Valsartan, independently of AT1 receptor or PPARγ, suppresses LPS-induced macrophage activation and improves insulin resistance in cocultured adipocytes

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Iwashita M, Sakoda H, Kushiyama A, Fujishiro M, Ohno H, Nakatsu Y, Fukushima T, Kumamoto S, Tsuchiya Y, Kikuchi T, Kurihara H, Akazawa H, Komuro I, Kamata H, Nishimura F, Asano T. Valsartan, independently of AT1 receptor or PPARγ, suppresses LPS-induced macrophage activation and improves insulin resistance in cocultured adipocytes. Am J Physiol Endocrinol Metab 302: E286–E296, 2012. First published November 1, 2011; doi:10.1152/ajpendo.00324.2011.—Macrophages are integrated into adipose tissues and interact with adipocytes in obese subjects, thereby exacerbating adipose insulin resistance. This study aimed to elucidate the molecular mechanism underlying the insulin-sensitizing effect of the angiotensin II receptor blocker (ARB) valsartan, as demonstrated in clinical studies. Insulin signaling, i.e., insulin receptor substrate-1 and Akt phosphorylations, in 3T3-L1 adipocytes was impaired markedly by treatment with tumor necrosis factor-α (TNFα) or in the culture medium of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages, and valsartan had no effects on these impairments. However, in contrast, when cocultured with RAW 264.7 cells using a transwell system, the LPS-induced insulin signaling impairment in 3T3-L1 adipocytes showed almost complete normalization with coadaption of valsartan. Furthermore, valsartan strongly suppressed LPS-induced productions of cytokines such as interleukin (IL)-1β, IL-6, and TNFα with nuclear factor-κB activation and c-Jun NH2-terminal kinase (JNK) phosphorylation in RAW 264.7 and primary murine macrophages. Very interestingly, this effect of valsartan was also observed in THP-1 cells treated with angiotensin II type 1 (AT1) siRNA or a peroxisome proliferator-activated receptor-γ (PPARγ) agonist as well as macrophages from AT1a receptor-knockout mice. We conclude that valsartan suppresses the inflammatory response of macrophages, albeit not via PPARγ or the AT1a receptor. This suppression appears to secondarily improve adipose insulin resistance.

angiotensin II type 1; peroxisome proliferator-activated receptor-γ; type 2 diabetes; angiotensin II receptor blockers; inflammation

INSULIN RESISTANCE PLAYS A CRITICAL ROLE in the development and worsening of type 2 diabetes mellitus. The most common cause of insulin resistance is obesity (9, 17, 20, 32). In the obese state, enlarged adipocytes and infiltrating macrophages exert effects on each other, triggering a vicious cycle that is involved in the pathogenesis of chronic inflammation and insulin resistance (16, 17, 29, 47, 49). Peroxisome proliferator-activated receptor-γ (PPARγ) agonists, of which the main target is adipocytes, are widely used to treat obesity-related insulin resistance (23, 27, 41). Ever since the end of the 20th century, much attention has been paid to the relationship between the renin-angiotensin system and insulin resistance. First, it was reported that angiotensin-converting enzyme (ACE) inhibitors enhance insulin sensitivity (14, 24). A previous study showed that in vivo ACE inhibitor administration increases the serum bradykinin concentration, which appears to delay dephosphorylation and increase phosphorylation of the insulin receptor. Thus, whereas ACE inhibitors were considered to enhance insulin sensitivity, angiotensin II receptor blockers (ARBs) had no effect on bradykinin metabolism (44). Nonetheless, many studies have shown ARBs to improve insulin resistance significantly in type 2 diabetic patients (2, 8, 22, 39).

Interestingly, some other ARBs, such as telmisartan, reportedly exert PPARγ agonist activity, raising the possibility that the insulin-sensitizing effects of ARBs are attributable to their PPARγ stimulatory activity (2, 37). However, valsartan exhibits an insulin-sensitizing effect (28), although it lacks PPARγ activity, as reported previously (2). To investigate the mechanism underlying valsartan-induced insulin-sensitizing effect, which is clearly independent of PPARγ activity, we employed a coculture system containing both adipocytes and macrophages. Since valsartan did not improve insulin signaling in 3T3-L1 adipocytes treated with tumor necrosis factor-α (TNFα) or conditioned medium from activated RAW 264.7 macrophages but markedly enhanced the signaling in adipocytes cocultured with RAW 264.7 macrophages, we speculate that the principle action of valsartan is suppression of the inflammatory response of macrophages.

Herein, we present evidence suggesting that the main target of valsartan is not adipocytes but macrophages. Furthermore, the insulin-sensitizing effect involves neither angiotensin II type 1 (AT1) receptors nor PPARγ.
MATERIALS AND METHODS

Materials. Anti-insulin receptor substrate-1 (IRS-1), anti-glucose transporter type 4 (GLUT4), and anti-Akt antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-insulin receptor-β subunit (IRβ; C-19), anti-inhibitor-kB (IkBα; C-21), anti-JNK (C-17), anti-p38 (C-20), anti-p65 (C-20), and anti-AT1 (N-10) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (Thr308), anti-phospho-p65 (Ser536), anti-phospho-c-Jun NH2-terminal kinase (JNK1), anti-extracellular signal-regulated kinase (ERK) 1/2, anti-phospho-p38, anti-phospho-IκB kinase (IKK) α/β (Ser176/180), and anti-IKKα antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-actin antibody and cycloheximide were purchased from Sigma (St. Louis, MO). The PPARγ inhibitor GW-9662 was purchased from Calbiochem (Gibbstown, NJ).

Animals. C57BL/6J (obtained from The Jackson Laboratory, Bar Harbor, ME) or AT1a-knockout (KO) mice (42) were housed under climate-controlled conditions with a 12:12-h light-dark cycle and were provided standard food and water ad libitum. All protocols were approved by the Institutional Review Board of Hiroshima University.

Cells and cell culture. Mouse 3T3-L1 preadipocytes [American Type Culture Collection (ATCC), Manassas, VA] were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Nissui Pharmaceuti- cal, Tokyo, Japan) containing 10% donor calf serum in an atmosphere of 10% CO2 at 37°C. Two days after the 3T3-L1 fibroblasts had reached confluence, differentiation was induced by treating the cells with DMEM containing 4 μg/ml dexamethasone, 0.5 mM 3-isobutyl-1-methyloxanthine, 200 nM insulin (Cell Science & Technology In- stitute, Sendai, Japan), and 10% fetal bovine serum (FBS) for 48 h, as described previously (1, 1a). Cells were fed DMEM supplemented with 10% FBS every other day and used as mature 3T3-L1 adipocytes on day 8 after the induction of differentiation.

The murine macrophage cell line RAW 264.7 was obtained from ATCC. RAW 264.7 and differentiated 3T3-L1 were cultured in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin.

Coculture of adipocytes and macrophages was performed using a transwell system (Corning, Acton, MA) with a 0.4-μm porous mem- brane to separate the upper and lower chambers. One × 106 differentiated 3T3-L1 cells were cultured in the lower chamber, whereas 5 × 105 RAW cells were cultured in the upper chamber (30).

Peritoneal macrophages were isolated from C57BL/6J mice or AT1a-KO mice. The mice were injected intraperitoneally with 2 ml of 4% thioglycoclate medium (Difco Laboratories, Detroit, MI). Four days later, the cells were harvested by injecting 5 ml of phosphate-buffered saline (PBS) into the peritoneal cavity with massage and then drawing the fluid containing macrophages back into the syringe. The cells were plated in RPMI 1640 (Nissui) medium supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Human THP-1 monocytic leukemia cells (ATCC) were also maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin.

The cells were treated with the indicated concentrations of Esche- richia coli LPS (Sigma) in the presence or absence of valsartan (Novartis Pharma, Basel, Switzerland) for the indicated times. Total RNA was isolated using Sepazol-RNA I (Nakalai Tesque, Kyoto, Japan), and 1 μg of RNA was reverse transcribed with Transcriptor Reverse Transcriptase (Roche). The amplification reaction was performed using SYBR Premix Ex Taq (Takara, Shiga, Japan) according to the manufacturer’s protocol. The primers were as follows: mouse IL-1β forward 5′-TCGCTCAAGGGTCACACAA3′-3′; mouse IL-1β reverse 5′-CATGAGGGAAGGGAACAC-3′; mouse TNFα forward 5′-GCCACACGCTTCTTCGCT-3′; mouse TNFα reverse 5′-GTCTGGCCGACATAGCTG-3′; mouse IL-6 forward 5′-GATGTCAACAACTGATATA-3′; mouse IL-6 reverse 5′-GCTTCTAGCCACTCTTCTG-3′; mouse Toll-like receptor (TLR4) forward 5′-CAGGTTGAAATGTTGCG-3′; mouse CD4 forward 5′-GGATTTTACTTCACTCACA-3′; mouse CD4 reverse 5′-GAGAAGCCAGAACACATCG-3′; mouse CD14 forward 5′-GGATTTTACTTCACTCACA-3′; mouse CD14 reverse 5′-GAGAAGCCAGAACACATCG-3′; mouse GAPDH forward 5′-TGACGTGCGCCCTGGAGAA-3′; mouse GAPDH reverse 5′-AGTGGACCAAGATTGCCCTTCG-3′; human IL-1β forward 5′-ACCAAATGTGGCCGTGGTTTT-3′; human TNFα forward 5′-GCCACACGCTTCTTCGCT-3′; human TNFα reverse 5′-GCCAT- TGCCACAGGGCC-3′; human IL-6 forward 5′-AAACCTCTCA- CTGGCCACCA-3′; human IL-6 reverse 5′-TCTGGCTCTGAAAC- AAAAGGT-3′; human TNFα forward 5′-GAAAGGTGAAAGTGCTTTGCC-3′; human TNFα reverse 5′-GAAAGGTGAAAGTGCTTTGCC-3′. Post-PCR melting curves confirmed the specificity of single-target amplification. Fold changes in the expressions of IL-1β, TNFα, and IL-6 relative to GAPDH were determined in triplicate.

ELISA. Reagents of TNFα, IL-1β, and IL-6 were measured in the supernatants of RAW 264.7 macrophages treated with LPS in the absence or presence of valsartan using ELISA kits (eBioscience, San Diego, CA) according to the manufacturer’s instructions. Absorbances at 450 nm were determined using a micro- plate reader (Bio-Rad Laboratories, Hercules, CA).

Plasma membrane sheet assay. After stimulation with or without 100 nM insulin for 30 min, plasma membrane sheets were prepared from 3T3-L1 adipocytes essentially by the methods of Robinson and colleagues (33, 34). Following the isolation of plasma membrane sheets, these purified membranes were scraped directly into Laemmli sample buffer and used for immunoblotting.

Transfection and luciferase reporter assay. One day before transfection, the cells were plated to achieve 80–90% confluence at the time of transfection. Cells were transfected with NF-κB luciferase reporter, activator protein-1 (AP-1) luciferase reporter plasmid (Clontech, Palo Alto, CA), and pRL-TK Vector (Promega, Madison, WI) to normalize transfection efficiency, using FuGENE HD Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer’s instructions. The cells were left overnight and then incubated with or without 10 ng/ml LPS or 20 μM valsartan for an additional 8 or 20 h. The cells were then washed with PBS, and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

Measurement of mRNA expression by real-time PCR. Cells were treated with 10 μg/ml LPS in the presence or absence of valsartan for the indicated times. Total RNA was isolated using Sepazol-RNA I (Nakalai Tesque, Kyoto, Japan), and 1 μg of RNA was reverse transcribed with Transcriptor Reverse Transcriptase (Roche). The amplification reaction was performed using SYBR Premix Ex Taq (Takara, Shiga, Japan) according to the manufacturer’s protocol. The primers were as follows: mouse IL-1β forward 5′-GATGTCAACAACTGATATA-3′; mouse IL-1β reverse 5′-CATCAGGGAAGGGAACAC-3′; mouse TNFα forward 5′-GCCACACGCTTCTTCGCT-3′; mouse TNFα reverse 5′-GTCTGGCCGACATAGCTG-3′; mouse IL-6 forward 5′-GATGTCAACAACTGATATA-3′; mouse IL-6 reverse 5′-GCTTCTAGCCACTCTTCTG-3′; mouse Toll-like receptor (TLR4) forward 5′-CAGGTTGAAATGTTGCG-3′; mouse CD4 forward 5′-GGATTTTACTTCACTCACA-3′; mouse CD4 reverse 5′-GAGAAGCCAGAACACATCG-3′; mouse GAPDH forward 5′-TGACGTGCGCCCTGGAGAA-3′; mouse GAPDH reverse 5′-AGTGGACCAAGATTGCCCTTCG-3′; human IL-1β forward 5′-ACCAAATGTGGCCGTGGTTTT-3′; human TNFα forward 5′-GCCACACGCTTCTTCGCT-3′; human TNFα reverse 5′-GCCAT- TGCCACAGGGCC-3′; human IL-6 forward 5′-AAACCTCTCA- CTGGCCACCA-3′; human IL-6 reverse 5′-TCTGGCTCTGAAAC- AAAAGGT-3′; human TNFα forward 5′-GAAAGGTGAAAGTGCTTTGCC-3′; human TNFα reverse 5′-GAAAGGTGAAAGTGCTTTGCC-3′. Post-PCR melting curves confirmed the specificity of single-target amplification. Fold changes in the expressions of IL-1β, TNFα, and IL-6 relative to GAPDH were determined in triplicate.

ELISA. Reagents of TNFα, IL-1β, and IL-6 were measured in the supernatants of RAW 264.7 macrophages treated with LPS in the absence or presence of valsartan were measured using ELISA kits (eBioscience, San Diego, CA) according to the manufacturer’s instructions. Absorbances at 450 nm were determined using a micro-plate reader (Bio-Rad Laboratories, Hercules, CA).

Plasma membrane sheet assay. After stimulation with or without 100 nM insulin for 30 min, plasma membrane sheets were prepared from 3T3-L1 adipocytes essentially by the methods of Robinson and colleagues (33, 34). Following the isolation of plasma membrane sheets, these purified membranes were scraped directly into Laemmli sample buffer and used for immunoblotting.
Statistical analysis. Data are expressed as means ± SE. Statistical analyses were performed using ANOVA or Student’s t-test. Values of P < 0.05 were considered significant.

RESULTS

Valsartan has no effect on TNFα-induced insulin signaling impairment in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with 10 ng/ml TNFα in the presence or absence of 10 μM valsartan for 24 h. The expression levels of IRβ, IRS-1, and GLUT4 were reduced markedly by TNFα treatment, and valsartan had no significant effect on these reductions in either control or TNFα-stimulated 3T3-L1 adipocytes. Insulin-induced phosphorylations of IRβ, IRS-1, and Akt were also reduced by incubation with TNFα, and no changes were observed with valsartan treatment (Fig. 1A). Our quantitative analysis of the immunoblotting results, using the LAS-3000 mini (FujiFilm), revealed decreased tyrosine phosphorylations of IRβ and IRS-1 to be attributable to decreased protein levels. These results are in a good agreement with those of other investigations (6, 19) showing that TNFα decreases expressions of IRβ, IRS-1, and GLUT4 without affecting the tyrosine kinase activity of IRβ.

The effect of valsartan on adipose differentiation was also examined compared with that of troglitazone (Fig. 1C). As reported previously, troglitazone significantly induced adipose differentiation of 3T3-L1 cells even in the absence of induction medium.

Fig. 1. The effects of TNFα and valsartan (Val) on expressions of insulin receptor-β subunit (IRβ), insulin receptor substrate (IRS-1), and glucose transporter type 4 (GLUT4) on insulin-induced phosphorylations of IRβ, IRS-1, and Akt and on adipose differentiation of 3T3-L1 cells. A and B: 3T3-L1 adipocytes were treated in the presence or absence of 10 ng/ml TNFα or 10 μM valsartan for 24 h. The cells were stimulated with or without 100 ng/ml insulin for 10 min, and the cell lysates were then subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The protein expression levels of IRβ, IRS-1, Akt, and GLUT4, the tyrosine phosphorylations of IRβ and IRS-1, and the phosphorylation of Akt at (Thr308) were measured by immunoblotting using the corresponding antibodies. A: representative blots are shown, and quantitative data from 4 independent experiments are presented as bar graphs. B: without insulin stimulation. C: 10 ng/ml TNFα, 10 μM troglitazone, and 10 μM valsartan were added as the indicated combinations to normal or adipose differentiation induction medium. Seven days after this induction, adipose differentiation of 3T3-L1 cells was examined by oil red O staining.
medium. In addition, TNFα treatment inhibited this adipose differentiation in the presence of induction medium, although troglitazone overcame this inhibitory effect of TNFα. In contrast, valsartan neither induced adipose differentiation of 3T3-L1 cells nor blocked the inhibitory effect of TNFα. These results suggest that valsartan exerts no PPARγ agonist activity, which is highly consistent with the findings of previous studies.

Valsartan does not normalize the insulin resistance nor NF-κB activation in 3T3-L1 adipocytes induced by medium containing LPS-stimulated macrophages or TNFα. As shown in Fig. 2A, RAW 264.7 macrophages were stimulated with or without 1 ng/ml LPS for 24 h, and this culture medium was added to 3T3-L1 adipocytes. The culture medium from LPS-stimulated RAW 264.7 macrophages significantly reduced insulin-induced phosphorylations of IRβ, IRS-1, and Akt (Thr308). It was also shown that valsartan treatment of 3T3-L1 cells exerted no significant effect on the insulin-signaling impairment produced by medium from LPS-stimulated RAW 264.7 macrophages (Fig. 2B and C). We also investigated effects on the GLUT4 expression level and insulin-stimulated GLUT4 translocation to the plasma membrane. Conditioned medium from RAW 264.7 macrophages decreased not only whole cellular GLUT4 content but also the amount of GLUT4 on the plasma membrane when stimulated with insulin. Valsartan did not normalize these impairments of GLUT4 (Fig. 2D). Conditioned medium and TNFα induced phosphorylation of IKKβ significantly, and coaddition of valsartan did not affect these phosphorylation levels. Similarly, valsartan did not alter the NF-κB transcriptional activity induced by conditioned medium or TNFα (Fig. 2E and F).

Valsartan normalized the insulin-signaling impairment in 3T3-L1 adipocytes produced by LPS-stimulated cocultured RAW 264.7 macrophages. In contrast, in the coculture system containing RAW 264.7 macrophages and 3T3-L1 adipocytes

![Figure 2](http://ajpendo.physiology.org/)

Fig. 2. Valsartan did not ameliorate either the insulin signaling or the NF-κB activation impairment in 3T3-L1 adipocytes caused by the culture medium containing LPS-stimulated macrophages or by TNFα. A: RAW 264.7 macrophages were stimulated with or without 1 ng/ml LPS for 24 h, and this culture medium was added to 3T3-L1 adipocytes with or without the addition of 20 μM Val for 1 h previously. B and D: the cells were stimulated with 100 ng/ml insulin for 10 min. The protein expressions or insulin-induced phosphorylations of IRβ, IRS-1, Akt (Thr308), and GLUT4 (membrane and whole cell) were examined by immunoblotting. Representative blots are shown. C and D: quantitative data from 4 independent experiments are presented as bar graphs. E: 3T3-L1 adipocytes transfected with p-NF-κB-Luc were stimulated with conditioned medium (CM) or TNFα for 8 h with or without 20 μM valsartan. Lysates of the cells were subjected to the luciferase assay. F: 3T3-L1 adipocytes were stimulated with CM or TNFα for 8 h with or without 20 μM valsartan. The protein expressions of phosphorylated IKKβ were examined with immunoblotting. Representative immunoblots from 4 independent experiments are shown, and quantified data are shown as bar graphs. *P < 0.05, **P < 0.01, and ***P < 0.001, Student’s t-test.
LPS treatment markedly impaired insulin signaling, as reflected by IRS-1 and Akt phosphorylations in 3T3-L1 adipocytes, but the coaddition of valsartan almost completely abolished the effect of LPS treatment (Fig. 3, B and C). Similarly, GLUT4 amounts in the whole cell or on the plasma membrane after insulin stimulation were markedly suppressed by LPS treatment, but coaddition of valsartan normalized these amounts significantly (Fig. 3D).

Valsartan inhibits LPS-induced cytokine productions by macrophages. Stimulation with 10 ng/ml LPS for 24 h markedly increased levels of IL-1β, TNFα, and IL-6 mRNA 12- to 16-fold. Coincubation with valsartan dose-dependently reduced these LPS-induced increases in cytokine mRNA expressions (Fig. 4A). Very similar results were obtained for primary cultured murine peritoneal macrophages. LPS treatment increased the levels of IL-1β, TNFα, and IL-6 mRNA ~40-, 100-, and 180-fold, respectively, and these increases were markedly suppressed by coincubation with valsartan (Fig. 4B). In fact, these cytokine levels in the medium secreted from RAW macrophages were shown to be almost completely suppressed by valsartan (Fig. 4C).

Valsartan inhibits LPS-induced NF-κB activation via p65 phosphorylation and JNK/AP-1 activity in RAW 264.7 cells. To ascertain the molecular mechanism underlying valsartan-induced suppression of cytokine production, NF-κB and AP-1 activations were investigated using the luciferase assay, since both are involved in inflammatory responses and productions of proinflammatory cytokines such as NF-κB (7, 52). As shown in Fig. 5A, left, LPS stimulation enhanced the transcriptional activity of NF-κB approximately sixfold, and valsartan strongly suppressed this increase. Similarly, LPS stimulation enhanced AP-1 transcriptional activity approximately three- to fourfold, and valsartan suppressed this increase (Fig. 5A, right).

Fig. 3. Val normalized the insulin-signaling impairment in 3T3-L1 adipocytes in LPS-stimulated cocultured RAW cells. A: using the coculture system containing both RAW 264.7 macrophages and 3T3-L1 adipocytes, the effects of LPS and Val on insulin signaling in 3T3-L1 adipocytes were examined. Cells were treated in the presence or absence of 1 ng/ml LPS or 10 μM Val for 24 h. B and D: the protein expressions or insulin-induced phosphorylations of IRβ, IRS-1, Akt, and GLUT4 (membrane and whole cell) were examined by immunoblotting. Representative blots are shown. Coculture (×) and (–) mean adipocytes cultured with and without macrophages, respectively. C: quantitative data from 4 independent experiments (33 conditions in B) are presented as bar graphs. *P < 0.05 and **P < 0.01, Student’s t-test.
The complex of p50 and phosphorylated p65 is reportedly involved in NF-κB activation, and we found LPS-induced phosphorylation of p65 to be significantly suppressed by valsartan (Fig. 5, B and C). IκBα degradation was also shown to be delayed by valsartan treatment. In addition, LPS induces activation of mitogen-activated protein kinase (MAPK) pathways. Therefore, we examined the effects of valsartan on LPS-induced phosphorylations of ERK1/2, p38 MAPK, and JNK1. It was revealed that valsartan had no apparent effects on the phosphorylations of ERK1/2 and p38 MAPK, whereas the phosphorylation of JNK1 was significantly suppressed by valsartan (Fig. 5, B and C). These immunoblotting data, shown in Fig. 5, B and C, are in a good agreement with those of the NF-κB and AP-1 assays presented in Fig. 5A.

Angiotensin II raises cytokine mRNA levels slightly in RAW 264.7 macrophages. RAW 264.7 macrophages were stimulated with various concentrations of angiotensin II, and maximal effects were obtained at 100 μM. Angiotensin II at this concentration increased slightly the expressions of TNFα and IL-6 mRNA to approximately two- to threefold those of the control, although these changes were not statistically significant (Fig. 4D). Angiotensin II did not affect the IL-1β mRNA level. These results indicate the effect of angiotensin II to be far weaker than that of LPS.

Valsartan does not exert its effect via the AT1a receptor or PPARγ. To examine whether or not valsartan exerts its effect via AT1 receptors, we carried out two separate experiments, one using macrophages from AT1a-KO mice and the other using THP-1 cells treated with AT1 siRNA. First, it was shown that LPS stimulation raised the levels of cytokine mRNAs in macrophages from AT1a-KO mice to the same levels as those in control mice. In addition, very interestingly, in macrophages from AT1a-KO mice, valsartan markedly suppressed LPS-induced increases in cytokine mRNA levels, essentially the same effect that this drug had in control mice (Fig. 6). Taking the previous report showing no expression of AT1b in mouse macrophages (26) into consideration, it is likely that AT1 is not required for valsartan to exert its effect.

Subsequently, we compared the effect of valsartan on cytokine production by control siRNA- and AT1 siRNA-treated...
THP-1 cells. Since THP-1 is a human cell line, it expresses only one isoform of AT1. The two siRNAs against human AT1 each markedly suppressed the expression of AT1 protein in THP-1, as shown by immunoblotting (Fig. 7), but the effects of valsartan on LPS-induced cytokine production were not altered.

We also investigated whether or not the effect of valsartan was inhibited by a PPARγ antagonist, GW-9662. The suppressive effect of valsartan on LPS-induced cytokine production was not inhibited by GW-9662 treatment (Fig. 8). Taking these observations together, it is reasonable to conclude that neither AT1 nor PPARγ is necessary for valsartan to exert its effects.

**DISCUSSION**

Adipocytes have long been regarded as a storage tissue for excess nutrition, but it is now widely recognized that adipocytes secrete numerous factors that regulate metabolism, appetite, inflammation, and so on (15, 18, 36). As adipocyte size increases, properties change. For example, small adipocytes secrete adiponectin or leptin, whereas...
enlarged adipocytes secrete several proinflammatory cytokines, such as IL-1β, IL-6, and TNFα, although large adipocytes may not be more insulin resistant than small ones (48). Recently, a tendency for enlarged adipocytes to undergo apoptosis, followed by macrophage infiltration around these enlarged adipocytes, was reported (13, 35, 40). This relationship between macrophages and adipocytes reportedly plays a central role in the pathogenesis of obesity-associated chronic inflammation and insulin resistance (16, 17, 29, 47, 49).

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Fig. 6. Effects of Val on cytokine productions by macrophages from angiotensin II type 1 (AT1) receptor-knockout (KO) mice. Macrophages were prepared from normal and AT1a-KO mice. These macrophages were stimulated with 10 ng/ml LPS for 4 h with or without 100 μM Val. Total mRNA was prepared from the cells, and TNFα IL-1β and IL-6 mRNA levels were examined using real-time PCR. Representative data from 5 independent experiments are shown. Black bars, wild type; open bars, AT1a-KO. **P < 0.01 and ***P < 0.001, Student’s t-test.

Fig. 7. Effects of AT1 knockdown on Val-induced suppression of cytokine productions from THP-1 cells. THP-1 cells were treated with 100 nM PMA for 24 h and then transfected with one of the controls or one of the two siRNAs (siAT1 no. 1 and siAT1 no. 2) against human AT1, using Lipofectamine RNAiMAX Reagent (Invitrogen). Forty-eight hours after the transfection of 20 μM siRNA, the cells were stimulated with 10 ng/ml LPS for 8 h with or without 100 μM Val. A: cell lysates were electrophoresed and subjected to immunoblotting with anti-human AT1 antibody. N, negative. B: total mRNA was prepared from the cells, and TNFα IL-1β and IL-6 mRNA levels were examined using real-time PCR. Representative data from 5 independent experiments are shown. Black bars, control; open bars, siAT1 no. 1; striped bars, siAT1 no. 2. **P < 0.01 and ***P < 0.001, Student’s t-test.
Based on the aforementioned pathogenic relationship, halting the vicious cycle involving macrophages and adipocytes would be an important treatment strategy for insulin resistance. As PPARγ agonists, thiazolidinediones (TZDs) affect mainly adipocytes, regulating cytokine productions and thereby improving insulin sensitivity (23, 27, 41). Interestingly, some ARBs have significant PPARγ agonist activity, as shown by the induction of adipose differentiation of 3T3-L1 cells (2, 3, 12, 37). This has been taken as support for the hypothesis that the insulin-sensitizing effect of ARBs is attributable to this PPARγ agonist activity. If this is the case, ARBs without PPARγ activity should fail to exert an insulin-sensitizing effect. Valsartan reportedly has no PPARγ agonist activity (2, 43), and we confirmed that it does not induce adipose differentiation of 3T3-L1 cells (Fig. 1C). However, clinical studies have demonstrated that valsartan improves insulin sensitivity in patients with type 2 diabetes (28).

Accordingly, this study was carried out to ascertain the mechanism by which an ARB lacking PPARγ agonistic activity improves insulin sensitivity. First, we confirmed that valsartan has no direct effect, resulting in either the induction of adipose differentiation or normalization of insulin signaling impaired by TNFα in 3T3-L1 cells (Fig. 1, A–C), observations that agree with the previously demonstrated lack of PPARγ agonist activity of valsartan (2, 43). In contrast, valsartan was clearly shown to exert a strong inhibitory effect on LPS-induced cytokine production by macrophages (Fig. 4, A–C). LPS is a ligand for TLR4, and the serum concentration of LPS is reportedly increased in obese subjects (5). It was also reported that a high-fat diet increases the absorption of LPS from the gut into blood, which may contribute to the pathogenesis of high-fat diet-induced insulin resistance (25, 45). In addition, free fatty acids also bind to TLR4 and exert activity similar to that of LPS. Thus, reducing the response to LPS or free fatty acids would contribute to the normalization of chronic inflammation observed in obese and diabetic patients.

ACE inhibitors and ARBs are known to have anti-inflammatory actions and to prevent new-onset diabetes (4, 11, 21, 28, 31). Angiotensin II was shown to be a proinflammatory mediator, causing the release of reactive oxygen species and stimulating the production of inflammatory cytokines such as TNFα and IL-6 (46). However, the AT1a receptor expression level in macrophages is reportedly low. In addition, stimulation of macrophages with angiotensin II induced only a slight increase in cytokine production (Fig. 4D), suggesting that signals from the AT1 receptor play a minimal role in cytokine production. Thus, we suspected the strong inhibitory effect of valsartan on cytokine production in macrophages to be independent of the angiotensin II pathway. In fact, we obtained the surprising finding that macrophages from AT1a-KO mice, reportedly expressing no AT1b (26), still respond to valsartan to a degree similar to those from control mice (Fig. 6). In addition, the effect of valsartan on LPS-induced cytokine expressions in THP-1 was not altered by either AT1 siRNA or PPARγ antagonist treatment (Figs. 7 and 8). Thus, it is very likely that valsartan acts on another target molecule different from the AT1a receptor or PPARγ, which is involved in suppressing NF-κB activation associated with p65 phosphorylation and reducing JNK/AP-1 activation (Fig. 5), and the resultant secretions of cytokines such as IL-1β, IL-6, and TNFα. We examined the effects of valsartan on LPS-induced mRNA expressions of TLR4 and CD14, key factors in LPS-induced inflammatory responses, but valsartan had no effects on these expressions (data not shown), ruling out the involvement of TLR4 or CD14 in the molecular mechanism of the anti-inflammatory effect of valsartan. Although further investigation is required, we speculate that several proteins might be a target of valsartan. For example, valsartan may affect LPS-induced activation/assembly of signal molecules leading to NF-κB activation, including some ubiquitin ligases (e.g., TNF receptor-associated factor 6) and kinases (e.g., transforming growth factor-β-activated kinase 1) (10).

Identification of this novel target of valsartan, one of the ARBs without PPARγ activity, is a future research goal.

Taking our results and those of previous studies together, we can conclude that valsartan has mechanisms of action different from those of TZDs or telmisartan with PPARγ activity, leading to improved insulin sensitivity of adipose tissues (37,
43). Although TZDs act directly on adipocytes and suppress proinflammatory cytokine secretion from these cells, TZDs also induce adipose tissue enlargement and obesity. There is also concern that long-term TZD administration might induce osteoporosis (20, 38, 51), since TZDs induce the differentiation of bone marrow stem cells into adipocytes rather than bone cells. In contrast, although valsartan improves the functions of adipose tissues, this effect is mediated by coexisting macrophages. It appears that the insulin-sensitizing effect of valsartan on adipocytes is very likely not a direct action but rather one that occurs via altered functions of macrophages infiltrating adipose tissues. Thus, we can reasonably conclude that valsartan induces neither proliferation nor enlargement of adipocytes. If this is the case, valsartan would presumably improve insulin sensitivity without inducing obesity or osteoporosis. We have clearly shown that, at a minimum, valsartan, a non-AT1 receptor antagonist, improves insulin sensitivity without inducing obesity or osteoporosis (20, 38, 51), since TZDs induce the differentiation of bone marrow fat cells isolated from the same individual. Diabetologia 50: 1716 –1722, 2007.


VALSARTAN SUPPRESSES INFLAMMATION INDEPENDENTLY OF AT1R


