

Valsartan restores inflammatory response by macrophages in adipose and hepatic tissues of LPS-infused mice

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Inflammation involving adipose tissue is regarded as one of the major molecular mechanisms underlying obesity-related insulin resistance. Recent studies have suggested a series of angiotensin II receptor blockers (ARBs) to improve insulin resistance or protect against the development of diabetes mellitus. We previously demonstrated that valsartan suppresses the inflammatory response of macrophages. Interestingly, however, this effect did not occur via peroxisome proliferator-activated receptor (PPAR) γ or the AT1a receptor. This suppression appears to secondarily lead to amelioration of insulin resistance and reductions in abnormal gene expressions in adipocytes. In addition to these *in vitro* findings, we herein demonstrate the *in vivo* effects of valsartan, using mice constitutively infused with lipopolysaccharide (LPS) for 4 weeks. Oral administration of valsartan to LPS-infused mice normalized the increased expressions of inflammatory cytokines in adipose and liver tissues. These results raise the possibility that valsartan not only contributes to normalization of obesity-related insulin resistance, but is also beneficial for the treatment of other diseases with inflammation related to the metabolic syndrome such as atherosclerosis and non-alcoholic steatohepatitis. Further study is necessary to clarify these issues.

Introduction

There are numerous factors causing insulin resistance leading type 2 diabetes mellitus, but the most common is obesity.^{1–4} Recent studies suggest that infiltrating macrophages in adipose tissue are involved in the pathogenesis of chronic inflammation and induce insulin resistance in obese states.^{2,5–8} The interactions between enlarged adipocytes and activated macrophages result in a vicious cycle causing chronic inflammation not only in adipose tissue but also systemically, as shown by elevated serum C-reactive protein (CRP), increased inflammatory cytokines, reduced adiponectin and so on.

Besides the local events in adipose tissues, the serum concentration of lipopolysaccharide (LPS) is reportedly increased in obese subjects, which would give rise to chronic inflammation and insulin resistance, since LPS-infused mice reportedly showed impaired glucose tolerance.⁹ LPS is a ligand for Toll like receptor 4 (TLR4), and activation of the TLR4 pathway reportedly exacerbates inflammation and insulin resistance with increased inflammatory cytokine expressions. In addition, interestingly, it was shown that a high fat diet (HFD) weakens the barrier function of the gut epithelium and thereby increases the absorption of LPS from the gut into the circulation. This

mechanism could be responsible for the high LPS in sera from obese subjects, and might thus contribute to the pathogenesis of HFD-induced or obesity-related insulin resistance.^{10,11} Furthermore, it is noteworthy that free fatty acids also bind to TLR4 and exert activity similar to that of LPS. Based on these previous findings, we speculated that reducing the response to LPS or free fatty acids would normalize the chronic inflammation observed in obese and diabetic patients.

Since the association of type 2 diabetes mellitus and hypertension is well known, considerable attention has been paid to the insulin-sensitizing effects of many anti-hypertensive drugs. First, it was reported that angiotensin converting enzyme (ACE) inhibitors enhance insulin sensitivity,^{12,13} by raising the concentration of serum bradykinin. Although angiotensin II receptor blockers (ARBs) do not affect bradykinin metabolism, many clinical studies have revealed ARBs to significantly reduce insulin resistance or protect against the development of type 2 diabetes mellitus.^{14–17} Some ARBs, such as telmisartan, have weak but significant peroxisome proliferator-activated receptor (PPAR) γ agonistic activity, as they enhance adipose differentiation of 3T3-L1 cells.^{14,18–20} PPAR γ agonists also have an anti-inflammatory role, as shown by their inhibitory effects on the production of

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inflammatory cytokines such as tumor necrosis factor (TNF)- α , in turn promoting the production of adiponectin and thereby normalizing obesity-related insulin resistance.²¹⁻²³ Thus, the insulin-sensitizing effect of ARBs is considered to possibly be attributable to their PPAR γ agonist activity. However, valsartan, one of the most widely used ARBs, also exhibits an insulin-sensitizing effect²⁴ despite lacking PPAR γ activity.¹⁴

In our previous *in vitro* study, we obtained evidence suggesting that the main target of valsartan is not adipocytes but, rather, macrophages.²⁵ LPS-induced insulin signaling impairment in 3T3-L1 adipocytes co-cultured with RAW264.7 cells showed almost complete normalization with co-addition of valsartan. Furthermore, valsartan strongly suppressed LPS-induced productions of cytokines, such as interleukin (IL)-1 β , IL-6 and TNF- α with nuclear factor (NF) κ B activation and c-jun N-terminal kinase (JNK) phosphorylation, in RAW264.7 and primary murine macrophages. Surprisingly, this effect of valsartan was also observed in THP-1 cells treated with siRNA of AT1, the human angiotensin II receptor gene, or a PPAR γ antagonist, as well as macrophages from AT1a receptor knockout mice. Herein, we present evidence of the *in vivo* effects of valsartan on adipose and liver tissues of LPS-infused mice and discuss the beneficial aspects of valsartan-induced anti-inflammatory effects.

Results

Body weights and plasma glucose levels. Body weights of all mice rose slightly for 4 weeks and the differences between groups were not significant (Fig. 1A). While fasting plasma glucose levels differed slightly among the groups after 4 weeks of LPS or PBS infusion, the differences did not reach statistical significance (Fig. 1Bi). There were no significant differences in GTT results among the groups (Fig. 1Bii).

mRNA levels of inflammatory factors in adipose tissue, liver tissue and serum. We determined the expressions of the main inflammatory factors involved in metabolic diseases after 4 weeks of low-dose LPS (1.0 pg/g/h) infusion. The expressions of TNF- α and IL-6 mRNA were increased in adipose tissue from LPS-infused mice as compared with PBS-infused control mice. In valsartan-administered LPS-infused mice, however, expressions of these inflammatory cytokines were significantly suppressed as compared with mice receiving only LPS infusion (Fig. 1C). Similarly, LPS-induced expression of TNF- α in the liver was significantly suppressed by valsartan (Fig. 1D).

Subsequently, the effect of a higher dose (1.0 ng/g/h) of LPS for 4 weeks was also investigated. None of the groups showed alterations in body weight or fasting glucose concentrations (data not shown). Increased expressions of hepatic TNF- α and IL-6 with high-dose LPS were significantly suppressed by valsartan (Fig. 1E), results very similar to those obtained with infusion of a lower dose (1.0 pg/g/h) of LPS. While the serum concentration of TNF- α in mice infused with LPS (1.0 ng/g/h) for 4 weeks was markedly elevated as compared with that of the control mice, oral administration of valsartan tended to normalize LPS-induced TNF- α elevations, but the difference between the two doses was not significant (Fig. 1F).

Discussion

As the association of type 2 diabetes mellitus is well recognized, anti-hypertensive drugs are very frequently administered to diabetic patients. Patients having both type 2 diabetes mellitus and hypertension often have obesity and hyperlipidemia, which are features of the metabolic syndrome. Though not all of the molecular mechanisms underlying the development of metabolic syndrome are as yet clear, recent reports suggest an important role of adipose tissue inflammation.^{5,7,8} The enlargement of adipocytes and infiltration of macrophages are critical in this process, but an elevated serum LPS concentration is also likely to contribute to this inflammation.

In our previous studies using the co-culture system of macrophages and adipocytes, valsartan, one of the widely-used ARBs, was demonstrated to suppress the inflammatory response of macrophages and thereby restore impaired insulin signaling and reduce abnormal gene expressions of adipocytes.^{25,26} Based on these previous *in vitro* studies, this study was performed to ascertain *in vivo* effects of valsartan on the inflammation affecting adipose and liver tissues of mice continuously infused with LPS. Although 300 μ g/kg/d LPS-infused mice reportedly showed significant increases in the expressions of TNF- α and IL-6 mRNA in adipose tissue, liver and muscle, as well as hepatic insulin resistance,⁹ we employed physiologically low and high concentrations of LPS as an *in vivo* model of inflammation, and obtained essentially the same results. LPS-induced elevations in TNF- α and IL-6 expressions were observed in both adipose tissue and the liver. In addition, oral administration of valsartan blunted LPS-induced elevations of TNF- α and IL-6 in adipose tissues (Fig. 1C), observations in agreement with our previous *in vitro* findings.²⁵

Similar to the macrophages recruited into adipose tissue, Kupffer cells, resident hepatic macrophages, also play a pathogenic role in hepatic insulin resistance induced by a HFD.²⁷ TNF- α released by Kupffer cells in response to physiological concentrations of LPS²⁸ plays a central role in the insulin resistance of hepatocytes.²⁹ This study also revealed that valsartan suppressed LPS-induced TNF- α expression in the liver, regardless of whether a high or a low concentration of LPS was used (Fig. 1D and E). Recently, several ARBs have been shown to be effective for the treatment of non-alcoholic steatohepatitis (NASH),³⁰ and LPS from the gut was found to be involved in the pathogenesis of NASH.³¹ Taking these reports together, it is reasonable to speculate that the anti-inflammatory effect of valsartan contributes to not only improved insulin action or metabolism but also to therapy for NASH or other inflammatory diseases, although the suppressive effect on serum TNF- α concentrations exerted by valsartan was less marked than that on hepatic TNF- α mRNA levels.

Several other ARBs have already been reported to have anti-inflammatory effects. These effects are believed to be exerted through their PPAR γ agonist actions on macrophages. However, surprisingly, at least in the case of valsartan, which has already been reported to have no PPAR γ agonist activity,^{9,14} the anti-inflammatory effect on macrophages was shown to be independent of either PPAR γ or the AT1 receptor, based on findings obtained in AT1 KO mice and cells treated with PPAR γ or

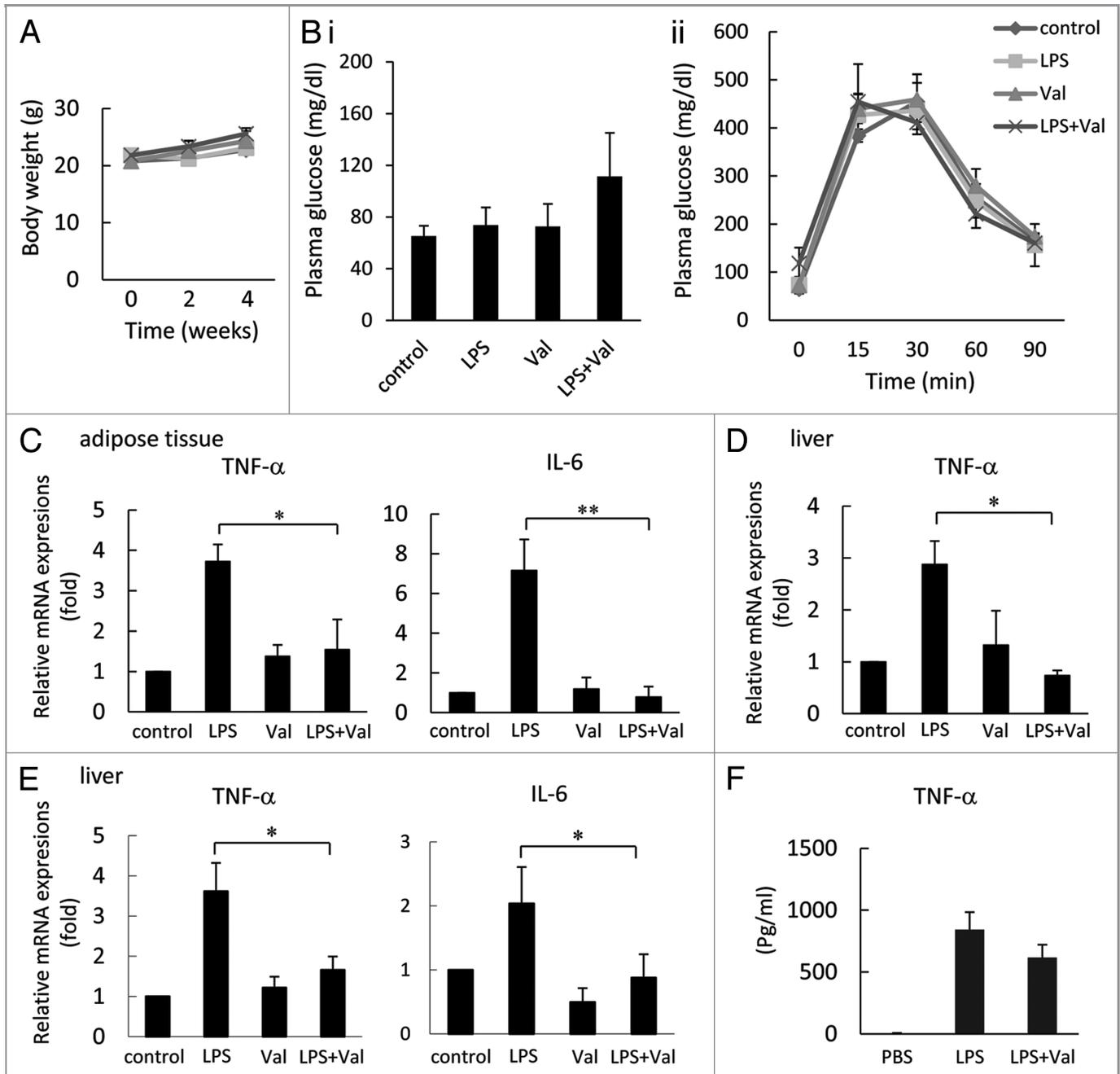


Figure 1. Effects of continuous LPS-infusion and valsartan (Val)-administration. Six-week-old LDL-R KO mice fed a HFD were injected with lose-dose (1.0 pg/kg/h) LPS continuously using osmic minipumps, with or without oral administration of valsartan, for 4 weeks. Each group consists of 6 mice. (A) Body weights. "Time-0" means before treatment. (Bi) Fasting plasma glucose levels. GTTs (ii) of 10-week-old mice kept on the HFD for 4 weeks. (C and D) Total mRNA was prepared from epididymal adipose and liver tissues. TNF- α and IL-6 mRNA levels were examined using real-time PCR. (E and F) LDL-R KO mice fed a HFD were injected with high-dose (1.0 ng/kg/h) LPS, with or without oral administration of valsartan, for 4 weeks, and hepatic TNF- α and IL-6 mRNA levels and serum concentrations of TNF- α were measured. Data are means \pm s.e.m. * $p \leq 0.05$, ** $p \leq 0.01$, Student's t-test.

AT1 siRNA or PPAR γ antagonist.²⁵ This suggests the presence of another target molecule on which valsartan exerts an anti-inflammatory effect, although further study is necessary to clarify this issue. The relationship between macrophages and adipocytes plays important roles in obesity-induced inflammatory changes and insulin resistance.^{2,5-8} We have reported the effects of valsartan using adipocytes co-cultured with LPS-treated

macrophages, from two viewpoints; altered gene expression patterns and insulin signaling.^{25,26} Regarding the gene expression pattern of adipocytes co-cultured with macrophages, DNA microarray analysis revealed that the profiles of gene expressions localized downstream from NF κ B, thyroid receptor and activator protein (AP) in adipocytes were altered by co-incubation with LPS-stimulated macrophages, and that these alterations were generally

normalized by co-incubation with valsartan. In particular, the expression patterns of caspases, integrins, matrix metalloproteinases and adipogenic genes, altered by co-culture with LPS-treated macrophages, were generally normalized by valsartan treatment. In light of these data, it is reasonable to consider valsartan to ameliorate inflammation, apoptosis and fibrotic changes of adipose tissue.

In addition, while valsartan does not improve insulin signaling in 3T3-L1 adipocytes treated with TNF- α or conditioned medium from activated RAW 264.7 macrophages, it was demonstrated to markedly enhance signaling in adipocytes co-cultured with RAW 264.7 macrophages. Thus, we speculated that the principle action of valsartan is suppression of the inflammatory responses of macrophages, since valsartan inhibits inflammatory cytokine production, thereby suppressing the NF- κ B activation associated with p65 phosphorylation and reducing c-Jun NH₂-terminal kinase/AP-1 activation in macrophages.²⁵

Taking our results and those of previous studies together, it is reasonable to assume that valsartan has mechanisms of action different from those of thiazolidinediones (TZDs) or telmisartan with PPAR γ activity, and that these actions improve the insulin sensitivity of adipose tissues.^{20,25,32} Although TZDs act directly on adipocytes and suppress pro-inflammatory cytokine secretion from these cells, TZDs induce adipose tissue enlargement and obesity as well. There is also concern that long-term TZD administration might lead to osteoporosis,^{3,33,34} 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 co-existing macrophages. It appears that the insulin-sensitizing effect of valsartan on adipocytes is very likely not a direct action, but rather one which occurs via altered functions of macrophages infiltrating adipose tissues. The unknown target of valsartan might be a key to establishing novel treatments for insulin resistance (Fig. 2).

Materials and Methods

Animals. Six-week-old low density lipoprotein receptor (LDL-R) knockout (KO) mice (obtained from Jackson Laboratory) were used. The animals were housed under climate-controlled conditions with a 12-h light/dark cycle and were provided a HFD (7.5% carbohydrate, 24.5% protein and 60% fat) purchased from Oriental Yeast Co. Ltd. and water ad libitum. All protocols were approved by the institutional review board of Hiroshima University.

Pumps delivering either PBS or LPS. Osmic minipumps (Alzet Model 1004; Alzet) were implanted intraperitoneally under Somnopenyl (pentobarbital sodium, 25 mg/kg body weight, Kyoritsu Shoji Co.) anesthesia. The pumps were filled either with phosphate buffered saline (PBS) or LPS (*Escherichia coli* 055:B5; Sigma) to infuse 1.0 pg or 1.0 ng/kg/h for 4 weeks. Body weights were monitored every other week.

Glucose tolerance test. Glucose tolerance tests (GTTs) were performed as follows: 10 h-fasted mice were injected with glucose

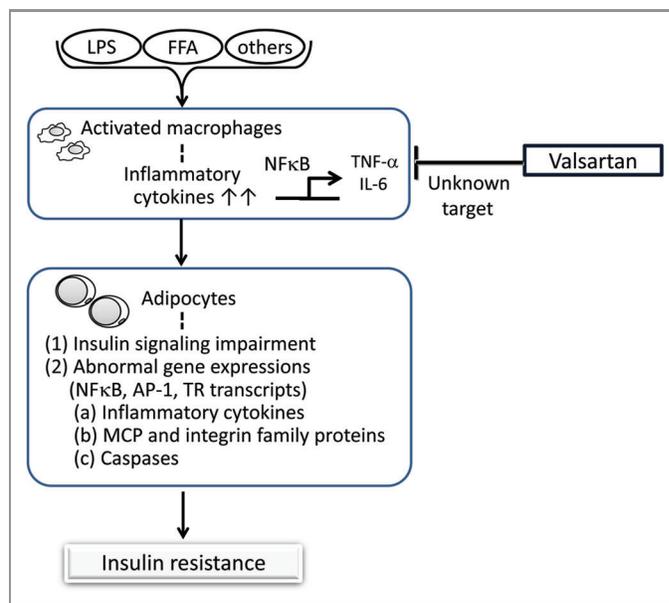


Figure 2. Valsartan improves insulin resistance by suppressing inflammation independently of PPAR γ .

(2 g/kg glucose, 10% glucose solution) into the peritoneal cavity. Blood glucose was determined with a glucose meter (Roche) using 3.5 μ l of blood collected from the tip of the tail vein.

Measurement of mRNA expression by real-time PCR. Epididymal adipose and liver tissues were collected and frozen at -80°C . Total RNA was extracted from these tissues using Sepazol-RNA I (NakaLai Tesque), and 1 μ g of RNA was reverse transcribed with Transcriptor Reverse Transcriptase (Roche). The amplification reaction assay was performed using SYBR Premix EX Taq (Takara) according to the manufacturer's protocol. The primers were as follows: mouse TNF- α forward, 5'-GCCACC ACGCTCTTCTGTCT-3', mouse TNF- α reverse, 5'-GTCTGG GCCATAGAAGTAT-3', mouse IL-6 forward, 5'-GATGCT ACCAACTGGATATAATC-3', mouse IL-6 reverse, 5'-GGT CCTTAGCCACTCCTTCTGTG-3', mouse GAPDH forward, 5'-TGACGTGCCGCTGGAGAAA-3', mouse GAPDH reverse, 5'-AGTGTAGCCCAAGATGCCCTTCAG-3'. Post-PCR melting curves confirmed the specificity of single-target amplification. Fold changes in the expressions of TNF- α and IL-6 relative to GAPDH were determined in triplicate.

Enzyme linked immunosorbent assay (ELISA). Serum TNF- α levels were measured using ELISA kits (eBioscience), according to the manufacturer's instructions. Absorbances at 450 nm were determined using a microplate reader (Biorad Laboratories).

Statistical analysis. Data are expressed as means \pm SE. Statistical analyses were performed using Student's t-test. Values of $p < 0.05$ were considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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