

ORIGINAL ARTICLE

Valsartan ameliorates the constitutive adipokine expression pattern in mature adipocytes: a role for inverse agonism of the angiotensin II type 1 receptor in obesity

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Angiotensin (Ang) II receptor blockers (ARBs) alleviate obesity-related insulin resistance, which suggests an important role for the Ang II type 1 receptor (AT1R) in the regulation of adipocytokines. Therefore, we treated mature 3T3-L1 adipocytes with 50 $\mu\text{mol l}^{-1}$ of valsartan, a selective AT1R blocker without direct agonism to peroxisome proliferator-activated receptor (PPAR)- γ . In the absence of effective concentrations of Ang II, unstimulated mature adipocytes expressed and secreted high levels of interleukin (IL)-6. This constitutive proinflammatory activity was attenuated by the suppression of extracellular signal-regulated kinase phosphorylation by valsartan but was unaffected by the Ang II type 2 receptor blocker PD123319. COS7 cells co-transfected with AT1R and IL-6, which expressed NF- κB but lacked PPAR- γ , showed no constitutive but substantial ligand-dependent IL-6 reporter activity, which was counteracted by valsartan. Valsartan preserved cytosolic I κB - α and subsequently reduced nuclear NF- κB 1 protein expression in mature adipocytes. Interestingly, valsartan did not increase PPAR- γ messenger RNA expression *per se* but enhanced the transcriptional activity of PPAR- γ in mature adipocytes; this enhancement was accompanied by upregulation of the PPAR coactivator (PGC)-1 α . Moreover, T0090907, a PPAR- γ inhibitor, increased IL-6 expression, and this increase was attenuated by valsartan. Indeed, addition of valsartan without direct PPAR- γ agonism increased adiponectin production in mature adipocytes. Together, the findings indicate that valsartan blocks the constitutive AT1R activity involving the NF- κB pathway that limits PPAR- γ activity in mature adipocytes. Thus, inverse agonism of AT1R attenuates the spontaneous proinflammatory response and enhances the constitutive insulin-sensitizing activities of mature adipocytes, which may underlie the beneficial metabolic impacts of ARBs.

Hypertension Research (2014) 37, 621–628; doi:10.1038/hr.2014.51; published online 6 March 2014

Keywords: adiponectin; constitutive activity; diabetes mellitus; interleukin-6; peroxisome proliferator-activated receptor- γ

INTRODUCTION

Mature adipocytes produce anti-inflammatory and insulin-sensitizing cytokines, including adiponectin.¹ However, in response to abnormal metabolic stimuli in obesity, mature adipocytes predominantly produce proinflammatory adipocytokines including interleukin (IL)-6,^{2,3} which eventually leads to the infiltration of macrophages into the adipose tissue.⁴ Adipocytes in the visceral⁵ and perivascular⁶ depots are particularly responsive to such metabolic abnormalities. Interestingly, however, mature adipocytes, even in the unstimulated state, produce some of these proinflammatory adipocytokines.⁷ Indeed, a heightened proinflammatory state of the adipocytes residing in the perivascular adipose tissue surrounding the coronary arteries has been reported.⁶

Adipocytes possess their own local renin-angiotensin system (RAS), comprising angiotensinogen, renin, angiotensin-converting enzyme and several angiotensin (Ang) II receptors, including the type 1 and type 2 receptors.¹ Of these, the Ang II type 1 receptor (AT1R) is more abundantly expressed than the other receptors.¹ According to the classical concept of the RAS, Ang II binds to Ang II receptors and induces downstream effects that cause pathologies such as cardiovascular remodeling and insulin resistance. However, exploiting the structural flexibility and instability of the AT1R, recent studies have shown compelling evidence that there is constitutive Ang II-independent activity of the AT1R in cardiomyocytes and cultured cells under mechanical stress.⁸ This constitutive activity results in increases in inositol phosphate

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Received 3 July 2013; revised 21 October 2013; accepted 17 November 2013; published online 6 March 2014

production, the phosphorylation of extracellular signal-regulated kinase (ERK) and cardiac hypertrophy,⁹ which then contributes to cardiovascular remodeling. Therefore, it is conceivable that AT1R in adipocytes could produce similar constitutive proinflammatory adipocytokine activity.

The NF- κ B complex is composed of five subunits. The inhibitor of NF- κ B (I κ B)- α inhibits the translocation of this transcription factor complex into the nucleus.¹⁰ In response to various stimuli, I κ B- α is subjected to proteasomal degradation, which allows the NF- κ B complex to move into the nucleus and initiate the transcription of its target genes, which include IL-6.¹⁰ Reconciliation of these molecular connections strongly suggests that the constitutive activity of AT1R can sufficiently activate NF- κ B in adipocytes, thereby inducing a proinflammatory milieu in otherwise normal adipocytes.²

The nuclear transcription factor peroxisome proliferator-activated receptor (PPAR)- γ has numerous regulatory roles in various cell types including adipocytes.¹¹ However, the components of NF- κ B can also limit PPAR- γ activity through several mechanisms. These include suppression of PPAR- γ messenger RNA (mRNA) expression,¹² interference with DNA binding activity,¹³ and downregulation of PPAR coactivator (PGC)-1 α ,¹⁴ a coactivator of PPAR- γ .¹⁵ Therefore, AT1R blockade, through inhibition of the NF- κ B pathway, may de-repress (restore) PPAR- γ activity.

Valsartan is one of a family of drugs called angiotensin II receptor blockers (ARBs), which are widely used to treat patients with hypertension, heart failure and myocardial infarction.¹⁶ In addition to treating these conditions, valsartan mitigates obesity-induced production of several proinflammatory adipocytokines from the visceral adipose tissues.¹⁷ Indeed, although valsartan is not a PPAR γ agonist,^{18,19} a subanalysis of a clinical trial revealed that it prevented new-onset diabetes mellitus (DM).²⁰ Notably, valsartan has strong inverse agonistic effect on AT1R.²¹ Thereby, valsartan (and other inverse agonists) can produce opposite responses to those produced by the agonists, particularly for constitutively activated receptors.⁸ Although inverse agonists expand therapeutic potentials, particularly in cardiovascular medicine, their precise roles in adipocytes have not been well defined.

We hypothesized that: (1) without any external stimuli, AT1R in mature adipocytes could be activated constitutively, which would enhance IL-6 expression via the NF- κ B pathway; (2) as an inverse agonist, valsartan would hinder the activity of the receptor and thus attenuate IL-6 expression; and (3) putative cross-talk between the NF- κ B pathway and the PPAR- γ pathway might upregulate PPAR- γ target molecules even through this non-PPAR- γ agonistic AT1R blocker.

METHODS

Cell culture and the induction of differentiation

3T3-L1 preadipocytes (JCRB Cell Bank, Tsukuba, Ibaraki, Japan) were cultured and induced to differentiate into adipocytes as described previously but with slight modifications.²² Briefly, the cells were grown in DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Nihon Bioreagents, Tokyo, Japan) and 1% penicillin-streptomycin-glutamine (Invitrogen, Grand Island, NY, USA). Two days after reaching confluence, the cells were induced to adipogenic differentiation using the same medium with the addition of 0.5 mmol l⁻¹ 3-isobutyl-1-methylxanthine, 1 μ mol l⁻¹ dexamethasone and 170 nmol l⁻¹ insulin (all from Sigma-Aldrich) for 2 days, followed by medium containing 0.2 mmol l⁻¹ 3-isobutyl-1-methylxanthine, 0.4 μ mol l⁻¹ dexamethasone and 170 nmol l⁻¹ insulin for 2 days, then only 170 nmol l⁻¹ insulin for the next 2 days. The day of induction was designated D0 (day 0), and unless otherwise indicated, adipocytes at the 8th day after induction (D8) were used for the study. The cells were treated with valsartan

(Tokyo Chemical Industries, Tokyo, Japan), dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Osaka, Japan) as a control, PD123319 (Wako Pure Chemical Industries), MG132, T0090907 and bisindolylmaleimide (all from Cayman Chemicals, Ann Arbor, MI, USA) for the various times indicated. Importantly, during the intervention period (from D8) serum-free medium containing 0.5% bovine serum albumin (BSA; Sigma-Aldrich) was used instead of fetal bovine serum to avoid possible contamination of the bioactive molecules in the serum, and the medium was replenished daily. For consistency in the assays that compared D0 and D7, the medium was additionally supplemented with 0.5% BSA throughout the adipogenic differentiation process, and was replenished daily.

Biochemical measurements

The release of IL-6 and adiponectin into the culture medium was measured using enzyme-linked immunosorbent assay kits (mouse IL-6 ELISA Ready-SET-Go, eBioscience, San Diego, CA, USA, and Human/Mouse/Rat Adiponectin Enzyme Immunoassay Kit, RayBiotech, Norcross, GA, USA, respectively). The Ang II level was also measured using the Angiotensin II EIA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA).

Cell viability assay

Cell viability was assessed in cells grown in 96-well plates using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric cell proliferation assay kit (MTT, Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions.

RNA isolation and qRT-PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and first-strand cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was carried out by real-time PCR in a Step ONE Plus Real-Time PCR System (Life Technologies, Tokyo, Japan) using the primers reported previously for IL-6,⁴ phosphoenolpyruvate carboxykinase 1,²³ PGC-1 α ²³ and adiponectin²⁴ with Fast SYBR Green Master Mix (Applied Biosystems); and PPAR- γ (Applied Biosystems) was measured with Premix Ex Taq (Probe qPCR), ROX plus (Takara Bio, Tokyo, Japan). Cyclophilin B²⁴ was used as an internal control. The mRNA expression is presented as fold change over the DMSO-treated groups.

Western blotting

Eight days after the adipogenic induction (D8), the mature adipocytes were serum starved with 0.5% BSA overnight and pretreated with MG132, bisindolylmaleimide or DMSO as a control for 1 h, followed by treatment with DMSO or valsartan with or without these inhibitors for the indicated times. Western blotting was performed using conventional methods as described previously.²² Approximately 20 μ g of the whole-cell lysate proteins were subjected to 10% SDS-PAGE and were then transferred to an Immobilon-P membrane (Millipore, County Cork, Ireland). The membranes were blocked with 5% skimmed milk and incubated with an anti-phospho-p44/42 MAPK, pERK (1:1000, Cell Signaling Technology, Danvers, MA, USA), or, anti-ERK1, ERK (1:1000, BD Biosciences, San Jose, CA, USA) antibody. The cytoplasmic and nuclear fractions were extracted using an NE-PER Kit (Thermo Fisher Scientific, Rockford, IL, USA) following the manufacturer's instructions. Approximately 20 μ g of the cytoplasmic extracts were resolved and probed with anti-I κ B- α (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA) followed by anti- β -tubulin (1:1000, Cell Signaling Technology) antibodies. Subsequently, ~20 μ g of the nuclear proteins were resolved and probed with an anti-NF- κ B1 antibody (1:2500, Novus Biological, Littleton, CO, USA), followed by an anti-Lamin A/C antibody (1:1000, Cell Signaling Technology). For the COS7 cells (JCRB Cell Bank), whole-cell lysates were probed with an anti-PPAR- γ antibody (1:1000, Cell Signaling Technology), or anti-NF- κ B1 followed by β -actin (1:4000, Sigma-Aldrich) antibodies. The amounts of the proteins were quantified by densitometry, and the band intensities were standardized to their respective internal controls.

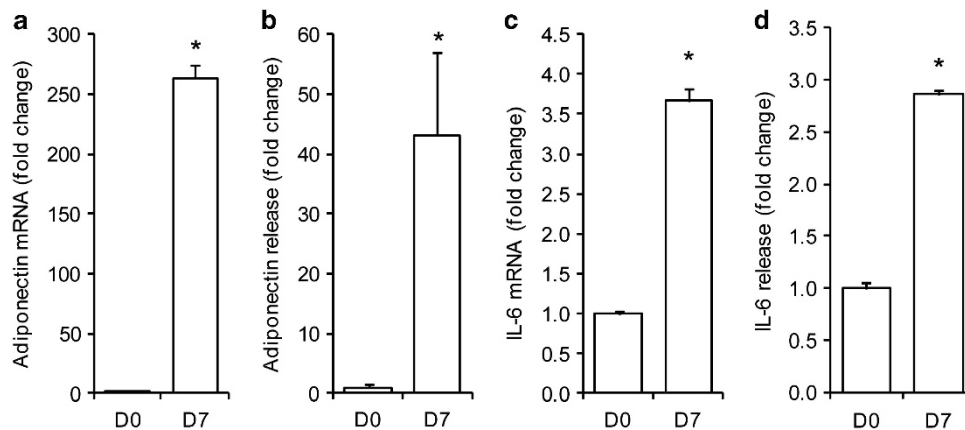


Figure 1 Changes in adiponectin and interleukin-6 (IL-6) production during adipogenesis. 3T3-L1 preadipocytes were induced to differentiate into adipocytes. The messenger RNA (mRNA) expression of (a) adiponectin and (c) IL-6 assessed by quantitative reverse transcriptase PCR and the concentrations in the culture medium of (b) adiponectin and (d) IL-6 by enzyme-linked immunosorbent assay were compared between preadipocytes immediately before induction (D0) and mature adipocytes at the seventh day of adipogenesis (D7). The mRNA expression was normalized to that of cyclophilin B. $n=3$. * $P<0.05$ vs. D0 by Student's *t*-test.

Plasmid constructs and transfection

The study procedures using recombinant DNA were approved by the Recombinant DNA Usage Committees at Kagawa University. A mouse AT1Ra expression plasmid (pAT1R) was constructed by amplifying the full-length cDNA using the following oligonucleotides: 5'-GATTGGGGTACCCCCAC CATGGCCCTTAACCTTCTAC-3' and 5'-GATACGGGATCCCGATACTC CACCTCAGAACAAAGACGCA-3' as a *Kpn* I-*Bam*HI fragment, and ligating it between the same sites of the pcDNA 3.1/myc-His C vector (pcDNA; Invitrogen). An IL-6 promoter fragment (mIL-6-Luc), extending from 1,200 to 10 bp upstream of the mouse IL-6 gene was obtained from the 3T3-L1 cell DNA using the following oligonucleotides: 5'-GATTGGGGTACCCCAAAC CAGGAAGTCTG-3' and 5'-GGAAGATCTTCGGAATTGACTATCGTT CTTGG-3' as a *Kpn* I-*Bgl*II fragment, and ligating it between the same sites of the pGL4.16[*luc2CP/Hygro*] reporter vector (Promega, Madison, WI, USA). The fragment contained a putative NF- κ B binding site (5'-TGGGATTTCCTCA-3', from -123 to -111 bp relative to the ATG) as confirmed by the TFSEARCH program.²⁵

The transient transfection of these genes into COS7 cells was performed with Fugene 6 (Roche Applied Science) using a 3:1 ratio of Fugene 6 (μ l): DNA (μ g) following the manufacturer's instructions. Briefly, ~9000 COS7 cells were seeded into each well of a 96-well plate 24 h before the transfection. The next day, the cells were transfected with the empty pcDNA (100 ng) or pAT1R (100 ng) vectors. Both groups were co-transfected with mIL-6-Luc (100 ng) and the internal control *Renilla* luciferase pGL4.74[*hRluc/TK*] vector (5 ng; Promega). After 24 h, the cells were treated with DMSO, valsartan or Ang II alone or in combination for an additional 24 h. Similar to 3T3-L1 cells, during this intervention period, serum-free medium containing 0.5% BSA and 1% penicillin-streptomycin-glutamine was used and replenished daily. Luciferase activity was measured using a dual luciferase reporter assay system (Promega, San Luis Obispo, CA, USA) following the manufacturer's instructions.

Statistical analyses

All experiments were performed in triplicate. For comparisons between two groups, a two-tailed Student's *t*-test was used; for comparisons among multiple groups, an analysis of variance followed by a Tukey's test was performed for *post hoc* comparisons if appropriate. Differences were considered significant if $P<0.05$. The error bars in the graphs denotes the s.e. of the mean.

RESULTS

Mature adipocytes express the proinflammatory marker IL-6 in the basal state

The confluent 3T3-L1 preadipocytes (D0) were induced to differentiate for 7 days (D7). By this time, most of the preadipocytes had

turned into mature adipocytes. As indicators of adipocyte maturation, the mRNA expression of adiponectin and its release into the medium were assessed. Marked increases in adiponectin expression and secretion confirmed the maturation of the D7 adipocytes (Figures 1a and b). However, the mRNA expression of IL-6, a proinflammatory adipocytokine, and its concentration in the culture medium were also significantly increased at D7 (Figures 1c and d), suggesting that a proinflammatory milieu also develops during adipocyte maturation.

Ang II secreted by mature adipocytes was too low to induce the autocrine activation of AT1R and thereby to induce IL-6 expression

To determine the effect of Ang II on IL-6 expression in mature adipocytes after 8 days of adipogenic induction (D8) and when the medium had been switched to serum-free albumin, mature adipocytes were treated with increasing concentrations of Ang II for 2 days. The IL-6 protein release was not increased at doses of $1.0 \mu\text{mol l}^{-1}$ or lower, but was significantly increased by $10 \mu\text{mol l}^{-1}$ (Supplementary Figure S1). Therefore, we chose to use $10 \mu\text{mol l}^{-1}$ of Ang II in this study. Moreover, we measured the Ang II concentration in the culture medium at D7 to identify any possible autocrine effect on IL-6 of locally released Ang II or possible contamination from the fetal bovine serum. The concentration was $0.38 \pm 0.03 \text{ ng ml}^{-1}$ (equivalent to $0.29 \pm 0.02 \text{ pmol l}^{-1}$), which was far below that required to increase IL-6 expression. These findings suggest that the effects, if any, on basal IL-6 expression of Ang II released from the adipocytes or contained in the culture medium would be negligible.

High-dose valsartan reduced constitutive and Ang II-induced IL-6 expression and secretion in mature adipocytes

Mature adipocytes at D8 were treated with valsartan at doses from 0.1 to $50 \mu\text{mol l}^{-1}$ for 2 days to assess ligand-independent action. The mRNA expression of IL-6 was suppressed to below control levels with a dose-dependent trend even at the lower doses of valsartan, whereas secretion of IL-6 into the medium was significantly reduced only at $50 \mu\text{mol l}^{-1}$ (Supplementary Figures S2a and b). These observations suggested that valsartan was an inverse agonist of AT1R and that the dose of $50 \mu\text{mol l}^{-1}$ was suitable for demonstrating this effect, as assessed by both IL-6 transcription and secretion. To further confirm the attenuation of the constitutive activity of AT1R by $50 \mu\text{mol l}^{-1}$

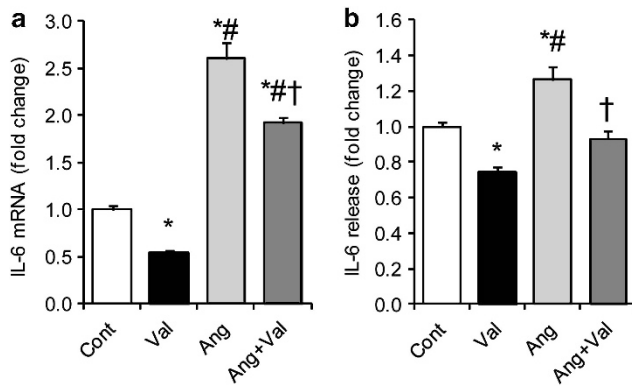


Figure 2 Effects of valsartan and angiotensin II on interleukin-6 (IL-6) transcription and secretion in mature adipocytes. 3T3-L1 adipocytes 8 days after adipogenic induction were treated with dimethyl sulfoxide (Cont), $50\ \mu\text{mol l}^{-1}$ valsartan (Val), $10\ \mu\text{mol l}^{-1}$ angiotensin II (Ang) alone or a combination (Ang+Val) for 2 days. (a) The messenger RNA (mRNA) expression and (b) release in the culture medium of IL-6 were assessed by quantitative reverse transcriptase PCR and enzyme-linked immunosorbent assay, respectively. The mRNA expression was normalized to that of cyclophilin B. $n=3$. * $P<0.05$ vs. Cont, # $P<0.05$ vs. Val, and † $P<0.05$ vs. Ang II (analysis of variance followed by a Tukey's *post hoc* test). Cont, control.

valsartan, we measured the phosphorylation of ERK²⁶ at D8 (Supplementary Figure S3). Valsartan alone significantly reduced the pERK/ERK ratio. As expected, blocking protein kinase C with bisindolylmaleimide reduced the phosphorylation of ERK, and it was further reduced by valsartan. Thus, $50\ \mu\text{mol l}^{-1}$ of valsartan blocked the constitutive activity of AT1R. This dose also caused significant classical inhibition of Ang II-induced activation and subsequent increases in IL-6 mRNA expression and secretion (Figures 2a and b).

Notably, the MTT assay confirmed that neither valsartan nor Ang II at the tested doses were cytotoxic to the adipocytes (Supplementary Figure S2c).

Valsartan reduced IL-6 expression via AT1R independently of AT2R

Subsequently, the adipocytes were treated with the specific AT2R blocker PD123319 ($10\ \mu\text{mol l}^{-1}$) alone or with $50\ \mu\text{mol l}^{-1}$ valsartan. PD123319 had no additional effects on IL-6 mRNA expression or secretion whether used alone or in combination with valsartan (Figures 3a and b). This finding suggested that there was no autocrine activation of the AT2R and that the suppression of the constitutive production of IL-6 by valsartan was independent of the AT2R.

To investigate the role of AT1R in the expression of IL-6 more precisely, we used a reporter gene, in which the luciferase gene is driven by an IL-6 promoter (mIL-6-Luc), in COS7 cells that had no detectable expression of AT1R or angiotensinogen.⁹ We further determined that the cells lacked PPAR- γ but retained NF- κ B activity (Supplementary Figure S3). The COS7 cells were transfected with an empty (pcDNA) vector or AT1R (pAT1R)-expressing vector along with mIL-6-Luc. AT1R was then activated by Ang II in the presence or absence of valsartan. The reporter activity was unaffected in all the empty vector-treated groups. Notably and dissimilarly to the result in the mature adipocytes, the valsartan did not decrease baseline IL-6 transcription in the unstimulated AT1R-transfected COS7 cells, which suggested that no constitutive AT1R activity (as measured by IL-6 expression) was present in AT1R-transfected COS7 cells. In the AT1R-transfected group, Ang II increased the activity to approximately fivefold, and valsartan treatment significantly reduced

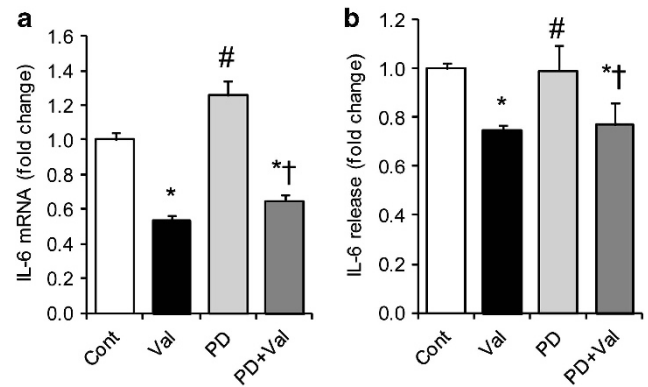


Figure 3 Effects of angiotensin II type 1 receptor (AT1R) and AT2R on basal and valsartan-induced interleukin-6 (IL-6) expression. 3T3-L1 adipocytes 8 days after adipogenic induction were treated with dimethyl sulfoxide (Cont), $50\ \mu\text{mol l}^{-1}$ valsartan (Val), $10\ \mu\text{mol l}^{-1}$ PD123319 alone (PD) or a combination (PD+Val) for 2 days. (a) The messenger RNA (mRNA) expression and (b) release in the culture medium of IL-6 were assessed by quantitative reverse transcriptase PCR and enzyme-linked immunosorbent assay, respectively. The mRNA expression was normalized to that of cyclophilin B. $n=3$. * $P<0.05$ vs. Cont, # $P<0.05$ vs. Val, † $P<0.05$ vs. PD (analysis of variance followed by a Tukey's *post hoc* test). Cont, control.

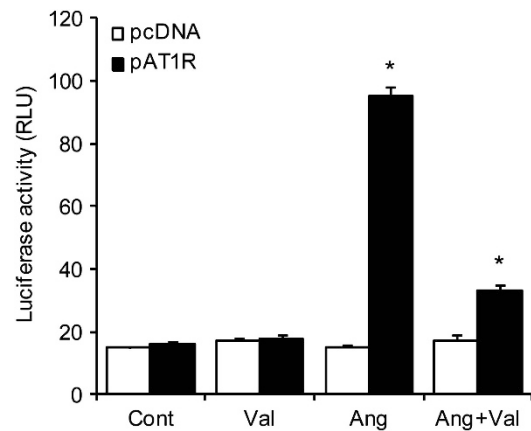


Figure 4 Involvement of angiotensin II type 1 receptor (AT1R) in angiotensin II (Ang II)-induced interleukin-6 (IL-6) transcription. COS7 cells were transfected with an AT1R-expressing vector (pAT1R) or empty vector (pcDNA) along with a firefly luciferase reporter vector containing the IL-6 promoter region and *Renilla* luciferase vector as an internal control. Next day, the cells were treated with dimethyl sulfoxide (Cont), $50\ \mu\text{mol l}^{-1}$ valsartan (Val), $10\ \mu\text{mol l}^{-1}$ Ang II alone (Ang) or a combination (Ang+Val) for 1 day, then IL-6 transcription was assessed by the luciferase activity. RLU, relative light units. $n=3$. * $P<0.05$ vs. all groups. (analysis of variance followed by a Tukey's *post hoc* test). Cont, control.

this activity (Figure 4). Whereas these observations confirmed that activated AT1R could induce transcription of IL-6 and that valsartan specifically blocked this activity to mitigate IL-6 production, they also suggested that the constitutive IL-6 expression occurred only in cells in which both PPAR- γ and NF- κ B systems were functional.

Valsartan-induced repression of IL-6 expression was accompanied by reduced nuclear translocation of NF- κ B

To elucidate the possible molecular mechanisms involving NF- κ B in the valsartan-induced attenuation of constitutive IL-6 expression, we used a selective proteasomal inhibitor, MG132, which prevents I κ B- α

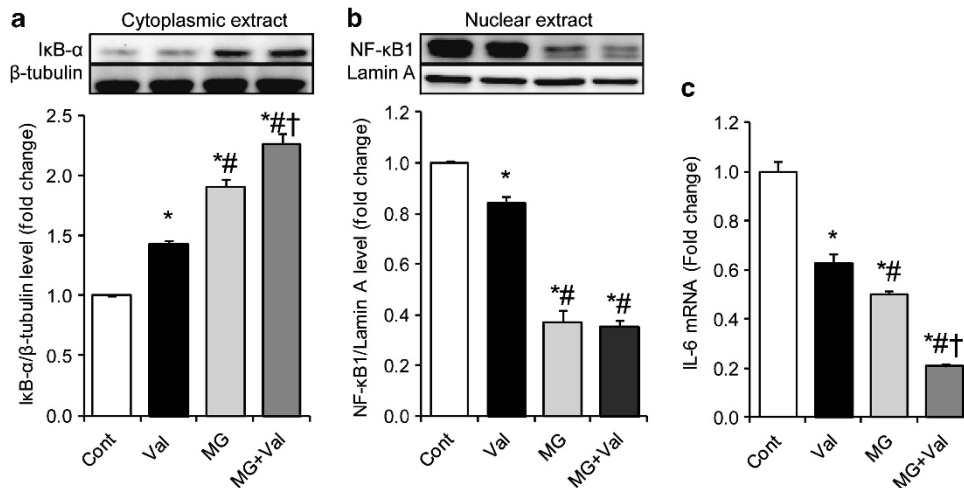


Figure 5 Effects of valsartan on constitutive IκB-α activity for interleukin-6 (IL-6) expression and on nuclear NF-κB1 protein level. 3T3-L1 adipocytes 8 days after adipogenic induction were treated with dimethyl sulfoxide (Cont), 50 μmol l⁻¹ valsartan (Val), 10 μmol l⁻¹ MG132 alone (MG) or a combination (MG + Val) for (a, b) 1 day or (c) for 2 days. (a) Protein levels of IκB-α and the loading control, β-tubulin, in the cytoplasmic extract; and (b) those of NF-κB1 and Lamin A in the nuclear extract were assessed by western blot. Representative blots are shown, and the graph shows the results of a densitometric quantification in which (a) IκB-α and (b) NF-κB1 protein levels were normalized to those of β-tubulin and Lamin A, respectively. (c) The mRNA expression of IL-6 was assessed by quantitative reverse transcriptase PCR. *n* = 3. **P* < 0.05 vs. Cont, #*P* < 0.05 vs. Val, †*P* < 0.05 vs. MG (analysis of variance followed by a Tukey's *post hoc* test). Cont, control.

degradation and thus inhibits translocation of NF-κB to the nucleus. A western blot was performed for IκB-α in the cytoplasmic extracts and for NF-κB in the nuclear extracts collected from the mature adipocytes after 1 day of treatment. Valsartan alone preserved the cytosolic IκB-α protein content similarly to MG132, and the preservation was augmented by valsartan and MG132 in combination (Figure 5a). Moreover, valsartan alone or in combination with MG132 reduced the nuclear content of NF-κB1 similarly to MG132 alone when compared with the control cells (Figure 5b). These observations imply that valsartan prevented translocation of NF-κB1 to the nucleus by preserving IκB-α, which eventually would reduce IL-6 transcription. Indeed, treatment with valsartan alone significantly reduced IL-6 mRNA expression and further enhanced the downregulation of IL-6 mRNA expression by MG132 (Figure 5c).

Valsartan upregulated the coactivator PGC-1α to augment PPAR-γ activity

Next, to evaluate the possible cross-talk between the NF-κB pathway and the PPAR-γ pathway, we explored the additional effects of valsartan on PPAR-γ. Although the mRNA expression of PPAR-γ was unchanged (Figure 6a), expression of PGC-1α was significantly upregulated by valsartan (Figure 6b). The upregulation of this coactivator was accompanied by a significant increase in the mRNA expression of the PPAR-γ target gene, phosphoenolpyruvate carboxykinase 1 (Figure 6c).

To further examine the effects of valsartan on the cross-talk between the NF-κB and PPAR-γ pathways, we treated the mature 3T3-L1 cells with valsartan or T0070907 (a specific inhibitor of PPAR-γ) alone or in combination. Although T0070907 alone significantly upregulated IL-6 expression, the addition of valsartan strongly repressed this effect to below the control level. This suggests that PPAR-γ can suppress IL-6 expression and might also rule out the direct agonism of PPAR-γ by valsartan (Supplementary Figure S5).

These results strongly suggest that valsartan indirectly augments PPAR-γ activity by upregulating its coactivator, PGC-1α, via inhibition of the NF-κB pathway, and that inhibition of constitutive IL-6 production by valsartan may involve negative cross-talk from the

NF-κB pathway to the PPAR-γ pathway. Indeed, valsartan significantly augmented the production of adiponectin, another PPAR-γ target molecule¹¹ that has anti-inflammatory actions,²⁷ both at the mRNA expression level (Figure 6d) and at the level of the protein released into the culture medium (Figure 6e).

DISCUSSION

Adipose tissue inflammation constitutes a vital mechanism for the development of metabolic disorders in obese individuals.³ However, adipocytes, particularly the perivascular deposits around the coronary arteries, show a proinflammatory phenotype even in the normal subjects,⁶ suggesting that some intrinsic factors may activate this process in the basal state. In the present study, we demonstrated for the first time that the constitutive activity of AT1R in adipocytes leads to an increase in basal IL-6 production and that valsartan, an ARB, can attenuate this activity in a manner attributable to inverse agonism.

Thus, the major findings of the present study are as follows: (1) valsartan inhibited constitutive IL-6 production in mature 3T3-L1 adipocytes; (2) COS7 cells transfected with AT1R but lacking AT2R or PPAR-γ did not show constitutive IL-6 transcription but exhibited active IL-6 transcription in response to Ang II, which was attenuated by valsartan; (3) valsartan's inverse agonism to AT1R in mature adipocytes was coupled with preserved IκB-α and reduced nuclear NF-κB content; (4) AT2R blockade did not alter constitutive IL-6 production or its valsartan-induced suppression in mature adipocytes; and (5) valsartan indirectly augmented PPAR-γ transcriptional activity by upregulating PGC-1α under negative regulation by the NF-κB pathway, which resulted in the increased production and secretion of adiponectin in mature adipocytes.

Ligand-independent constitutive activity of AT1R induces IL-6 in mature adipocytes

Mature adipocytes express several key components of the RAS,¹ the activation of which results in production of Ang II in mature adipocytes.²⁸ Therefore, it could be assumed that basal IL-6 expression in the unstimulated adipocytes could be mediated by

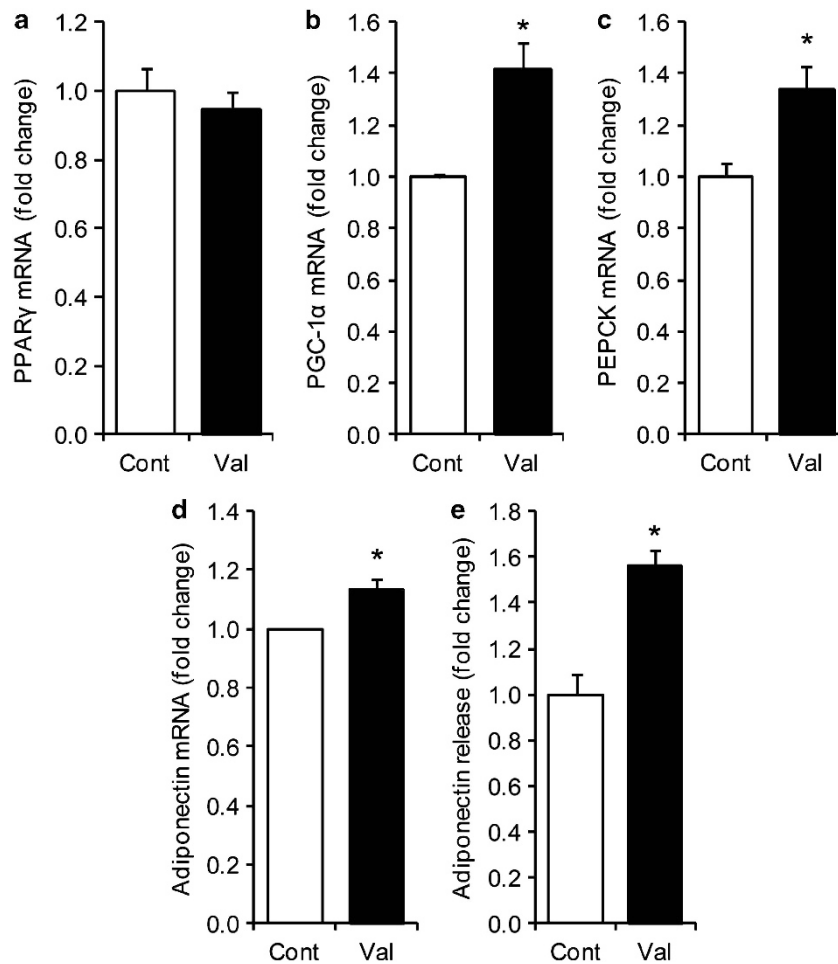


Figure 6 Effects of valsartan on peroxisome proliferator-activated receptor (PPAR)- γ target molecules in mature adipocytes. 3T3-L1 adipocytes 8 days after adipogenic induction were treated with dimethyl sulfoxide (Cont) or $50\ \mu\text{mol l}^{-1}$ valsartan (Val) for 2 days. Messenger RNA (mRNA) expression of (a) PPAR- γ , (b) PGC-1 α , (c) phosphoenolpyruvate carboxykinase (PEPCK) and (d) adiponectin was assessed by quantitative reverse transcriptase PCR. mRNA expression was normalized to that of cyclophilin B. (e) The release of adiponectin in the culture medium was assessed by enzyme-linked immunosorbent assay. $n=3$. * $P<0.05$ vs. Cont by Student's t -test. Cont, control.

endogenous Ang II via the AT receptors.¹ However, we observed that 3T3-L1 adipocytes did not produce IL-6 in response to Ang II at a concentration of up to $1.0\ \mu\text{mol l}^{-1}$ (Supplementary Figure S1). In this regard, in the current study, the cells produced substantially less Ang II ($0.38 \pm 0.03\ \text{ng ml}^{-1}$, equivalent to $0.29 \pm 0.02\ \text{pmol l}^{-1}$), which was similar to the amount produced by mature human adipocytes derived from mesenchymal stem cells ($0.2\ \text{ng ml}^{-1}$).²⁸ Thus, the amount of endogenously produced Ang II was far lower than that required to stimulate IL-6 production. Therefore, we argue that the increase in IL-6 production during the maturation of adipocytes was independent of local Ang II.

Mechanical stretch can activate AT1R and thereby trigger intracellular downstream mechanisms that result in proinflammatory and proliferative milieus in cardiomyocytes⁸ and vascular smooth muscle cells.^{9,21} It is possible that progressive lipid storage and cell enlargement during adipogenic differentiation or maturation might mechanically activate AT1R in adipocytes independently of its ligands.

High-concentration valsartan effectively attenuates the basal IL-6 expression in mature 3T3-L1 adipocytes

Recently, valsartan was shown to reduce the expression of several proinflammatory adipocytokines including IL-6 in isolated mouse

visceral adipocytes¹⁷ and to reverse the anti-adipogenic effects of Ang II.²⁹ A recent clinical trial on hypertensive patients with type 2 diabetes mellitus focused on a high dose (640 mg per day) of valsartan and demonstrated that the high dose effectively reduced albuminuria without any significant adverse effects.³⁰ Thus, because valsartan is well tolerated over a wide dose range,¹⁶ it is worth examining the potential of higher concentrations of valsartan based on the inverse agonism observed in adipocyte biology. Indeed, we observed that $50\ \mu\text{mol l}^{-1}$ of valsartan effectively reduced basal IL-6 production after 2 days of administration (Figure 2, and Supplementary Figures S2a and b), without any cytotoxic effect (Supplementary Figure S2c). Although the above-mentioned clinical study did not report the serum concentrations of valsartan, in an exploration of increasing doses, it would have reached $\sim 50\ \mu\text{mol l}^{-1}$ ($20\ \mu\text{g ml}^{-1}$ equivalent),³¹ which is similar to the dose we demonstrate here to be effective compared with the low-concentration ($10\ \mu\text{mol l}^{-1}$)¹⁸ in adipocytes.

Constitutive activity of AT1R is blocked by the inverse agonism of valsartan

The expression of several AT receptors, such as AT1R, AT2R and AT4R, has been reported in adipocytes.³² However, the functional

relevance of AT2R in mature adipocytes is less clear, and the role of AT4R is not yet well defined.³² Consistent with its local abundance, AT1R is considered to be the major AT receptor that is actively involved in various RAS activities in adipocytes.¹ Our results also showed that blocking AT2R using PD123319 could not modulate the IL-6 expression and that combined with the valsartan treatment had no additional effects (Figure 3). This observation suggests that the relative contribution of AT2R to IL-6 expression, if any, is negligible in these cells. Therefore, we assume that the valsartan-induced anti-inflammatory effect was mostly mediated through AT1R.

Recent studies have shown compelling evidence of inverse agonism by AT1R blockers⁹ that attenuates the constitutive activity of the receptors to below the basal level. Here we have demonstrated that valsartan reduced IL-6 production below the baseline in unstimulated adipocytes (Figure 2). We also observed reduced ERK phosphorylation by valsartan to below basal levels. Thus, we argue that the constitutive activity of AT1R is responsible for the increased IL-6 expression in the basal state, which can be reversed by the inverse agonism of valsartan to AT1R.

Notably, during the interventions, 3T3-L1 cells were cultured in serum-free medium supplemented with 0.5% BSA, which ruled out the presence of any external occult stimulators interfering with the findings.

Valsartan shows a relatively higher affinity for the AT1R than for the AT2R,³¹ which leads to the reputational advantage of valsartan based on highly selective blockage of AT1R and relative stimulation of AT2R by freed Ang II. However, because valsartan attenuated IL-6 production with negligible amounts of endogenous Ang II in the medium, and because selective AT2R blockade by PD123319 did not augment IL-6 production or attenuate valsartan-induced IL-6 downregulation (Figure 3), this is unlikely to be the mechanism by which of IL-6 production is attenuated in mature adipocytes.³³

Valsartan inactivates NF- κ B signaling to reduce IL-6 expression

The end-point of the classical Ang II–AT1R–NF- κ B–IL-6 pathway is the proteasomal degradation of I κ B- α and the release of the RelA–NF- κ B1 complex. The complex is then translocated to the nucleus where it induces IL-6 transcription.¹⁰ Therefore, blocking of the I κ B- α degradation should reduce IL-6 expression. As anticipated, treating adipocytes with the potent and selective proteasomal inhibitor, MG132, prevented I κ B- α degradation and inhibited NF- κ B translocation to the nucleus (Figure 5b) and markedly reduced IL-6 expression (Figure 5c). In this study, we made the novel observation that valsartan similarly increased cytosolic I κ B- α while reducing the nuclear NF- κ B1 content (Figures 5a and b). Indeed, the IL-6 mRNA expression was significantly reduced by both valsartan and MG132 and was reduced even more so in combination (Figure 5c). Thus, these findings strongly suggest that the inverse agonism of AT1R by valsartan inhibits I κ B- α degradation and reduces nuclear translocation of NF- κ B1, thereby reducing the transcription of IL-6.

Of note, valsartan did not reduce baseline IL-6 mRNA expression in AT1R-transfected COS7 cells (Figure 4), which retain NF- κ B but lack PPAR- γ (Supplementary Figure S4). Thus, the constitutive activity of AT1R manifests as IL-6 expression only when both the PPAR- γ and NF- κ B pathways are functional in the cells. Therefore, we speculate that the cross-talk between the I κ B/NF- κ B pathway and the PPAR- γ pathway in mature adipocytes is involved in the observed constitutive AT1R activity, as measured by IL-6 production.

Valsartan augments PPAR- γ activity to increase the expression and secretion of adiponectin

Interestingly, valsartan enhanced constitutive PPAR- γ transcription activity in mature adipocytes (Figures 6c and d). Unlike irbesartan³⁴ or telmisartan,³⁵ valsartan is not a direct PPAR- γ agonist.¹⁸ Direct non-cellular experiments showed that among several ARBs, valsartan and olmesartan had no effect on PPAR- γ activation up to at least 50 μ mol l⁻¹, which was used in the present study.¹⁹ In our study, PGC-1 α , which is essential for the transcriptional activity of PPAR- γ ,¹⁵ was upregulated by valsartan (Figure 6b). We speculate that PPAR- γ transcriptional activity could, at least in part, remain suppressed by the AT1R–NF- κ B activity downregulating PGC-1 α , a process that can be reversed by blockade of AT1R. Indeed, valsartan, which inhibited NF- κ B, significantly upregulated PGC-1 α expression and augmented PPAR- γ transcriptional activity without altering the expression of PPAR- γ mRNA itself (Figure 6a). Enhancement of the expression and secretion of adiponectin in mature adipocytes (Figures 6d and e) would be another benefit of valsartan for obese patients in addition to limiting IL-6 release to ameliorate the proinflammatory milieu.

Limitations of this study

There are several limitations of this study. First, the doses of Ang II and valsartan were fixed at relatively high levels; other combinations at the lower doses would have yielded different insights. Second, we attributed the absence of constitutive activity of AT1R (measured as IL-6 expression in AT1R-transfected COS7 cells) to the lack of PPAR- γ . The precise mechanisms for the constitutive activity of AT1R should be explored using immediate markers of AT1R activity that are located upstream of the NF- κ B or PPAR- γ pathways in the future. Similarly, hypothetical mechanisms of possible cross-talk between PPAR- γ and angiotensin pathways need to be studied. Finally, this study assessed only a part of the intracellular angiotensin signaling. AT4R may have a role and the NADPH oxidase complex, a major player in this system, remains to be studied in the future.

Clinical implications

Several ARBs are advantageous because their chemical properties happen to have a high affinity to the ligand binding site of PPAR- γ , resulting in adiponectin increases or improved lipid metabolism in addition to the lowering of blood pressure. However, RAS inhibitors are generally reported to ameliorate metabolic abnormalities in hypertensive patients, improving insulin resistance and preventing new-onset DM. In the present study, possibly advantageous cross-talk between AT1R blockage and PPAR- γ activity by an ‘inverse agonistic’ and ‘PPAR- γ -neutral’ ARB is shown in unstimulated mature adipocytes that would underlie the additional beneficial effects of this class of agents for obese patients.

CONCLUSIONS AND PERSPECTIVES

We have demonstrated a previously unappreciated role for the constitutive activity of AT1R in inducing proinflammatory IL-6 expression in mature adipocytes, which valsartan effectively ameliorated by its inverse agonism on this receptor. The present study highlights that the basal AT1R activity should be taken into account as an additional precipitating factor for the accentuated proinflammatory nature of mature adipocytes, which is an important therapeutic target.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to MA Hossain, C Takahashi and E Takahashi for their constructive suggestions and invaluable support in various aspects of the study and A Yamagami, Y Fujita and T Oka for their excellent technical suggestions and assistance. This study was partially supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science No. 24591057, awarded to KO.

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Supplementary Information accompanies the paper on Hypertension Research website (<http://www.nature.com/hr>)