Original Article

Blockade of Endogenous Proinflammatory Cytokines Ameliorates Endothelial Dysfunction in Obese Zucker Rats

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To study the role of endogenous proinflammatory cytokines in endothelial dysfunction in diabetes, we administered semapimod, an inhibitor of proinflammatory cytokine production, to obese Zucker (OZ) rats, and examined its effect on endothelium-dependent vasorelaxation. Endothelium-dependent vasorelaxation induced by acetylcholine and adrenomedullin (AM) was significantly reduced in OZ rats compared to a control group of lean Zucker rats. Semapimod significantly restored endothelium-dependent vasorelaxation in OZ rats. This effect of semapimod was well correlated with the reduction in the serum concentrations of tumor necrosis factor-α (TNF-α), interleukin-6, and C-reactive protein, as well as with the recovery of AM-induced Akt phosphorylation and cGMP production. Furthermore, acute administration of TNF-α significantly suppressed endothelium-dependent vasorelaxation and AM-induced cGMP production. These results implicate endogenous proinflammatory cytokines, especially TNF-α, in endothelial dysfunction in diabetes, and indicate that blockade of these cytokines will be a promising strategy for inhibiting the progression of vascular inflammation. (*Hypertens Res* 2008; 31: 737–743)

***Key Words*: diabetes, endothelial dysfunction, proinflammatory cytokines, adrenomedullin**

Introduction

Diabetes mellitus is now well known as a major risk factor for cardiovascular diseases (*1*, *2*). More than 90% of diabetic patients are classified as type 2, which is characterized by obesity and insulin resistance. It is also well known that endothelial dysfunction is associated with a variety of pathophysiological states such as hypertension, hyperlipidemia, diabetes, smoking, and atherosclerosis, and that endothelial dysfunction can be an early marker of the pathophysiological state of blood vessels (*3*–*6*). Although the precise mechanisms of endothelial dysfunction remain unclear, reactive oxygen species (ROS) seem to play a role in that process (*4*–*7*).

Several lines of evidence suggest that insulin resistance results from the action of proinflammatory cytokines released from the adipose tissue, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 (*8*, *9*). Interestingly, these cytokines are known to induce endothelial dysfunction when administered exogenously (*10*, *11*). Because these cytokines increase ROS production in blood vessels (*11*, *12*), it is possible that these cytokines are involved in endothelial dysfunction.

To examine the role of endogenous proinflammatory cyto-

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Table 1. Baseline Parameters of 4 Groups of Zucker Rats

	LZ	LZ/Sem	OZ	OZ/Sem
BW (g)	383.3±9.9	367.9±13.7	616.0±11.7*	605.0±9.9*
HR (bpm)	400.1±18.3	401.8±9.9	421.3±10.6	417.2±8.6
sBP (mmHg)	138.5±1.6	138.1±1.5	167.7±1.9*	159.9±6.1*
BS (mg/dL)	104.8±2.5	92.3±12.9	217.2±6.1*	223.3±12.1*
TG (mg/dL)	95.7±18.0	94.4±4.2	383.7±57.0*	335.3±68.4*
TC (mg/dL)	87.8±9.9	88.0±2.2	200.0±18.2*	201.5±53.7*

LZ, lean Zucker; OZ, obese Zucker; Sem, semapimod-treated; BW, body weight; HR, heart rate; sBP, systolic blood pressure; BS, blood sugar; TG, triglyceride; TC, total cholesterol. Data are mean±SEM. *$p<0.05$ *vs*. LZ rats.

Table 2. Serum and Tissue TNF-α Concentrations of 4 Groups of Zucker Rats

	LZ	LZ/Sem	OZ	OZ/Sem
Serum (pg/mL)	19.8±4.0	21.7±3.8	31.8±2.7*	17.5±1.7$^{\#}$
Visceral fat (pg/100 μg protein)	87.1±5.5	84.5±8.8	404.1±105.4*	136.3±32.3$^{\#}$
Aorta (pg/100 μg protein)	43.1±4.0	41.6±5.2	90.8±7.6*	47.1±5.6$^{\#}$
Heart (pg/100 μg protein)	208.4±29.6	236.4±23.3	295.6±41.6	272.1±44.5
Lung (pg/100 μg protein)	47.8±4.1	38.9±6.1	46.4±8.9	44.9±10.6

TNF-α, tumor necrosis factor-α; LZ, lean Zucker; OZ, obese Zucker; Sem, semapimod-treated. Data are mean±SEM. *$p<0.05$ *vs*. LZ rats. $^{\#}p<0.05$ *vs*. OZ rats.

Table 3. Serum and Tissue IL-6 Concentrations of 4 Groups of Zucker Rats

	LZ	LZ/Sem	OZ	OZ/Sem
Serum (pg/mL)	15.0±0.9	17.3±2.3	23.3±1.6*	20.0±2.1$^{\#}$
Visceral fat (pg/100 μg protein)	35.3±4.7	33.5±3.1	89.3±1.7*	33.0±0.7$^{\#}$
Aorta (pg/100 μg protein)	54.7±11.2	48.9±10.1	57.6±13.9	41.6±7.0
Heart (pg/100 μg protein)	463.1±72.1	478.3±25.1	494.6±72.9	518.4±151.1
Lung(pg/100 μg protein)	38.7±3.1	41.0±2.1	39.3±8.0	32.6±6.9

IL-6, interleukin-6; LZ, lean Zucker; OZ, obese Zucker; Sem, semapimod-treated. Data are mean±SEM. *$p<0.05$ *vs*. LZ rats. $^{\#}p<0.05$ *vs*. OZ rats.

kines such as TNF-α and IL-6 in endothelial dysfunction observed in a diabetic state, we administered semapimod to obese Zucker (OZ) rats, a model of type 2 diabetes, and examined its effect on endothelium-dependent vasorelaxation. Semapimod (formerly known as CNI-1493) is a tetravalent guanylhydrazone compound that inhibits the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (*13*). Semapimod reportedly does not suppress the production of transforming growth factor-β or the expression of major histocompatibility complex class II antigens (*13*). Furthermore, it does not affect total cellular protein synthesis, suggesting the specificity of its effects (*13*). To induce endothelium-dependent vasorelaxation, we used acetylcholine (ACh) and adrenomedullin (AM). AM is a peptide that has a potent vasorelaxant effect (*14*). Recently, using AM gene knockout mice, it has been demonstrated that endogenous AM is involved in blood pressure control, because AM+/− mice show significantly higher blood pressure than wild-type mice (*15*). We have shown that AM partially induces vasorelaxation in an endothelium-dependent manner, and that AM-induced endothelium-dependent vasorelaxation is mediated by the phosphatidylinositol-3 kinase (PI3K)/Akt-dependent pathway (*16*).

In this study, we examined whether or not the blockade of endogenous proinflammatory cytokines using semapimod would ameliorate endothelial dysfunction in OZ rats and, if so, what its mechanism is.

Methods

Reagents

Semapimod was kindly supplied by Cytokine Pharma-Sciences (King of Prussia, USA). Phosphospecific anti-Akt antibody, which recognizes catalytically active Akt, was obtained from New England BioLabs (Beverly, USA). Anti-

Akt antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Human recombinant TNF-α was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animals

Male OZ rats and the control group of age-matched lean Zucker (LZ) rats were purchased from Charles River Laboratories (Wilmington, USA). They were fed a standard chow and had free access to water. LZ and OZ rats were divided into two groups: an untreated group and a group treated with semapimod. The rats in the treated group received 5 mg/kg/d of semapimod intraperitoneally for 2 weeks before the tension experiments. The untreated rats received the same amount of normal saline. All animal studies were performed in accordance with the guidelines for animal care of the University of Tokyo.

Ex Vivo Experiments

Sixteen-week-old OZ and LZ rats were used for the experiments. The effects of ACh and AM on the tension of rat aortic rings were examined as previously described (*16*). Aortic rings were precontracted with 10^{-5} mol/L prostaglandin F2α. Acute administration of TNF-α was performed by adding TNF-α at a final concentration of 5 ng/mL directly into the incubation chamber 20 min before the tension experiments. In some experiments, rat aortas were placed in tubes containing oxygenated Krebs-Ringer bicarbonate solution at 37°C and incubated with AM before protein extraction.

Enzyme-Linked Immunosorbent Assay

The concentrations of TNF-α and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BioSource International, Camarillo, USA). The rat visceral fat, thoracic aorta, heart, and lungs were homogenized on ice in a Triton X-100 cell lysis buffer (*17*). After centrifugation for 20 min at 4°C, the supernatant was used for the assay. Serum concentration of C-reactive protein (CRP) was also measured using an ELISA kit (Alpha Diagnostic International, San Antonio, USA).

Western Blot Analysis

Protein extracts were prepared from rat aortas as previously described (*16*). Western blot analysis was performed as previously described (*17*). Fifty micrograms of each protein extract was used in the Western blot analysis.

Measurement of cGMP Production

cGMP production in the rat aortas was measured as previously described (*16*). In some experiments, rat aortas were preincubated with TNF-α for 20 min.

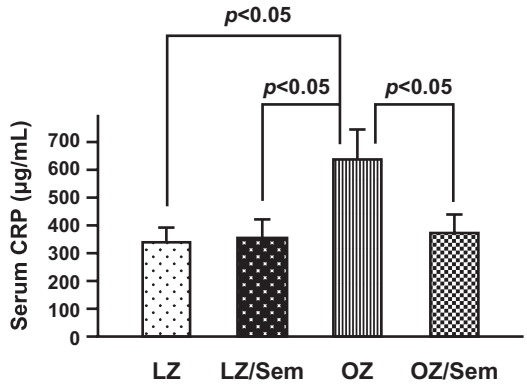

Fig. 1. *Serum CRP concentrations in LZ and OZ rats treated with or without semapimod (Sem) (n = 6).*

Statistical Analyses

Values are reported as means±SEM. The statistical analyses were performed using analysis of variance followed by the Student-Newman-Keul's test. p values of <0.05 were considered statistically significant.

Results

Physical and Metabolic Features of OZ and LZ Rats

Body weight and systolic blood pressure, measured by the tail-cuff method, were significantly higher in 16-week-old OZ rats than in age-matched LZ rats. Serum glucose, triglycerides, and total cholesterol were significantly higher in OZ rats than in LZ rats. Semapimod had no significant effects on these parameters in LZ or OZ rats (Table 1). There were no significant differences in heart rate among the four groups.

Serum and Tissue Levels of Adipocytokines

The concentrations of TNF-α in serum, visceral fat, and thoracic aorta were significantly higher in OZ rats than in LZ rats; this increase was significantly suppressed by treatment with semapimod (Table 2). In contrast, the concentrations of TNF-α in the heart and lungs did not differ significantly among the four groups. Semapimod did not have any significant effect on TNF-α levels in LZ rats. The concentrations of IL-6 in serum and visceral fat were significantly higher in OZ rats than in LZ rats, and these increases were significantly suppressed by treatment with semapimod (Table 3). In contrast to the concentration of TNF-α, IL-6 levels in the thoracic aorta did not significantly differ between LZ and OZ rats. IL-6 levels in the heart and lungs did not differ significantly among the four groups. Semapimod did not have any significant effect on IL-6 levels in LZ rats.

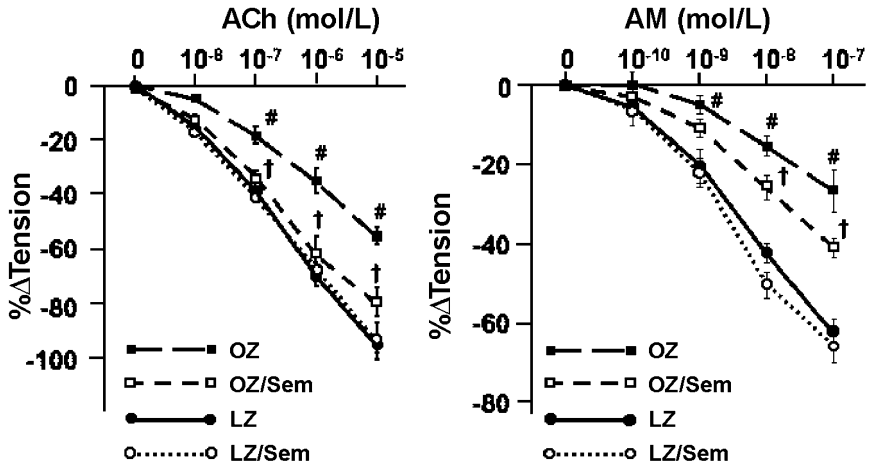

Fig. 2. *Semapimod (Sem) restores endothelium-dependent vasorelaxation in OZ rats. Aortic rings with intact endothelium were precontracted with prostaglandin F2α, and vasorelaxation in response to ACh (left) and AM (right) was examined. $^{\#}p < 0.05$ vs. LZ rats, and $^{\dagger}p < 0.05$ vs. OZ rats (n = 10 each).*

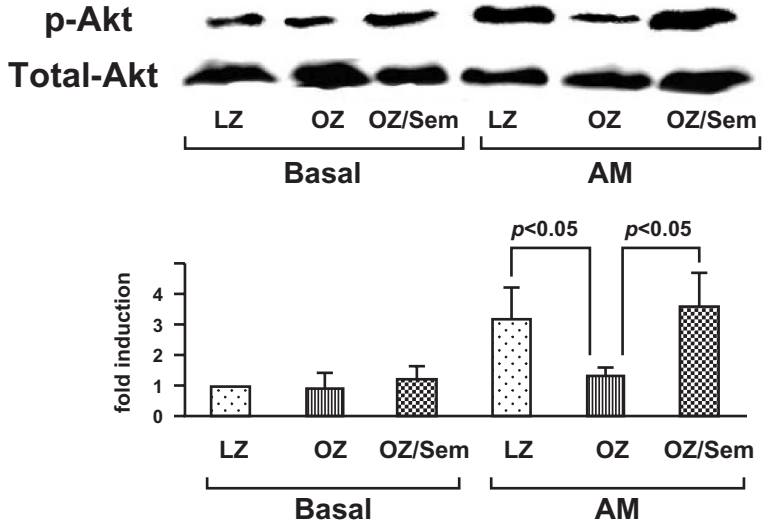

Fig. 3. *Semapimod restores Akt phosphorylation in response to AM in aortas prepared from OZ rats. Rat thoracic aortas from LZ rats (LZ), OZ rats (OZ), and OZ rats administered semapimod (OZ/Sem) were incubated with 10^{-7} mol/L AM for 15 min. Fifty micrograms of each protein extract was immunoblotted with a phospho-specific anti-Akt antibody (p-Akt), which recognizes catalytically active Akt, or anti-Akt antibody (Total-Akt), which recognizes total Akt1/2, regardless of whether Akt is phosphorylated or not. The relative intensity of each band is shown in the lower histogram (n = 5).*

Serum CRP Concentration

Serum CRP level was significantly higher in OZ rats than in LZ rats, and semapimod significantly suppressed CRP levels in OZ rats to a level similar to that observed in LZ rats. Semapimod did not affect CRP level in LZ rats (Fig. 1).

Endothelial Dysfunction Is Ameliorated in OZ Rats Treated with Semapimod

To assess endothelial function in OZ rats, we examined endothelium-dependent vasorelaxation in response to ACh and AM in OZ and LZ rats. ACh-induced endothelium-dependent vasorelaxation was significantly diminished in aortic rings prepared from OZ rats compared with that observed in aortic rings from LZ rats (Fig. 2, left). Semapimod significantly

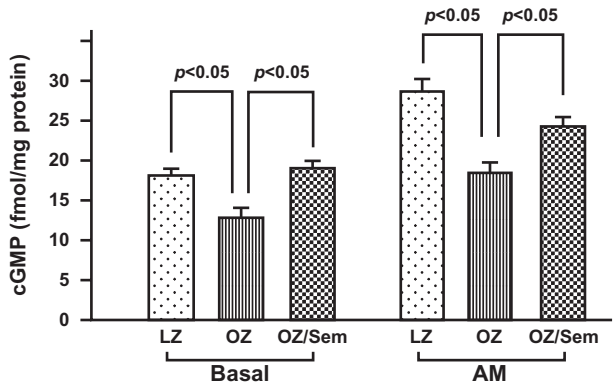

Fig. 4. *Semapimod restores cGMP production in aortas prepared from OZ rats. Rat thoracic aortas from LZ, OZ, and semapimod (Sem)-treated OZ rats were stimulated with 10^{-7} mol/L AM for 5 min, and cGMP was then measured (n = 6).*

restored ACh-induced endothelium-dependent vasorelaxation in OZ rats to a level similar to that observed in LZ rats. Semapimod did not affect ACh-induced endothelium-dependent vasorelaxation in LZ rats. We next examined AM-induced endothelium-dependent vasorelaxation. We have reported that AM induces both endothelium-dependent and endothelium-independent vasorelaxation (*16*). We have also shown that AM-induced endothelium-dependent vasorelaxation is diminished in OZ rats compared to LZ rats, whereas endothelium-independent vasorelaxation examined using endothelium-denuded aortic rings did not differ significantly between OZ and LZ rats. These findings suggest that endothelium-dependent vasorelaxation—but not endothelium-independent vasorelaxation—is impaired in OZ rats (*18*). As shown in Fig. 2 (right), AM-induced vasorelaxation of aortic rings with intact endothelium prepared from OZ rats was significantly lower than that of aortic rings from LZ rats, suggesting that AM-induced endothelium-dependent vasorelaxation was also diminished in OZ rats compared to LZ rats. Semapimod significantly restored AM-induced vasorelaxation in aortic rings with intact endothelium prepared from OZ rats, whereas it did not significantly affect AM-induced vasorelaxation in endothelium-denuded aortic rings from OZ rats (data not shown), suggesting that semapimod treatment restored AM-induced endothelium-dependent vasorelaxation—but not AM-induced endothelium-independent vasorelaxation. Semapimod did not affect AM-induced vasorelaxation in LZ rats.

AM-Induced Akt Phosphorylation and cGMP Production Are Restored by Semapimod in OZ Rats

We have previously shown that AM induces endothelium-dependent vasorelaxation *via* the PI3K/Akt- and nitric oxide (NO)/cGMP–dependent pathways in rat aortas (*16*). Thus, we examined Akt phosphorylation in the aortas of OZ and LZ rats. As we have shown, because semapimod did not affect a variety of parameters, such as serum TNF-α and CRP levels, and endothelium-dependent vasorelaxation in LZ rats, we examined its effect only in OZ rats. AM-induced Akt phosphorylation peaked around 15 min poststimulation (data not shown). Thus, the aortas were stimulated with 10^{-7} mol/L AM for 15 min in this experiment. Akt phosphorylation observed in the basal (nonstimulated) condition did not differ significantly between LZ and OZ rats. AM-induced Akt phosphorylation was significantly lower in aortas from OZ rats than in those from LZ rats (Fig. 3). AM-induced Akt phosphorylation was significantly restored when OZ rats were treated with semapimod.

We also measured cGMP production in the aortas as a marker of NO production. cGMP production under both nonstimulated basal and AM-stimulated conditions was significantly lower in OZ rats than in LZ rats (Fig. 4). Semapimod significantly restored cGMP production in OZ rats under both basal and AM-stimulated conditions.

Acute Administration of TNF-α Impairs Endothelium-Dependent Vasorelaxation and AM-Induced cGMP Production

We also examined the effect of acute administration of TNF-α on endothelium-dependent vasorelaxation using LZ rats. Preincubation with TNF-α significantly suppressed ACh-induced endothelium-dependent vasorelaxation (Fig. 5). It also significantly suppressed AM-induced vasorelaxation in aortic rings with intact endothelium, although it did not significantly affect AM-induced vasorelaxation in endothelium-denuded aortic rings (data not shown). Furthermore, sodium nitroprusside, at a dose of 10^{-6} mol/L, induced 100% relaxation of TNF-α–treated and –untreated aortic rings (data not shown). These results suggested that acute administration of TNF-α impairs endothelial function, and that this result was not caused by the toxic effects of TNF-α on the relaxation capacity of smooth muscle cells.

We also examined the effect of TNF-α administration on AM-induced cGMP production in aortas of LZ rats. TNF-α significantly suppressed cGMP production under both basal and AM-stimulated conditions (Fig. 6).

Discussion

In this study, we have shown that endothelium-dependent vasorelaxation was impaired in OZ rats compared with LZ rats. This impairment was associated with increased concentrations of TNF-α and IL-6 in plasma and adipose tissues as well as with increased serum CRP level. This impairment was also correlated with diminishment of AM-induced Akt phosphorylation and cGMP production. Semapimod treatment significantly restored endothelium-dependent vasorelaxation in OZ rats. This recovery was associated with a decreased concentration of adipocytokines in plasma and adipose tis-

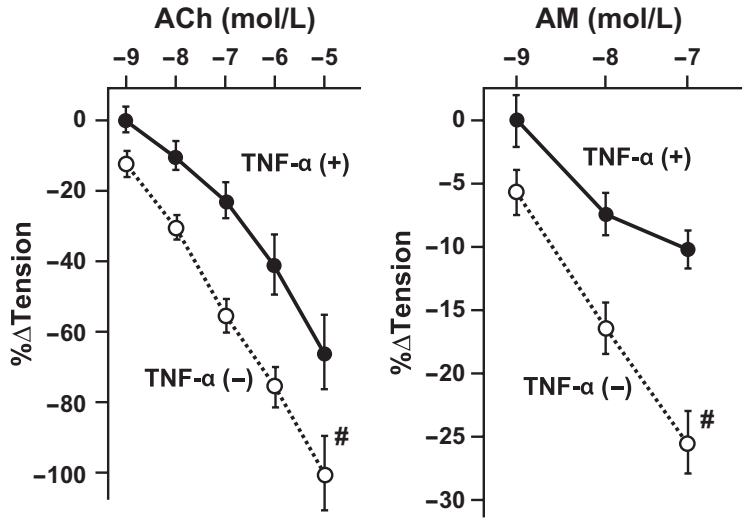

Fig. 5. *Acute administration of TNF-α impairs endothelium-dependent vasorelaxation. Aortic rings with intact endothelium prepared from LZ rats were preincubated with 5 ng/mL of TNF-α for 20 min and precontracted with prostaglandin F2α. Vasorelaxation in response to ACh (left) and AM (right) was examined.* $^{\#}p < 0.05$ *vs. TNF-α treatment (n = 6).*

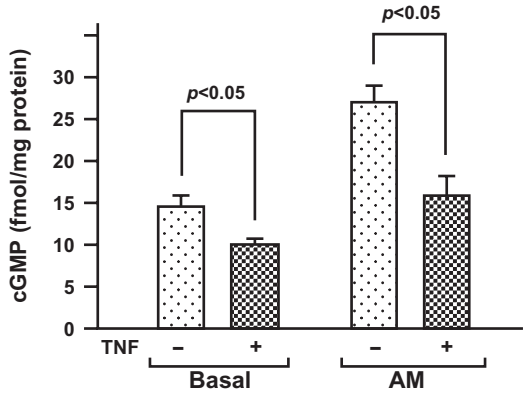

Fig. 6. *Acute administration of TNF-α (TNF) impairs cGMP production in response to AM in aortas from LZ rats. Rat thoracic aortas with intact endothelium prepared from LZ rats were preincubated with 5 ng/mL of TNF-α for 20 min and stimulated with* 10^{-7} *mol/L AM for 5 min before cGMP measurement (n = 6).*

sues, and with decreased serum CRP. This recovery was also correlated with the restoration of AM-induced Akt phosphorylation and cGMP production. Furthermore, acute exogenous administration of TNF-α significantly suppressed endothelium-dependent vasorelaxation and AM-induced cGMP production. These results suggested that endogenous proinflammatory cytokines, especially TNF-α, were implicated in endothelial dysfunction in OZ rats. These results were compatible with those of a recent publication, which reported that endothelium-dependent vasorelaxation was restored in diabetic mice ($Lepr^{db}$ mice) null for TNF-α as compared with $Lepr^{db}$ mice (*19*). There are several possible mechanisms by which proinflammatory cytokines induce endothelial dysfunction. First, proinflammatory cytokines such as TNF-α stimulated the production of ROS in blood vessels and reduced NO bioavailability. It is well known that TNF-α and IL-6 stimulate ROS production in blood vessels (*11*, *20*). In fact, it was recently reported that TNF-α stimulates ROS production *via* the NADPH oxidase–dependent pathway in blood vessels (*19–21*). Second, TNF-α might induce endothelial dysfunction by inhibiting the expression and activation of endothelial NO synthase (eNOS) (*22*, *23*). Third, TNF-α might induce endothelial dysfunction by stimulating CRP expression. It is well known that TNF-α stimulates CRP expression, and that CRP stimulates ROS production and decreases eNOS expression (*24–27*). Thus, CRP might mediate TNF-α–induced endothelial dysfunction. Fourth, although the precise mechanism remains unclear, our results suggested that TNF-α inhibited AM-induced endothelium-dependent vasorelaxation *via* suppression of AM-induced Akt phosphorylation and cGMP production. Thus, TNF-α might induce endothelial dysfunction, partly *via* the inhibition of AM-induced signal transduction. This inhibitory effect of TNF-α on AM-induced signal transduction seems to occur at a level downstream of the AM receptor expression, because, as we reported previously, cAMP production in the thoracic aorta did not differ significantly between LZ and OZ rats under both basal and AM-stimulated conditions (*18*), suggesting that the AM receptor's functional activities did not differ between LZ and OZ rats. Future studies are necessary to elucidate the mechanisms by which AM-induced signal transduction is impaired in diabetes.

In summary, endothelium-dependent vasorelaxation was impaired in OZ rats and semapimod restored endothelial

function, probably by inhibiting the production of endogenous proinflammatory cytokines such as TNF-α. Blockade of endogenous proinflammatory cytokines will be a novel strategy to inhibit endothelial dysfunction and progression of atherosclerosis-related diseases such as coronary heart disease.

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