Candesartan Inhibits Carotid Intimal Thickening and Ameliorates Insulin Resistance in Balloon-Injured Diabetic Rats

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Abstract—This study investigates the effects of candesartan, an angiotensin II type 1 receptor blockade, on carotid arterial intimal thickening and glucose tolerance in balloon-injured male Wistar fatty rats and their littermates (Wistar lean rats). Candesartan was orally administered to 12-week-old rats for 21 days, and age-matched rats without the agent were used as the respective controls. Balloon catheterization in the left common carotid artery was performed on day 7, and the artery was removed on day 14 for histological analysis. Compared with the area ratios of the neointima/media in fatty rats without treatment, the ratios in fatty rats treated with candesartan at 1 mg · kg⁻¹ · d⁻¹ and lean rats without treatment were significantly decreased to 65%; on the other hand, the ratios of fatty rats treated with candesartan at 10 mg · kg⁻¹ · d⁻¹ and lean rats treated with 1 mg · kg⁻¹ · d⁻¹ were reduced to 35%, and those of lean rats treated with 10 mg · kg⁻¹ · d⁻¹ were reduced to 28%. The administration of candesartan also decreased the level of plasma glucose time- and dose-dependently in fatty rats. In an intragastric glucose load, the levels of both glucose and insulin at 30 and 60 minutes were significantly decreased when fatty rats were treated with candesartan at 10 mg · kg⁻¹ · d⁻¹. In cultured vascular smooth muscle cells from fatty rats, insulin-stimulated Akt (New England Biolabs) phosphorylation and 2-deoxy-D-glucose uptake were inhibited to 59% and 68%, respectively, by angiotensin II, but the effects were ameliorated by the administration of candesartan. We conclude that candesartan could be effective for the suppression of vascular smooth muscle cell growth dose-dependently in Wistar fatty and lean rats. Furthermore, the agent could improve insulin resistance in Wistar fatty rats. (Hypertension. 2001;38:1255-1259.)

Key Words: angiotensin II receptors, angiotensin II rats, balloon injury, insulin resistance

Multiple epidemiological and clinical studies have established that diabetes mellitus and hypertension are the most crucial risk factors in the pathogenesis of atherosclerosis, including cardiovascular disease and subsequent sudden death. Considerable evidence supports the view that insulin resistance, its concomitant compensatory hyperinsulinemia, and the related glucose intolerance are associated with hypertension, although the mechanisms of interaction still need to be completely clarified. Therefore, for the prevention of atherosclerosis and diabetic complications, it is necessary to identify the hypotensive agents that are beneficial for glucose metabolism.

The renin-angiotensin system plays a key role in the pathogenesis of hypertension and atherosclerosis. In this pathological process, the universal underlying abnormality, which is mainly concerned with alterations in the structure and function of vascular smooth muscle cells (VSMCs), is enhanced by circulating and locally generated angiotensin (Ang) II, which affects the contractility and growth of VSMCs and the sympathetic nervous system. Recent studies have reported that Ang II has pleiotropic effects on the proliferation of VSMCs, ie, the activation of mitogen-activated protein kinase and the induction of growth factors and proinflammatory cytokines. In contrast, several investigators, although not all, have clinically shown that Ang II may contribute to glucose tolerance and the progression of diabetic cardiovascular and renal complications. Thus, it is possible that the interaction between hyperglycemia and the renin-angiotensin system may have an important role in the progression of atherosclerosis in diabetic patients, although how these factors contribute to VSMC growth and transmit their signals intracellularly in diabetic conditions has not been fully elucidated.

Therefore, we set out to clarify whether treatment with an Ang II type 1 (AT₁) receptor blockade can suppress a process occurring early in atherosclerosis or the glucose metabolism in diabetes. To explore this possibility, we investigated the effects of candesartan (TCV-116), one of the AT₁ receptor

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blockades, on balloon-injured carotid arterial intimal thickening and glucose metabolism by using male Wistar fatty rats (fa/+, a genetically established obese-hyperglycemic animal model for type II diabetes mellitus,\textsuperscript{12} and their littermates (Wistar lean rats, Fa/−).

Methods
The experiments were approved by the Committee on Ethics of Animal Experiments and conducted by the Guidelines for Animal Experiments, Yamagata University School of Medicine.

Balloon Catheterization
Twelve-week-old male Wistar fatty and lean rats were each divided into 3 groups (group L, TCV-116 at 1 mg · kg\(^{-1}\) · d\(^{-1}\) [\(n=7\)]; group H, TCV-116 at 10 mg · kg\(^{-1}\) · d\(^{-1}\) [\(n=7\)]; and untreated group [\(n=7\)]). TCV-116 suspended in 5% gum arabic solution was administered in 5% gum arabic solution was administered at 1 or 10 mg/kg PO once a day for 21 days. Balloon catheterization in the left common carotid artery was performed on day 7 by using a 2F Fogarty balloon catheter (Baxter), as reported previously.\textsuperscript{16} After 14 days, the balloon-injured arteries were rapidly removed for histological analysis, including immunohistological staining.\textsuperscript{14}

Other Measurements
Blood pressures and heart rate were measured by the tail-cuff method. Paroxysmal plasma glucose (PPG) was measured before and after balloon catheterization at 7-day intervals.

Intragastric Glucose Load
Other 12-week-old male Wistar fatty and lean rats were prepared for the intragastric glucose load. After a 24-hour fast, a blood sample was drawn from the cannulated right jugular vein; then 1 g/kg glucose was administered into the stomach orally, as reported previously.\textsuperscript{15} Blood samples were also taken at 30 and 60 minutes after the load. Then, these rats were divided into 2 groups (group H, TCV-116 at 10 mg · kg\(^{-1}\) · d\(^{-1}\) [\(n=6\)] and untreated group [\(n=6\)]; the above-mentioned suspension with TCV-116 was administered for 21 days, and the glucose load was performed again.

Cell Culture
VSMCs were harvested from the aortas from 12-week-old male Wistar fatty and lean rats as described previously.\textsuperscript{13}

Immunoblot Analysis
After being starved with Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical) containing 0.5% BSA (Sigma Chemical Co) for 24 hours, VSMCs were preincubated with either serum-free DMEM or several concentrations of CV-11974, an active metabolite of TCV-116, for 1 hour. Then the cells were stimulated by 2% FBS (GIBCO-BRL) for another 24 hours or with 10\(^{-7}\) mol/L insulin and/or Ang II for another 5 minutes. The following procedure was performed as reported previously.\textsuperscript{16} The membranes were incubated with antibodies phosphospecific for extracellular signal-regulated kinase (ERK) 1/2 and/or Akt (New England Biolabs) at 4°C overnight and then with secondary antibody at room temperature for 1 hour. The antibody binding sites were visualized by use of enhanced chemiluminescence (ECL, Amersham).

2-DOG Uptake
2-Deoxy-D-glucose (2-DOG) uptake was measured as described previously\textsuperscript{17} with minor modifications. After being preincubated with a Krebs-Ringer phosphate-HEPES buffer with 0.5% BSA for 30 minutes, VSMCs were treated with 10\(^{-7}\) mol/L CV-11974 for another 30 minutes. Then the cells were stimulated with 10\(^{-7}\) mol/L insulin and/or Ang II for 1 hour at 37°C. Unlabeled 2-DOG and [\(^{[3}H]\)]-2-DOG (100 mmol/L, 0.5 mCi per well, New England Nuclear) were added and incubated for 5 minutes. The cells were solubilized, and the radioactivity was determined.

Statistical Analysis
Results are expressed as mean±SEM. Statistical significance was estimated by ANOVA, and the differences were considered to be significant at \(P<0.05\).

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results
Effect of TCV-116 on Body Weight, Hemodynamic Parameters, and Glucose Tolerance
As shown in the Table, treatment with TCV-116 did not modify the body weight in either Wistar fatty or lean rats. Although there were no changes in either systolic or diastolic blood pressure between fatty and lean rats without TCV-116, these levels were decreased dose-dependently by treatment with the agent. However, TCV-116 did not cause changes in heart rate in any of the 6 groups. Treatment with TCV-116 decreased the levels of both PPG and immunoreactive insulin (IRI) in fatty rats and even of IRI in the lean group H in a dose-dependent manner. In Wistar fatty rats, treatment with TCV-116 decreased the levels of PPG time- and dose-dependently (Figure 1A). Compared with the level of PPG of the fatty untreated group, those of the fatty group H after day 7 and group L after day 14 significantly declined. In contrast, the levels of PPG in lean rats were not affected by the agent. To clarify the effect of TCV-116 on glucose tolerance, intragastric glucose loads were performed in fatty and lean rats.
rats before and after the administration of TCV-116. The levels of plasma glucose and IRI were not different between the 2 groups in either fatty or lean rats in the first glucose load (data not shown). Interestingly, in the fatty rats, the levels of both plasma glucose and IRI in group H were significantly decreased at 30 and 60 minutes in the second glucose load compared with the levels in the untreated group (Figure 1B).

Effects of TCV-116 on I/M Ratio and of CV-11974 on Phosphorylated ERK 1/2 in Cultured VSMCs

The intimal thickening layers consisted of abundant infiltrated cells and intercellular matrix in the elastica-Masson staining. Compared with the area ratio of the neointima/media layer (I/M ratio) in the lean untreated group, that in the fatty untreated group was significantly higher (Figure 2A). In addition, we could observe that treatment with TCV-116 resulted in a distinct change in the size of intimal thickening in both Wistar fatty and lean rats dose-dependently. Compared with the value of the I/M ratio of the fatty untreated group, that of the fatty rats treated with TCV-116 decreased significantly to 65% in group L and to 35% in group H. In lean rats, the I/M ratios of the treated groups also showed a significant decrease compared with those of the control groups in a dose-dependent manner, and the value in group H was even reduced to 28% of that of the fatty untreated group. In contrast, there was no difference among any of the groups in the area of the medial layer in either catheterized or intact common carotid arteries. Immunohistological staining with anti-α-smooth muscle actin antibody (Sigma), used for the detection of VSMC-specific structural protein, showed that diffusely positive immunoreactivities completely occupied the intimal thickening layers of all preparations; these results were equivalent to the values of the I/M ratio (data not shown).

In addition, by 2% FBS stimulation for 24 hours, the level of phosphorylated ERK 1/2 in cultured VSMCs from fatty rats was significantly increased to 146% compared with the level in VSMCs from lean rats. Then, the levels of phosphorylated ERK 1/2 were inhibited by the addition of CV-11974 dose-dependently in both fatty and lean rats (Figure 2B).

Effect of CV-11974 on Akt Phosphorylation and Glucose Uptake in Cultured VSMCs

To investigate the effect of CV-11974 on glucose metabolism in vitro, we examined the levels of phosphorylated Akt in cultured VSMCs. Insulin at 10⁻⁷ mol/L increased serine phosphorylation of Akt 29-fold in fatty rats and 22-fold in lean rats compared with the control level in lean rats, and these phosphorylations were not affected by treatment with 10⁻⁷ mol/L CV-11974 (Figure 3A). The insulin-stimulated Akt phosphorylation in fatty rats, compared with lean rats, was significantly decreased by 17%. Although Ang II at 10⁻⁷ mol/L decreased the insulin-stimulated Akt phosphorylations by 11% in lean rats and by 41% in fatty rats, the addition of CV-11974 elevated the phosphorylation by 47% in lean rats and 87% in fatty rats compared with each control. In contrast, Ang II itself did not affect Akt phosphorylation.

We next examined the effect of CV-11974 on 2-DOG uptake in cultured VSMCs. As shown in Figure 3B, the level of 2-DOG uptake in untreated fatty rats was decreased by 26% compared with that in untreated lean rats. Insulin at 10⁻⁷ mol/L increased 2-DOG uptake to 127% in lean rats and to 144% in fatty rats compared with each control, and 10⁻⁷ mol/L CV-11974 did not affect these uptakes. Although Ang II at 10⁻⁷ mol/L significantly decreased the insulin-stimulated 2-DOG uptake by 22% in lean rats and 32% in fatty rats,
treatment with CV-11974 elevated the 2-DOG uptake by 26% in lean rats and 45% in fatty rats compared with each control.

Discussion

In the present study, we have clearly demonstrated that TCV-116 has an inhibitory effect on the early events of atherosclerotic formation by direct suppression of VSMC growth in a strict dose-dependent manner. We have also shown that it ameliorates insulin resistance, especially in diabetic rats. These favorable results suggest that TCV-116 could be one of the most beneficial hypotensive agents for patients who have both hypertension and diabetes.

First, we examined the in vivo effect of TCV-116 to determine whether it could suppress rat intimal thickening induced by balloon catheterization or improve glucose tolerance in Wistar fatty and lean rats. Surprisingly, treatment with TCV-116 could ameliorate insulin resistance in fatty rats in time- and dose-dependent manners (Table and Figure 1), suggesting that the suppression of Ang II signal transduction pathways by TCV-116 can improve the glucose metabolism without any change of body weight in the diabetic condition. In addition, treatment with TCV-116 could decrease the values of the I/M ratio in both fatty and lean rats dose-dependently, and the change was more drastic in diabetic fatty rats than in lean rats (Figure 2A). Although the I/M ratio in the nondiabetic lean rats was dose-dependently decreased by treatment with TCV-116, the changes in the I/M ratio by treatment with TCV-116 was more drastic in diabetic fatty rats than in lean rats. It is likely that both the amelioration of insulin resistance and the hypotensive effect by TCV-116 might be considered sufficient to influence the marked reduction of I/M ratio in diabetic fatty rats, inasmuch as the levels of AT₁ receptors in VSMCs were more elevated (by ≈25%) in fatty rats than in lean rats by immunoblot analysis (data not shown). Interestingly, CV-11974 itself could inhibit the levels of phosphorylated ERK 1/2 dose-dependently in VSMCs from both fatty and lean rats (Figure 2B), suggesting that treatment with TCV-116 has a significant inhibitory effect on the content of VSMCs in the balloon-injured neointima. Although we could not clarify the mechanism(s) in the present study, we speculate that the agent may be able to influence intracellular calcium concentration in VSMCs, because Ang II can phosphorylate PYK2, a Ca²⁺-dependent tyrosine kinase, through the AT₁ receptor in rat VSMCs. ¹⁸

Several investigators have recently shown that losartan cannot affect the improvement of insulin resistance in rats and humans. ¹⁹ In the present study, we showed the improvement of glucose tolerance by treatment of TCV-116 by using the intragastric glucose load (Figure 1B). The discrepancy between their results and ours may be caused by the differences in experimental condition and/or type of AT₁ receptor blockade. In contrast, recent studies have shown that TCV-116 is clinically superior to losartan in reducing blood pressure by tight binding and slow dissociation to the AT₁ receptor. ²¹,²² Furthermore, Higashiura et al ²³ have demonstrated that treatment with the agent increases insulin sensitivity assessed by the M-value in patients with essential hypertension. These results may have enhanced the amelioration of insulin resistance in the present study.

Ang II has been reported to inhibit insulin signaling at multiple levels, including insulin receptor substrates and phosphatidylinositol 3-kinase (PI3-kinase) in VSMCs, ²⁴ leading to increased levels of plasma insulin and the subsequent aggravation of insulin resistance. In contrast, Nickenig et al ²⁵ have revealed that insulin itself enhances AT₁ receptor gene expression and, subsequently, Ang II signaling, ²⁵ which suggests that the upregulation of Ang II signaling further prevents insulin signaling and additionally modulates vascular tone. Kuboki et al ²⁶ have revealed that the inhibition of PI3-kinase by insulin resistance reduces both the gene expression and production of endothelial NO synthase. ²⁶ Therefore, it is reasonable to assume that these complicated interactions further contribute to the progression of atherosclerosis in the insulin-resistant state. To clarify the effect of TCV-116 on the insulin-signaling pathway, we examined the levels of phosphorylated Akt, downstream from PI3-kinase by insulin resistance (Figure 3A). In agreement with a previous report, ²⁷ the insulin-stimulated phosphorylation of Akt was significantly reduced in VSMCs from insulin-resistant fatty rats compared with that from lean rats (Figure 3A). In addition, the levels of insulin-stimulated Akt phosphorylation were partially inhibited by Ang II in VSMCs from both fatty and lean rats, and these levels were significantly elevated by the addition of CV-11974. Furthermore, a recent report has shown that insulin-stimulated Akt participates in glucose transporter 4 translocation in insulin-targeted cells. ²⁸ In the present study, as shown in Figure 3B, the insulin-stimulated 2-DOG uptake was significantly reduced in VSMCs from fatty rats compared with the uptake in...
VSMCs from lean rats. In addition, the insulin-stimulated 2-DG uptake was significantly decreased by Ang II in VSMCs from both fatty and lean rats, and these levels were significantly elevated by CV-11974, indicating that Ang II subsequently results in preventing insulin-stimulated glucose transporter 4 translocation via Akt phosphorylation in VSMCs. These results strongly suggest that interactions between Ang II and the insulin-signaling systems may play a crucial role in the regulation of vascular physiology and the pathogenesis of insulin resistance.

Conversely, Henriksen et al. have recently shown that angiotensin-converting inhibitors, but not eprosartan, another AT1 receptor blocker, can enhance insulin-stimulated glucose transport activity by mediating bradykinin-B2 receptors and subsequent NO production in resected skeletal muscle from obese Zucker rats. The discrepancy of their results with ours may be caused by differences in experimental conditions and/or the amount of AT1 receptors in tissues, inasmuch as the expression of AT1 receptors can be clearly detected in VSMCs by immunohistological staining with anti–AT1 receptor antibodies, but it cannot be detected as clearly or it cannot be detected at all in skeletal muscle (data not shown).

In conclusion, in the present study, we have established that TCV-116 has pleiotropic effects not only on the inhibition of intimal thickening in balloon-injured arteries by attenuating the amount of VSMCs but also on the amelioration of insulin resistance. The suppression of intimal thickening by the agent was more remarkable in diabetic rats. These results provide a new insight into the potential cellular mechanisms in the vasculature (such as syndrome X) whereby treatment with TCV-116 may have clinical benefits and contribute to the prevention of atherosclerosis.

References
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