice compared with control mice when fed a high-fat diet (16).

It has been reported that the AT1 receptor is associated with obesity. AT1 KO mice show increased expression of browning markers, such as UCP1, peroxisome proliferator-activated receptor γ (PPARγ), and TMEM26, in inguinal white adipose tissue, but their total body weight and adipose tissue mass are also increased (44). On the other hand, in agreement with our results, losartan prevents diet-induced obesity in mice and decreases the adiposity index with enhanced energy expenditure, resulting

Figure 7. Losartan induced browning effects in vivo in C57BL/6J mice. C57BL/6J mice (6 weeks old, male) were administered vehicle (0.9% saline) or losartan (10 or 30 mg/kg, po, once daily, n = 6 per group) for 2 weeks. Body weight, food intake, and plasma glucose levels were measured before and after treatment (A) and oral glucose tolerance testing was performed after overnight fasting and area under the curve (AUC) was shown in the lower panel (B). Serum levels of apelin, adiponectin, TNF, triglycerides, and free fatty acids were quantified in treated mice (C). *p < 0.05 versus vehicle.
from the induction of thermogenic beige adipocytes in subcutaneous adipose tissues (29). In the present study, using a C57BL/6J mouse model, the effects of losartan on body weight were minimal, possibly because of the leanness of this animal model. However, the expression of browning markers was clearly enhanced, in association with the up-regulation of apelin in adipose tissues and in the plasma of losartan-treated mice. Therefore, the in vivo blockade of the AT1 receptor by losartan may play a vital role in the up-regulation of apelin, leading to adipose browning. However, the role of the AT2 receptor in adipose tissue remains controversial. AT2 receptor deficiency protects against high-fat-diet–induced obesity (45). Meanwhile, AT2 receptor activation suppresses norepinephrine-induced UCP1 production and cellular respiration in inguinal adipocytes and inhibits energy expenditure in vivo (46). On the contrary, a recent report by Than et al. (37) showed that AT2 receptor activation induces adipose browning and brown adipogenesis. Based on our observation that AT2 receptor knockdown partially reduced losartan-induced browning marker expression, with little effect on apelin induction in 3T3/L1 adipocytes, the browning effects of losartan may be elicited by dual mechanisms, i.e. primarily via AT1 receptor antagonism-induced apelin induction and, to a lesser extent, via AT2 receptor agonism by endogenous AT resulting from the AT1 receptor blockade.

In ob/ob mice, losartan was shown to reduce body and fat weights. Correspondingly, glucose intolerance was improved by losartan, which might also be attributed to the reduced body weight. Although increased expression of browning markers was confirmed in adipose tissues of ob/ob mice after 4 weeks of losartan treatment, the beneficial metabolic effects of losartan in ob/ob mice may not be solely due to its effects on adipose tissues. Apelin secreted from adipose tissues has been shown to enhance mitochondrial biogenesis, fatty acid oxidation, and insulin-stimulated glucose uptake in skeletal muscle, in a paracrine manner (47). Conclusive evidence of the role of apelin in mediating the actions of losartan was obtained using AAV-apeLIN shRNA to show that in vivo knockdown of apelin expression in adipose tissues masked the browning effects of losartan. Besides AT1 receptor blockade-mediated apelin induction, other mechanisms involved in losartan-induced browning might exist. Interestingly, recent studies demonstrated the UCP1 independent thermogenesis in beige fats involving sarco/endoplasmic reticulum Ca^{2+}-ATPase 2b–ryanodine receptor 2 (SERCA2b–RyR2)–mediated Ca cycling (48) and a creatine-driven substrate cycle (49), which may possibly be considered for further investigation on losartan action.

In conclusion, this study demonstrated that losartan induced adipocyte browning in vitro and in vivo, possibly via dual mechanisms, i.e. AT1 receptor antagonism, followed by apelin induction through Akt/AMPK activation as the key mechanism, and AT2 receptor agonism, resulting from the AT1 receptor blockade, independent of apelin induction, as an indirect mechanism. Because the use of the AT1 receptor blocker resulted in either weight loss or weight neutral in humans (50), a better

![Figure 8. In vivo browning effects of losartan in C57BL/6J mice. C57BL/6J mice (6 weeks old, male) were administered vehicle (0.9% saline) or losartan (10 or 30 mg/kg, po, once daily, n = 6 per group), and various adipose tissues were isolated after 2 weeks of treatment. The levels of browning markers (A) and apelin (B) in fat tissues were determined by real-time PCR (n = 6, repeated three times). UCP1 immunostaining (C, scale bar = 50 μm), H&E staining (D, scale bar = 50 μm), and MitoTracker staining (E, red labeling, scale bar = 50 μm) were performed. Separately, losartan-treated groups were exposed to 4 °C for 48 h, and rectal temperature was determined at the indicated times (F, upper graph). After sacrifice, ex vivo OCRs were determined using a dissolved oxygen meter and a probe, as described under “Experimental procedures” (F, lower graph). UCP1, apelin, and AT1 receptor immunostaining, and Western blotting were carried out using isolated fat tissues after cold exposure (G and H). *p < 0.05 versus vehicle. SCF, subcutaneous fat; VF, visceral fat; RF, reproductive fat; BF, brown fat.](image-url)
understanding of the detailed molecular mechanisms will lead to the development of novel anti-obesity therapeutics. Based on the current study, apelin modulation is a potentially novel therapeutic strategy for obesity and obesity-related morbidity.

Experimental procedures

Materials

DMEM, FCS, FBS, penicillin, and streptomycin were purchased from Gibco. Monoclonal antibodies against PRDM16, TMEM26, UCP1, and β-actin, and the polyclonal antibody against TFAM were from Santa Cruz Biotechnology. The polyclonal antibody against apelin was from Abcam; the polyclonal antibody against CideA was from Bioworld Technology; the polyclonal antibodies against Akt, phospho-Akt, AMPKa, phospho-AMPKa, STAT3, phospho-STAT3, and COX IV were from Cell Signaling. Peroxidase-conjugated secondary antibodies were from the Jackson Laboratory. Random oligonucleotide primers were from Promega. The Thunderbird SYBR qPCR Mix was from Toyobo, and the TOP script RT Dry MIX was from Enzynomics. Oligonucleotide primers specific for Akt, Ampk, apelin, AT1 receptor, AT2 receptor, Cidea, Gapdh, Pgc1α, Prdm16, Tmem26, β-actin, and Ucp1 were from Bioneer. AT, losartan, actinomycin D, cycloheximide, oligomycin A, and T3 were from Sigma-Aldrich. The apelin C terminus and AT enzyme immunoassay kits were from RayBiotech. C188-9 was from Selleck Chemicals.

Cell culture and differentiation

DMEM containing 10% FCS, penicillin (100 units/ml), and streptomycin sulfate (100 mg/ml) were used to culture 3T3-L1 preadipocytes (Korean Cell Line Bank, Seoul, Korea), which were maintained in a humidified 5% CO2 atmosphere at 37 °C. After reaching confluence, the cells were differentiated by incubation with differentiation medium (DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin) for 3 days. The differentiated cells were then cultured in DMEM containing 10% FBS and 10 μg/ml insulin for 2 days. The cells were maintained in DMEM supplemented with 10% FBS only and the medium was changed every 2 days. After 8 days of differentiation, the fully differentiated white adipocytes were treated with various concentrations of losartan (10–100 μM) or 50 nM T3 for 2 days or 6 days.

To isolate murine adipose-derived cells, subcutaneous adipose tissues were extracted from 6-week-old male C57BL/6J mice (ORIENT BIO), chopped into small pieces, and digested using a solution containing 15 ml of type XI collagenase (2 mg/ml; Gibco) for 1 h at 37 °C in a shaking incubator. After filtration through a 40-μm nylon strainer, stromal vascular fraction was centrifuged at 1,750 rpm for 10 min and resuspended in...
ACK Lysing Buffer (Gibco) for 5 min at room temperature to lyse the contaminating red blood cells. The solution was then centrifuged at 1,750 rpm for 10 min and the resulting supernatant was aspirated. Stromal vascular cells were cultured in T25 flasks with DMEM/F12 supplemented with 20% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 mg/ml) and differentiated to mature adipocyte as described for 3T3-L1 adipocytes.

**Immunostaining and MitoTracker staining**

Differentiated 3T3-L1 adipocytes were treated with losartan (10–100 μM) or 50 nM T3 for 6 days and then incubated with methanol for 30 min, 0.1% Triton X-100 for 1 h, and 5% normal goat serum for 1 h. The cells were detached and then incubated with anti-mouse UCP1 or anti-mouse apelin antibodies (Abcam, diluted 1:500) overnight at 4 °C. After incubation with a goat anti-rabbit IgG secondary antibody conjugated with HRP (Invitrogen; diluted 1:5,000) for 1 h at room temperature, the cells were washed three times with PBS and incubated with a mounting solution containing 4',6-diamidino-2-phenylindole. The fluorescence images of adipocytes were captured using an LSM 700 confocal laser-scanning microscope.

**Western blotting analysis**

Treated cells were washed three times with PBS and scraped off in extraction lysis buffer (PRO-PREP; iNtRON Biotechnology) with a protease inhibitor mixture, kept for 30 min on ice, and then centrifuged at 14,000 rpm for 30 min at 4 °C. Protein content was measured using a Bio-Rad protein assay kit. Each sample containing the same amount of protein was separated by 10 or 15% SDS-PAGE and then transferred onto PVDF membranes. The membranes were blocked for 1 h with 4% BSA in Tris-buffered saline–Tween (TBS-T) at room temperature and incubated with primary antibody (Santa Cruz Biotechnology) overnight at 4 °C. After washing three times with TBS-T, the membranes were incubated with HRP-conjugated secondary antibody (1:5,000; Bioworld Technology) for 1 h at room temperature. The membranes were washed three times with TBS-T and developed using enhanced chemiluminescence (Amersham Biosciences Life Science, Little Chalfont, UK). All protein levels were normalized to β-actin.

**RNA preparation and real-time PCR**

Total RNA was isolated from cells using an Easy Blue kit (iNtron Biotechnology). RNA was reverse-transcribed into
cDNA using TOPscript™ RT DryMIX (Enzynomics). PCR analyses were performed in duplicate for each sample, and the transcription levels of each gene were normalized to Gapdh. The sequences of the primers used in these experiments were as follows: CideA forward, 5’-AAACCATGCGCCAAGTA-GCC-3′ and reverse, 5’-AGGCCAGTTGTGATGACTAA-3′; Gapdh forward, 5’-AGTGTCGCTGGAAGCGTTTG-3′ and reverse, 5’-GGGCTGCGTTGATGACCAACA-3′; Pgc1α forward, 5’-TGGTGAA GGGTTA TCTTGG-3′ and reverse, 5’-GCCCTTGAA GGGTTA TCTTGG-3′; Prdm16 forward 5’-CCACCAGGAGACTTCAC-3′ and reverse, 5’-GGAGGA TCTCTGAGCTCGAA-3′; Tmem26 forward, 5’-TCCGTTG TGATCTCCCTGGTC-3′ and reverse, 5’-GCCGGAGAA GCCATTTGT-3′; and Ucp1 forward, 5’-ACTGCCACA CCTCGATC-3′ and reverse, 5’-CTTTGCCTACTCAGGA TTTG-3′.

Animal studies

Mice (C57BL/6J, 6 weeks of age, male) were purchased from ORIENT BIO and maintained for 1 week under constant conditions (temperature 23 ± 2 °C, humidity 40–60%, 12-h light/dark cycle) to acclimatize. They were allowed ad libitum access to a normal chow diet and tap water. For experiments, the mice were randomly divided into three groups (n = 6 per group). In group 1 (control group), animals were treated with 0.9% saline as the vehicle. In groups 2 and 3, animals were treated with losartan (10 or 30 mg/kg, respectively), dissolved in 100 μl of 0.9% saline. Treatments were done by oral gavage, once daily for 2 weeks. Food intakes were determined by subtracting food amounts left in cages from the amounts supplied. After 2 weeks of treatment, the mice were sacrificed by CO2 inhalation and adipose tissues were promptly isolated. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication 85-23, revised 2011) and were approved by the Animal Care and Use Committee of Gachon University, Seongnam, South Korea (approval number LCDI-2019-0093).

Luciferase reporter assay

The apelin promoter, as defined by the location corresponding to the transcription start site, was amplified with PCR using the following primers: forward 5’-GCCGGAACCCGAGTG-TAAAAGAACATCCAG-3′ and reverse 5’-GCCGAGGTGGCTGGCTGG-3′. The apelin promoter was subcloned into the pGL4.14 hygro (Promega). The plasmid pGL4.14-mAPLN promoter and 0.1 μg of pRL-TK expressing control Renilla luciferase vector using lipofectamine 2000 reagent (Invitrogen). After 24 h of transfection, the media was replaced with fresh DMEM for 6 h, followed by treatment with losartan (20 μM, 100 μM) for 24 h. Luciferase activity was measured using the Dual-Luciferase reporter assay kit (Promega) with a PerkinElmer luminometer.

siRNA silencing

Fully differentiated 3T3-L1 adipocytes were transfected with mouse apelin siRNA, Akt siRNA, AMPK siRNA, or a control siRNA (Santa Cruz Biotechnology). siRNA transfections were performed for 6 h using Opti-MEM containing a complex of Lipofectamine-RNAiMAX transfection reagent (Thermo Fisher Scientific) with siRNAs (50 nM). The cells were then incubated with DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 mg/ml) for another 1 day. The knockdown extent was confirmed by real-time PCR.

Measurements of OCR

OCR was analyzed using a high-sensitivity Mito-ID® extracellular O2 sensor kit (Enzo Life Sciences). White adipocytes grown in 96-well plates were treated with losartan (10, 20, 50, or 100 μM) to induce browning. After 6 days of treatment, adipocytes were placed in fresh DMEM medium for 30 min and treated with dissolved oxygen solution for 30 min, and each well was promptly sealed by adding two drops of pre-warmed high-sensitivity mineral oil. The cell plates were then read using a Victor® plate reader (emission/excitation 380 nm/650 nm;
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PerkinElmer and O₂ concentrations were measured. Uncoupled OCR was measured in 3T3/L1 adipocytes in the presence of oligomycin A (1 μg/ml) for 30 min.

**Immunohistochemistry**

Adipose tissues were fixed with 3.7% formaldehyde, washed with PBS, and embedded in paraffin. The paraffin blocks were sectioned using a sliding microtome (4–6 mm). Paraffin-embedded tissue sections were deparaffinized, rehydrated, permeabilized with 0.1% Triton X-100 (1 h, room temperature), and washed again with PBS. The sections were incubated with primary polyclonal antibodies against UCPI or apelin (Abcam, diluted 1:500) at 4 °C overnight. After washing with PBS, the sections were incubated with a biotin-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, diluted 1:500) and subsequently with HRP-conjugated streptavidin (Vector Laboratories) was used for visualization, and the specimens were counterstained with H&E.

**Cytokine assay**

To determine secreted apelin concentrations in conditioned media, the media from each sample were collected and subjected to an ELISA according to the manufacturer’s protocol (MyBioSource, MBS701195). Plasma levels of adiponectin, TNF, triglyceride, and free fatty acids were determined using commercially available kits (adiponectin, Abcam, ab108785; TNF, BD Biosciences, BD OptEIA560478; triglyceride, Cell Biolabs, STA-396; free fatty acid, BioVision, K612-100).

**Statistical analysis**

Data were analyzed using a Student’s t test for paired experiments or a two-way analysis of variance. Results are expressed as the means ± S.D. of three separate experiments, and p values < 0.05 were regarded as statistically significant.

**Data availability**

All data are included in this article. Raw datasets are available upon request.

**Author contributions**—D. Y. K., M. J. C., T. K. K., N. H. L., and O.-H. K. investigation; D. Y. K. writing-original draft; H. G. C. conceptualization; H. G. C. supervision; H. G. C. funding acquisition; H. G. C. writing-review and editing.

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**Abbreviations**—The abbreviations used are: AT, angiotensin II; Akt, protein kinase B; AMPK, AMP-activated protein kinase; UCP, uncoupling protein; APJ, angiotensin 1 receptor-related protein; TNFa, tumor necrosis factor α; PGC, peroxisome proliferator-activated receptor-γ coactivator; CIDEA, cell death inducing DNA fragmentation factor subunit α like effector A; TMEM, transmembrane protein; PRDM, PR domain; OCR, oxygen consumption rate; T3, triiodothyronine; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; ERK, extracellular signal-regulated kinase; SCF, subcutaneous fat; VF, visceral fat; RF, reproductive fat; AAV, adenovo-associated virus; TBS-T, Tris-buffered saline–TWEEN; TNF, tumor necrosis factor

**References**

1. Jeremic, N., Chaturvedi, P., and Tyagi, S. C. (2017) Browning of white fat: novel insight into factors, mechanisms, and therapeutics. *J. Cell. Physiol.* 232, 61–68 CrossRef Medline

2. Kim, S. H., and Plutzky, J. (2016) Brown fat and browning for the treatment of obesity and related metabolic disorders. *Diabetes Metab. J.* 40, 12–21 CrossRef Medline

3. Gilanz, V., Hu, H. H., and Kajimura, S. (2013) Relevance of brown adipose tissue in infancy and adolescence. *Pediatr. Res.* 73, 3–9 CrossRef Medline

4. Azhar, Y., Parmar, A., Miller, C. N., Samuels, I. S., and Rayalam, S. (2016) Physicochemicals as novel agents for the induction of browning in white adipose tissue. *Nutr. Metab.* 13, 89 CrossRef Medline

5. Samocha-Bonet, D., Dixit, V. D., Kahn, C. R., Leibel, R. L., Lin, X., Nieuworp, M., Pietilänen, K. H., Rabassa-Lloret, R., Roden, M., Scherer, P. E., Klein, S., and Ravussin, E. (2014) Metabolically healthy and unhealthy obese—the 2013 Stock Conference report. *Obes. Rev.* 15, 697–708 CrossRef

6. Harms, M., and Seale, P. (2013) Brown and beige fat: development, function and therapeutic potential. *Nat. Med.* 19, 1252–1263 CrossRef Medline

7. van Marken Lichtenbelt, W. D., Vanhommeghere, J. W., Smulders, N. M., Drossaerts, J. M., Kemering, G. J., Bouvy, N. D., Schrauwen, P., and Teule, G. J. (2009) Cold-activated brown adipose tissue in healthy men. *N. Engl. J. Med.* 360, 1500–1508 CrossRef

8. Virtanen, K. A., Lidell, M. E., Orava, J., Heglind, M., Westergren, R., Niemi, T., Taittonen, M., Laine, J., Savisto, N. J., Enerbäck, S., and Nuutila, P. (2009) Functional brown adipose tissue in healthy adults. *N. Engl. J. Med.* 360, 1518–1525 CrossRef Medline

9. Castan-Laurell, I., Dray, C., Attané, C., Duparc, T., Knauf, C., and Valet, P. (2011) Apelin, diabetes, and obesity. *Endocrine* 40, 1–9 CrossRef Medline

10. Ma, Y., Yue, Y., Ma, Y., Zhang, Q., Zhou, Q., Song, Y., Shen, Y., Li, X., Ma, X., Li, C., Hanson, M. A., Han, G. W., Sickmier, E. A., Swaminath, G., Zhao, S., et al. (2017) Structural basis for apelin control of the human apelin receptor. *Structure* 25, 858–866 CrossRef Medline

11. Yue, P., Jin, H., Aillaud, M., Deng, A. C., Azuma, J., Asagami, T., Kandu, R. K., Reaven, G. M., Quertermous, T., and Tsao, P. S. (2010) Apelin is necessary for the maintenance of insulin sensitivity. *Am. J. Physiol. Endocrinol. Metab.* 298, E59–E67 CrossRef Medline

12. Zhu, S., Sun, F., Li, W., Cao, Y., Wang, C., Wang, Y., Liang, D., Zhang, R., Zhang, S., Wang, H., and Cao, F. (2011) Apelin stimulates glucose uptake through the P3K/Akt pathway and improves insulin resistance in 3T3-L1 adipocytes. *Mol. Cell. Biochem.* 353, 305–313 CrossRef Medline

13.Than, A., Cheng, Y., Foh, L. C., Leow, M. K., Lim, S. C., Chua, Y. J., Kang, Y., and Chen, P. (2012) Apelin inhibits adipogenesis and lipolysis through distinct molecular pathways. *Mol. Cell. Endocrinol.* 362, 227–241 CrossRef Medline

14. Than, A., He, H. L., Chua, S. H., Xu, D., Sun, L., Leow, M. K., and Chen, P. (2015) Apelin enhances brown adipogenesis and browning of white adipocytes. *J. Biol. Chem.* 290, 14679–14691 CrossRef Medline

15. Guo, L., Li, Q., Wang, W., Yu, P., Pan, H., Li, P., Sun, Y., and Zhang, J. (2009) Apelin inhibits insulin secretion in pancreatic β cells by activation of PI3 kinase–phosphodiesterase 3B. *Endocr. Res.* 34, 142–154 CrossRef Medline
Adipocyte browning effects of losartan

32. Lee, J. Y., Takahashi, N., Yasubuchi, M., Kim, Y. I., Hashizaki, H., Kim, M. J., Sakamoto, T., Goto, T., and Kawada, T. (2012) Triiodothyronine induces UCP-1 expression and mitochondrial biogenesis in human adipocytes. Am. J. Physiol. Cell Physiol. 302, C463–C472 CrossRef Medline

33. Boucher, J., Masi, B., Davda, D., Gesta, S., Guigné, C., Mazzucottelli, A., Castan-Laurell, I., Tack, I., Knibiehler, B., Carpenté, C., Audigier, Y., Saulnier-Blache, J. S., and Valet, P. (2005) Apelin, a newly identified adipokine up-regulated by insulin and obesity. Endocrinology 146, 1764–1771 CrossRef Medline

34. Zhu, Y. P., Brown, J. R., Sag, D., Zhang, L., and Suttles, J. (2015) Adenosine 5’-monophosphate-activated protein kinase regulates IL-10-mediated anti-inflammatory signaling pathways in macrophages. J. Immunol. 194, 584–594 CrossRef Medline

35. Chang, R. S., Siegl, P. K., Clineschmidt, B. V., Mantlo, N. B., Chakravarty, P. K., Greenlee, W. J., Pitchett, A. A., and Lotti, V. J. (1992) In vitro pharmacology of L-158,809, a new highly potent and selective angiotensin II receptor antagonist. J. Pharmacol. Exp. Ther. 262, 133–138 Medline

36. Wu, H., Cheng, X. W., Hao, C., Zhang, Z., Yao, H., Murohara, T., and Dai, Q. (2014) Regulation of apelin and its receptor expression in adipose tissues of obesity rats with hypertension and cultured 3T3-L1 adipocytes. Exp. Anim. 63, 257–267 CrossRef Medline

37. Than, A., Xu, S., Li, R., Leow, M. K.-S., Sun, L., and Chen, P. (2017) Angiotensin type 2 receptor activation promotes browning of white adipose tissue and brown adipogenesis. Signal Transduct. Target. Ther. 2, 1702 CrossRef Medline

38. Dray, C., Knauf, C., Davda, D., Waget, A., Boucher, J., Buléon, M., Cani, P. D., Attané, C., Guigné, C., Carpenté, C., Burcelin, R., Castan-Laurell, I., and Valet, P. (2008) Apelin stimulates glucose utilization in normal and obese insulin resistant mice. Cell Metab. 8, 437–445 CrossRef Medline

39. Samy, D. M., Ismail, C. A., Emmam, A., Abdallah, D., and Dwedar, F. (2014) Induction of apelin by losartan in renal ischemia/reperfusion injury in rats - implication of endothelial nitric oxide synthase (eNOS) phosphorylation. J. Phys. Pharm. Adv. 4, 465–477 CrossRef Medline

40. Nishida, M., Okumura, Y., Oka, T., Toyikama, K., Ozawa, S., Itoi, T., and Hamaoka, K. (2012) The role of apelin on the alleviative effect of angiotensin receptor blocker in unilateral ureteral obstruction-induced renal fibrosis. Nephron Extra 2, 39–47 CrossRef Medline

41. Fukushima, H., Kobayashi, N., Takeda, H., Koguchi, W., and Ishimitsu, T. (2010) Effects of olmesartan on apelin/AP and Akt/endothelial nitric oxide synthase pathway in Dahl rats with end-stage heart failure. J. Cardiovasc. Pharmacol. 55, 83–88 CrossRef Medline

42. Lorente-Cebrián, S., Bustos, M., Martí, A., Martínez, J. A., and Moreno-Alia, M. J. (2010) Eicosapentaenoic acid up-regulates adiponectin secretion and gene expression in 3T3-L1 adipocytes. Mol. Nutr. Food Res. 54, S104–S111 CrossRef Medline

43. Wu, Y., Wang, X., Zhou, X., Cheng, B. L., Li, G., and Bai, B. (2017) Temporal expression of apelin/apolipoprotein receptor in ischemic stroke and its therapeutic potential. Front. Mol. Neurosci. 10, 1 CrossRef Medline

44. Tsukuda, K., Mogi, M., Iwami, J., Kanno, H., Nakaoka, H., Wang, X. L., Bae, H. Y., Shan, B. S., Kukida, M., Higaki, A., Yamauchi, T., Min, L. J., and Horiuichi, M. (2016) Enhancement of adipocyte browning by angiotensin II type 1 receptor blockade. PLoS ONE 11, e0167704 CrossRef Medline

45. Yvan-Charvet, L., Even, P., Lamandé, N., Ferré, P., and Quignoun-Boulangé, A. (2006) Prevention of adipose tissue depletion during food deprivation in angiotensin type 2 receptor-deficient mice. Endocrinology 147, 5078–5086 CrossRef Medline

46. Littlejohn, N. K., Keen, H. L., Weidemann, B. J., Clafin, K. E., Tobin, K. V., Markan, K. R., Park, S., Naber, M. C., Gourronc, F. A., Pearson, N. A., Liu, X., Morgan, D. A., Klingelhutz, A. J., Poonthoff, M. J., Rahmouni, K., et al. (2016) Suppression of resting metabolism by the angiotensin AT2 receptor. Cell Rep. 16, 1548–1560 CrossRef Medline

47. Frier, B. C., Williams, D. B., and Wright, D. C. (2009) The effects of apelin treatment on skeletal muscle mitochondrial content. Am. J. Physiol. Regul. Integr. Comp. Physiol. 297, R1761–R1768 CrossRef Medline
Adipocyte browning effects of losartan

48. Ikeda, K., Kang, Q., Yoneshiro, T., Camporez, J. P., Maki, H., Homma, M., Shinoda, K., Chen, Y., Lu, X., Marelich, P., Tajima, K., Ajuwon, K. M., Soga, T., and Kajimura, S. (2017) UCP1-independent signaling involving SEr-CA2b-mediated calcium cycling regulates beige fat thermogenesis and systemic glucose homeostasis. *Nat. Med.* 23, 1454–1465 CrossRef Medline

49. Kazak, L., Chouchani, E. T., Jedrychowski, M. P., Erickson, B. K., Shinoda, K., Cohen, P., Vetrivelan, R., Lu, G. Z., Laznik-Bogoslavski, D., Hasenfuss, S. C., Kajimura, S., Gygi, S. P., and Spiegelman, B. M. (2015) A creatine-driven substrate cycle enhances energy expenditure and thermogenesis in beige fat. *Cell* 163, 643–655 CrossRef Medline

50. Wharton, S., Raiber, L., Serodio, K. J., Lee, J., and Christensen, R. A. G. (2018) Medications that cause weight gain and alternatives in Canada: a narrative review. *Diabetes Metab. Syndr. Obes.* 11, 427–438 CrossRef Medline