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# Role of ceramide in TNF- $\alpha$ -induced impairment of endothelium-dependent vasorelaxation in coronary arteries

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**Zhang, David X., Fu-Xian Yi, Ai-Ping Zou, and Pin-Lan Li.** Role of ceramide in TNF- $\alpha$ -induced impairment of endothelium-dependent vasorelaxation in coronary arteries. *Am J Physiol Heart Circ Physiol* 283: H1785–H1794, 2002. First published July 11, 2002; 10.1152/ajpheart.00318.2002.—The present study tested the hypothesis that ceramide, a sphingomyelinase metabolite, serves as a second messenger for tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) to stimulate superoxide production, thereby decreasing endothelium-dependent vasorelaxation in coronary arteries. In isolated bovine small coronary arteries, TNF- $\alpha$  (1 ng/ml) markedly attenuated vasodilator responses to bradykinin and A-23187. In the presence of *N*<sup>G</sup>-nitro-L-arginine methyl ester, TNF- $\alpha$  produced no further inhibition on the vasorelaxation induced by these vasodilators. With the use of 4,5-diaminofluorescein diacetate fluorescence imaging analysis, bradykinin was found to increase nitric oxide (NO) concentrations in the endothelium of isolated bovine small coronary arteries, which was inhibited by TNF- $\alpha$ . Pretreatment of the arteries with desipramine (10  $\mu$ M), an inhibitor of acidic sphingomyelinase, tiron (1 mM), a superoxide scavenger, and polyethylene glycol-superoxide dismutase (100 U/ml) largely restored the inhibitory effect of TNF- $\alpha$  on bradykinin- and A-23187-induced vasorelaxation. In addition, TNF- $\alpha$  activated acidic sphingomyelinase and increased ceramide levels in coronary endothelial cells. We conclude that TNF- $\alpha$  inhibits NO-mediated endothelium-dependent vasorelaxation in small coronary arteries via sphingomyelinase activation and consequent superoxide production in endothelial cells.

tumor necrosis factor; lipids; nitric oxide; free radicals

ACCUMULATING EVIDENCE has indicated that tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a proinflammatory cytokine originally defined by its antitumoral activity, is an important mediator of many cardiovascular diseases, such as myocardial ischemia-reperfusion injury, chronic heart failure, atherosclerosis, and sepsis-associated cardiovascular disorders (4, 23, 51). The vascular endothelium is a major target for the actions of TNF- $\alpha$  in these diseases (47), in which plasma TNF- $\alpha$  levels are significantly elevated. TNF- $\alpha$  and other cytokines can decrease the release of endothelial nitric oxide (NO) and induce impairment of endothelium-dependent vasorelaxation in a variety of vascular beds (2, 5, 41, 46, 55,

61, 63). These cytokine-induced pathological changes have been proposed to be an important mechanism producing endothelial dysfunction in coronary circulation that occurs in myocardial ischemia-reperfusion (17, 41, 60). However, the signaling pathways that couple TNF- $\alpha$  stimulation to endothelial dysfunction remain largely unknown.

Ceramide, a sphingolipid, has been reported (19, 27, 33, 37, 49, 57) to serve as a second messenger for TNF- $\alpha$  and other cytokines in different cell types, where it is involved in numerous cellular processes, including cell growth and differentiation, apoptosis, and inflammatory responses. Recent studies (14, 42, 56) have indicated that ceramide also acts on vascular cells and participates in the regulation of ion channel activity, smooth muscle cell proliferation, endothelial cell apoptosis, and vascular tone. With respect to the vasomotor regulation, cell-permeable ceramides have been shown to induce a relaxation of the phenylephrine-contracted rat thoracic aorta, but produce contractions in canine cerebral arteries, rat mesenteric resistance and capacitance vessels, and bovine coronary resistance arteries (34, 44, 68, 69). More recently, studies from our laboratory (67) have shown that ceramide attenuates the endothelium-dependent vasorelaxation to bradykinin by increasing superoxide ( $O_2^{\cdot-}$ ) production and subsequently decreasing NO concentrations in vascular endothelial cells. Similar results have also been reported with the ceramide metabolite sphingosine (54).

In other studies (43, 53, 64), TNF- $\alpha$  or other cytokines have been found to increase intracellular ceramide in vascular endothelial cells, thereby resulting in inflammatory responses and apoptosis. It seems that ceramide-mediated signaling is importantly involved in the actions of cytokines in endothelial cells. However, it is unknown whether increased ceramide in the vascular endothelium contributes to the impairment of endothelium-dependent vasorelaxation induced by TNF- $\alpha$  or other cytokines. We hypothesized that ceramide may serve as an intracellular second messenger for TNF- $\alpha$  to stimulate  $O_2^{\cdot-}$  production, thereby contributing to coronary endothelial dysfunction induced

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by this cytokine during myocardial ischemia-reperfusion. To test this hypothesis, we determined the effect of TNF- $\alpha$  on NO-mediated endothelium-dependent vasorelaxation and examined the role of ceramide in mediating the action of TNF- $\alpha$  on endothelium-dependent vasorelaxation and NO production by use of isolated and pressurized small bovine coronary arteries and fluorescence microscopic NO measurement. We then observed the effects of TNF- $\alpha$  on intracellular ceramide production via sphingomyelinase in the endothelial cells of these arteries to further confirm TNF- $\alpha$ -induced activation of sphingomyelinase.

## MATERIALS AND METHODS

**Isolated small coronary artery preparation.** Fresh bovine hearts were obtained from a local abattoir. The left ventricular wall was rapidly dissected and immersed in ice-cold physiological saline solution (PSS) of the following composition (in mM): 119 NaCl, 4.7 KCl, 1.6 CaCl<sub>2</sub>, 1.17 MgSO<sub>4</sub>, 1.18 NaH<sub>2</sub>PO<sub>4</sub>, 24 NaHCO<sub>3</sub>, 0.026 EDTA, and 5.5 glucose (pH 7.4). Small intramural coronary arteries from the left anterior descending artery were carefully dissected and placed in cold PSS until cannulation. Segments of small arteries (100–200  $\mu$ m ID) were transferred to a water-jacketed perfusion chamber and cannulated with two glass micropipettes at their in situ length, as we described previously (44, 66). The outflow cannula was clamped, and the arteries were pressurized to 60 mmHg. The arteries were bathed in the PSS-equilibrated solution with 95% O<sub>2</sub>-5% CO<sub>2</sub> and maintained at pH 7.4 and 37°C. The internal diameter of the arteries was measured with a video system composed of a stereomicroscope (model MZ8, Leica), a charge-coupled device camera (model KP-MI AU, Hitachi), a video monitor (model VM-1220U, Hitachi), a video measuring apparatus (model VIA-170, Boeckeler Instrument), and a video printer (model UP890 MD, Sony). The arterial images were recorded continuously with a videocassette recorder (model M-674, Toshiba).

After a 1-h equilibration period, the arteries were precontracted by ~50% of their resting diameter with a thromboxane A<sub>2</sub> analog, U-46619. Once steady-state contraction was obtained, cumulative dose-response curves to the endothelium-dependent vasodilators bradykinin (10<sup>-10</sup>–10<sup>-6</sup> M), A-23187 (10<sup>-9</sup>–10<sup>-5</sup> M) or endothelium-independent vasodilator 1-[2-(aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA NONOate) (10<sup>-7</sup>–10<sup>-4</sup> M) were determined by measuring changes in the internal diameter. To study the effects of TNF- $\alpha$  on vasodilator response to bradykinin, A-23187, or DETA NONOate, TNF- $\alpha$  (0.1 and 1 ng/ml in PSS containing 0.001% BSA; bioactivity: EC<sub>50</sub> = 0.01 ng/ml using L929 cells for inhibition) (Sigma) was perfused into the lumen of cannulated arteries and incubated for 60 min, and dose-response curves to the vasodilators were redetermined. The doses of TNF- $\alpha$  chosen for these studies (15) were based on previous studies showing that it can produce endothelial dysfunction in cultured endothelial cells. In addition, 1 ng/ml of TNF- $\alpha$  is within the range of TNF- $\alpha$  levels detected in patients during myocardial ischemia-reperfusion (3, 50). To examine the role of NO, endogenous ceramide, or O<sub>2</sub><sup>-</sup> in TNF- $\alpha$ -induced endothelial dysfunction, the arteries were preincubated with a NO synthase (NOS) inhibitor N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) (100  $\mu$ M for 15 min), the acidic sphingomyelinase inhibitor desipramine (10  $\mu$ M for 60 min) (1, 5, 30), a cell-permeable O<sub>2</sub><sup>-</sup> scavenger tiron (sodium dihydroxybenzene disulfonate; 1 mM for 15 min), or

polyethylene glycol (PEG)-SOD (100 U/ml for 15 min), respectively. Dose responses to bradykinin and A-23187 in the absence or presence of TNF- $\alpha$  (1 ng/ml) were then redetermined. All drugs were added into the bath solution unless otherwise indicated. Between pharmacological interventions, the arteries were washed three times with PSS and allowed to equilibrate in drug-free PSS for 20–30 min. The vasodilator response was expressed as the percent relaxation of U-46619-induced precontraction based on changes in the internal diameter.

**Measurement of intracellular NO concentration in the endothelium.** A novel fluorescent NO indicator 4,5-diaminofluorescein diacetate (DAF-2DA) (39) was used to measure [NO] within the endothelial cells of freshly isolated small bovine coronary arteries as we described previously (67). DAF-2DA can readily enter the cells and be hydrolyzed by cytosolic esterases to DAF-2, which is trapped inside the cells. In the presence of NO and oxygen, a relatively nonfluorescent DAF-2 is transformed into the highly green fluorescent triazole form DAF-2T. Thus the increases in DAF-2T fluorescence represent the elevation of NO concentration ([NO]). Small intramural arteries (200–400  $\mu$ m ID) were carefully dissected as described above and transferred to a 35-mm Sylgard-coated dissecting dish containing ice-cold HEPES-buffered PSS composed of (in mM) 140 NaCl, 4.7 KCl, 1.6 CaCl<sub>2</sub>, 1.17 MgSO<sub>4</sub>, 1.18 NaH<sub>2</sub>PO<sub>4</sub>, 5.5 glucose, and 10 HEPES (pH 7.4). The arterial segment was cut open along its longitudinal axis and pinned onto the dish with lumen side upward. Care was taken not to disrupt the endothelium. The arterial segment was incubated with DAF-2DA (10  $\mu$ M, Calbiochem) in 1 ml PSS at room temperature for 30 min. The segments were then rinsed three times with PSS, and the dish was mounted on the stage of an epifluorescence microscope (Nikon Diaphot 200) equipped with a  $\times$ 20 objective and 490-nm excitation and 510- to 560-nm emission filters. Digital images were captured and analyzed using a personal computer-controlled charge-coupled device camera (SPOT RT monochrome, Diagnostic Instruments) and MetaMorph imaging and analysis software (Universal Imaging).

Bradykinin (10<sup>-6</sup> M) was added into the bath solution to stimulate NO production. To study the effect of TNF- $\alpha$  on endothelial [NO], the arteries were incubated with TNF- $\alpha$  (1 ng/ml) for 30 min before the response to bradykinin was determined. NO fluorescence was measured every 10 s in a single area of the endothelial layer. The specificity of endothelium-dependent NO synthesis in fluorescence measurement of NO was confirmed in our previous study using L-NAME blockade and endothelium-denuded coronary arteries (67). Results were expressed as the integrated fluorescence intensity within the area observed.

**Culture of bovine coronary arterial endothelial cells.** The bovine coronary arterial endothelial cells (BCAECs) were cultured as described previously (66). Briefly, the arteries were rinsed with medium 199 containing 5% FCS, 2% solution of antibiotics (penicillin-streptomycin-amphotericin B), 0.3% gentamycin, and 0.3% nystatin, as well as cleaned-off connective tissues. The lumen of the arteries was filled with 0.25% collagenase in medium 199 and incubated at 37°C for 30 min. The arteries were then flushed with medium 199, and the detached endothelial cells were collected, cultured in RPMI 1640 containing 25% FCS, 1% glutamine, and 1% antibiotic solution, and maintained in an incubator with 5% CO<sub>2</sub> in room air at 37°C. The endothelial cells were identified by their morphological appearance (i.e., cobblestone array) and by positive staining for von Willebrand factor antigen. All studies were performed using the cells of 3–4 passages.



**Ceramide assay.** Ceramide was determined by DG kinase assay as reported previously (58, 65). In brief, confluent BCAECs were quickly frozen with liquid N<sub>2</sub> and homogenized in 4 vol of 10 mM PBS. An aliquot of homogenates was used for the measurement of protein concentrations. The lipids were then extracted from the homogenates, dried under N<sub>2</sub>, and used for the analysis of ceramide within 72 h. An aliquot of dried lipid was solubilized by bath sonication into a detergent solution composed of 7.5% *n*-octyl- $\beta$ -D-glucopyranoside and 5 mM cardiolipin in 1 mM diethylenetriamine-pentaacetic acid and mixed with bacterial DG kinase (Calbiochem) and 4  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP to a final volume of 100  $\mu$ l. After incubation at 25°C for 3 h, the reaction was stopped by extraction of lipids with 600  $\mu$ l of chloroform-methanol (1:1 vol/vol), 20  $\mu$ l of 1% perchloric acid, and 150  $\mu$ l of 1 M NaCl. The lower organic phase was then recovered, washed twice with 1% perchloric acid, and dried with N<sub>2</sub>. The <sup>32</sup>P-labeled ceramide was separated from other lipids by thin-layer chromatography (TLC) with a solvent consisting of chloroform:acetone:methanol:acetic acid:water (10:4:3:2:1 vol/vol/vol/vol/vol). After visualization by autoradiography, the ceramide [<sup>32</sup>P] band was recovered by scraping and counted in a Beckman liquid-scintillation counter. The amount of ceramide in the lipid extracts was calculated from a standard curve constructed with known amounts of ceramide (type III, Sigma) and expressed as nanomoles per milligram of protein. To ensure that detected differences in ceramide concentrations were not due to the effects of different tissue samples on the activity of the DAG kinase *in vitro* assay, the phosphorylation of C<sub>6</sub> ceramide as an internal standard was determined in parallel, which was used to normalize ceramide concentrations in different treatment groups. To determine the effect of TNF- $\alpha$  on intracellular ceramide concentrations, the cells were treated with vehicle, TNF- $\alpha$  (1 ng/ml), or TNF- $\alpha$  + desipramine for the indicated times, followed by lipid extraction and DG kinase assay as described above.

**Sphingomyelinase assay.** The activity of sphingomyelinase was determined as reported previously (45, 65). Briefly, *N*-methyl-[<sup>14</sup>C]sphingomyelin was incubated with BCAEC homogenates (66), and the metabolites of sphingomyelin, [<sup>14</sup>C]choline phosphate and ceramide were determined. An aliquot of samples (20  $\mu$ g protein) was incubated with an acidic assay mixture containing 100 mM sodium acetate (pH 5.0), 0.05% Triton X-100, and 0.1 mM *N*-methyl-[<sup>14</sup>C]sphingomyelin (0.02  $\mu$ Ci) in a final volume of 100  $\mu$ l. After the mixture was incubated at 37°C for 15 min, the reaction was stopped by adding 1.5 ml of chloroform-methanol (2:1 vol/vol), followed by the addition of 0.2 ml of H<sub>2</sub>O. The samples were then vortexed and centrifuged at 3,000 revolutions/min for 5 min to separate the two phases. A portion of the top aqueous phase was transferred to scintillation vials and counted for the formation of [<sup>14</sup>C]choline phosphate in a Beckman liquid-scintillation counter. To determine the effect of TNF- $\alpha$  on sphingomyelinase activity, the cells were treated with vehicle, TNF- $\alpha$  (1 ng/ml), or TNF- $\alpha$  + desipramine for the indicated times, respectively.

**Statistics.** Data are presented as means  $\pm$  SE. The significant differences between and within multiple groups were examined using an analysis of variance for repeated measures, followed by a Duncan's multiple-range test. Student's *t*-test was used to evaluate the significant differences between two paired observations. *P* < 0.05 was considered statistically significant.

## RESULTS

**Effect of TNF- $\alpha$  on endothelium-dependent vasorelaxation in small coronary arteries.** Concentration-response curves of the endothelium-dependent vasodilators bradykinin or A-23187 were determined in small coronary arteries before and after TNF- $\alpha$  treatment. As shown in Fig. 1, bradykinin and A-23187 produced a concentration-dependent vasorelaxation in small coronary arteries (resting diameter 178  $\pm$  14  $\mu$ m ID). Incubation of arteries with TNF- $\alpha$  (1 ng/ml perfused into the lumen side) had little effect on the basal vascular tone, but markedly attenuated the vasodilator responses to bradykinin (Fig. 1A) and A-23187 (Fig. 1B). Perfusion of the arteries with TNF- $\alpha$  at a low dose (0.1 ng/ml) had no significant effect on these vasodilator responses.

To determine whether TNF- $\alpha$  acts through a NO-dependent mechanism in the inhibition of endothelium-dependent vasorelaxation, the arteries (170  $\pm$  14  $\mu$ m ID) were pretreated with the NOS inhibitor L-NAME (100  $\mu$ M) in the absence or presence of TNF- $\alpha$  (1 ng/ml). As shown in Fig. 2, L-NAME significantly in-

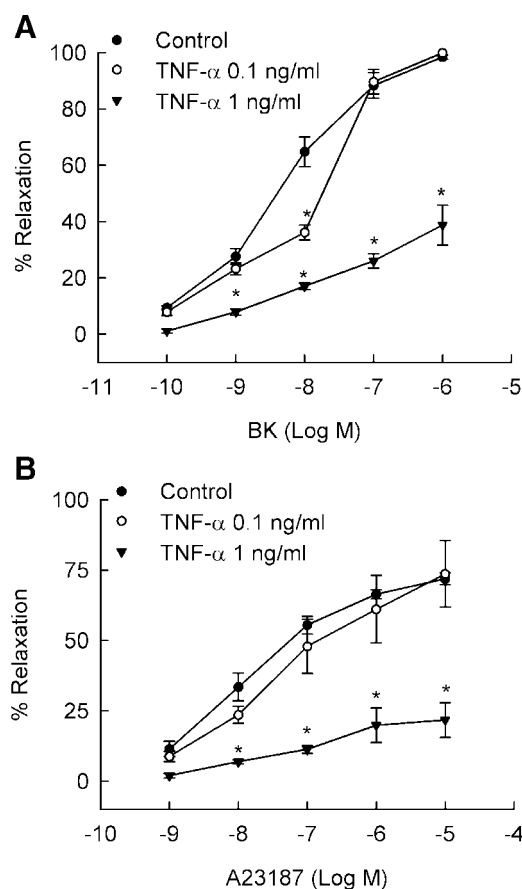


Fig. 1. Effect of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) on endothelium-dependent vasodilator responses to bradykinin (BK; A) and A-23187 (B) in bovine small coronary arteries. The arteries were precontracted with U-46619, followed by the determination of vasorelaxation to BK or A-23187. TNF- $\alpha$  (0.1 or 1 ng/ml) was perfused into the lumen of arteries and incubated for 60 min, and BK- or A-23187-induced vasodilator responses were redetermined. Values are means  $\pm$  SE; *n* = 6 arteries. \**P* < 0.05 vs. control.

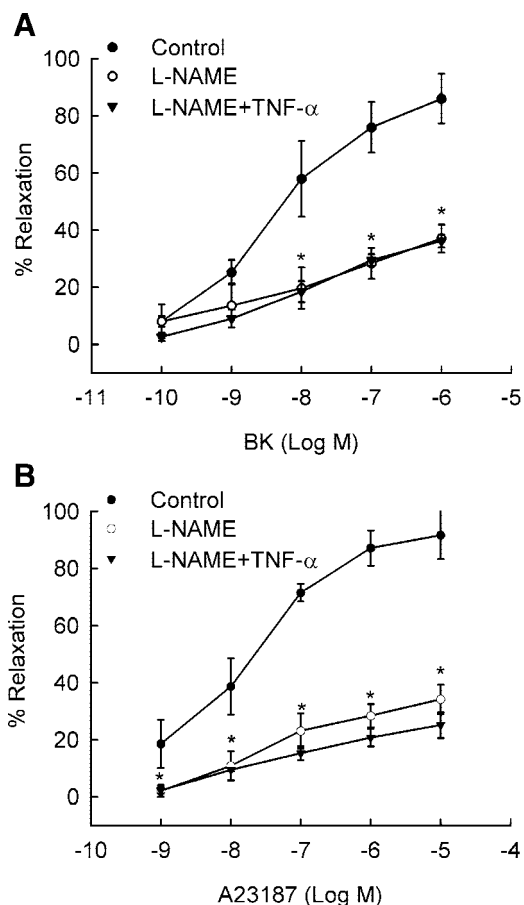


Fig. 2. Effect of  $N^G$ -nitro-L-arginine methyl ester (L-NAME) in bovine small coronary arteries in the presence or absence of TNF- $\alpha$  on vasodilator responses to BK (A) and A-23187 (B). The arteries were preincubated with L-NAME (100  $\mu$ M) for 15 min. TNF- $\alpha$  (1 ng/ml) was perfused into the lumen of arteries and incubated for 60 min. Values are means  $\pm$  SE;  $n = 6$  arteries. \* $P < 0.05$  vs. control.

hibited the vasodilator responses to bradykinin and A-23187. L-NAME alone caused no significant or slight vasoconstriction in resting arteries. This confirmed that the responses to these vasodilators were NO dependent. In the presence of L-NAME, TNF- $\alpha$  produced no further inhibition of bradykinin- or A-23187-induced vasorelaxation. The inhibition of vasorelaxation by TNF- $\alpha$  plus L-NAME was similar to that by TNF- $\alpha$  or L-NAME alone.

*Effect of TNF- $\alpha$  on endothelial [NO] in small coronary arteries.* The intracellular [NO] within endothelial cells was measured using isolated small coronary arteries. Figure 3A presents typical fluorescence microscopic images showing NO-induced DAF-2 green fluorescence within endothelial cells. Incubation of arteries with bradykinin ( $10^{-6}$  M) produced a marked increase in NO fluorescence. The addition of TNF- $\alpha$  (1 ng/ml) had no significant effect on basal NO fluorescence, but it markedly inhibited the bradykinin-induced increase in NO fluorescence. Figure 3B present representative traces of NO changes recorded, showing a dynamic change of [NO] in endothelial cells in response to bradykinin. TNF- $\alpha$  markedly blocked the bradykinin-in-

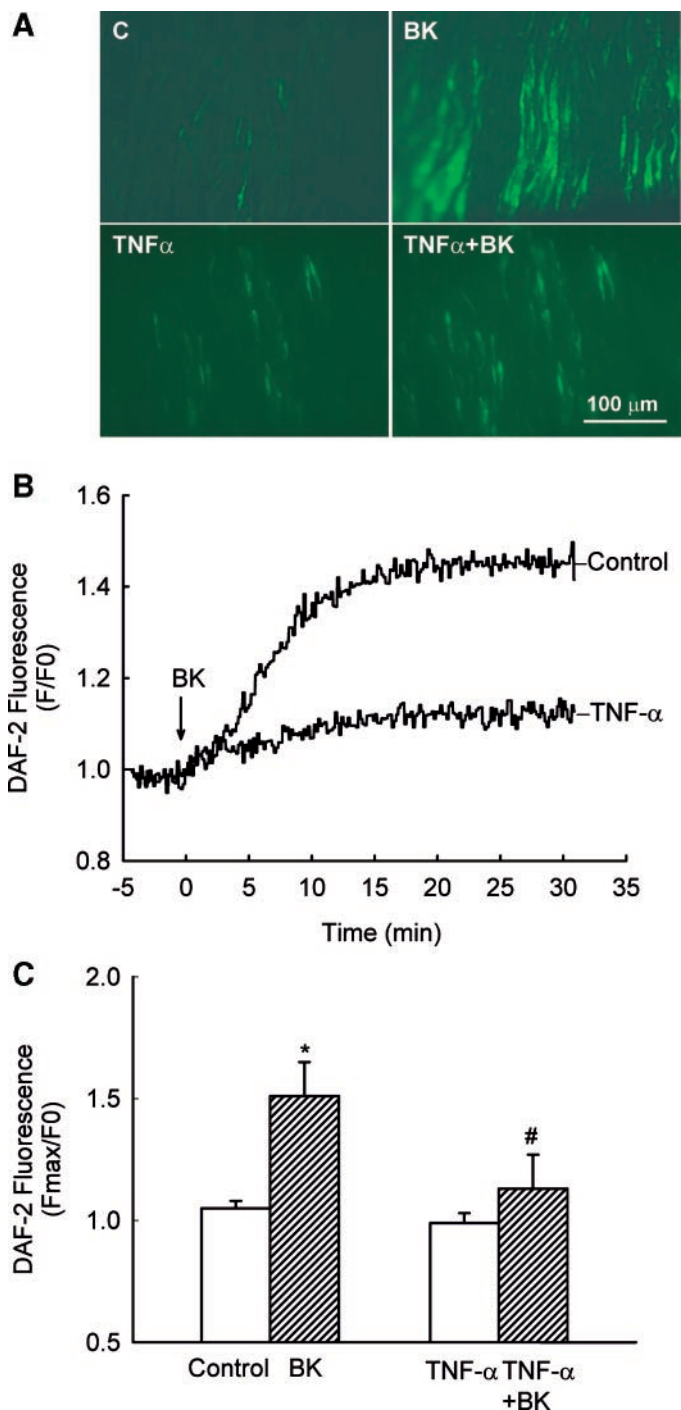


Fig. 3. Effect of TNF- $\alpha$  on bradykinin-induced nitric oxide (NO) increase in the endothelium of small coronary arteries. A: representative fluorescence images of NO in the endothelial cells taken at 30 min under control condition and after treatment with BK ( $10^{-6}$  M), TNF- $\alpha$  (1 ng/ml), or TNF- $\alpha$  + BK. B: representative dynamic recordings of NO fluorescence. C: summarized data of the maximal NO fluorescence. F/F<sub>0</sub>, relative fluorescence intensity to basal fluorescence; F<sub>max</sub>/F<sub>0</sub>, maximal fluorescence intensity to basal fluorescence. Values are means  $\pm$  SE;  $n = 7-10$  arteries. \* $P < 0.05$  vs. control; # $P < 0.05$  vs. BK.

duced NO increase. Figure 3C summarized maximal NO changes in response to bradykinin in control and TNF- $\alpha$ -pretreated arteries, respectively. TNF- $\alpha$  had no effect on basal [NO], as shown by the open bars. Bradykinin induced an increase in [NO] within endothelial cells, which was blocked by addition of TNF- $\alpha$  into bath solution.

**Effect of TNF- $\alpha$  on endothelium-independent vasorelaxation in small coronary arteries.** To confirm that TNF- $\alpha$  affects only endothelium-dependent vasorelaxation, we then determined the effects of TNF- $\alpha$  on the vasorelaxation induced by DETA NONOate, an endothelium-independent vasodilator, in small coronary arteries ( $195 \pm 16 \mu\text{m ID}$ ). It was found that DETA NONOate-induced vasorelaxation was not affected by TNF- $\alpha$  pretreatment (1 ng/ml) (Fig. 4). L-NAME had no significant effect on DETA NONOate-induced vasorelaxation.

**Effects of  $\text{O}_2^{\cdot-}$  scavengers on TNF- $\alpha$ -induced endothelial dysfunction.** We (67) previously reported that ceramide-induced impairment of endothelium-dependent vasorelaxation was associated with decreased bioavailability of NO due to  $\text{O}_2^{\cdot-}$  production in bovine small coronary arteries. To determine whether TNF- $\alpha$ -induced NO decrease and endothelial dysfunction are also due to increased  $\text{O}_2^{\cdot-}$  production, the arteries ( $180 \pm 6 \mu\text{m ID}$ ) were preincubated with the  $\text{O}_2^{\cdot-}$  scavengers tiron or PEG-SOD. As expected, tiron (1 mM) had no effect on either basal tone or vasodilator responses to bradykinin and A-23187 in the absence of TNF- $\alpha$ . However, it significantly reversed the inhibitory effect of TNF- $\alpha$  on the vasorelaxation to bradykinin or A-23187. The inhibitory effect of TNF- $\alpha$  was also restored by PEG-SOD (100 U/ml) (Fig. 5).

**Contribution of endogenous ceramide to TNF- $\alpha$ -induced endothelial dysfunction.** To explore whether the TNF- $\alpha$ -induced NO decrease and endothelial dysfunction are associated with the production of endogenous

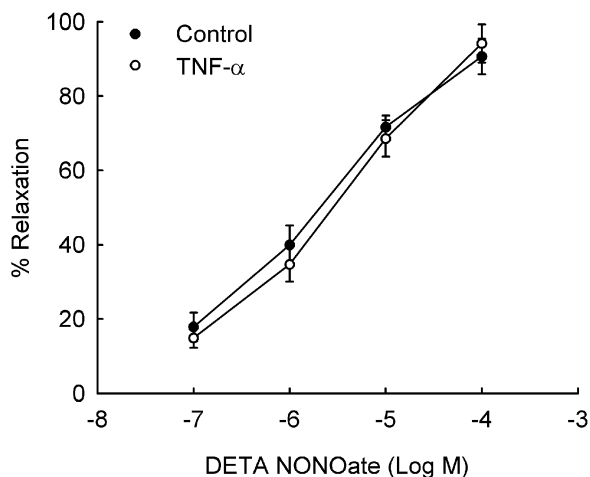


Fig. 4. Effect of TNF- $\alpha$  on endothelium-independent vasodilator response to 1-[2-(2-aminoethyl)-*N*-(2-ammonioethylamino)]diazene-1,2-diolate (DETA NONOate) in bovine small coronary arteries. TNF- $\alpha$  (1 ng/ml) was perfused into the lumen of arteries and incubated for 60 min. Values are means  $\pm$  SE;  $n = 6$  arteries. \* $P < 0.05$  vs. control.

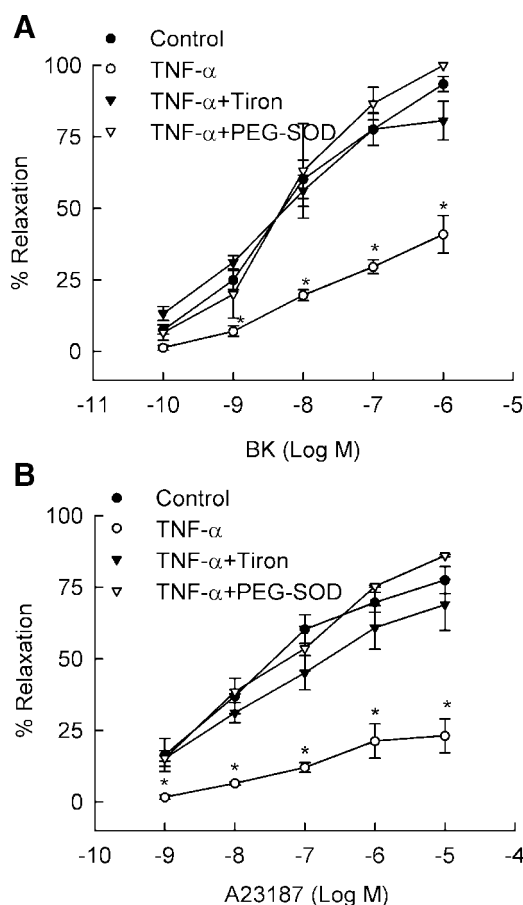


Fig. 5. Effect of the  $\text{O}_2^{\cdot-}$  scavengers tiron and polyethylene glycol (PEG)-superoxide dismutase (SOD) on TNF- $\alpha$ -induced impairment of vasodilator responses to BK (A) and A-23187 (B). The arteries were preincubated with tiron (1 mM) or PEG-SOD (100 U/ml) and then perfused with TNF- $\alpha$  (1 ng/ml) into the lumen of arteries and incubated for another 60 min. Values are means  $\pm$  SE;  $n = 6$  arteries. \* $P < 0.05$  vs. control.

ceramide, the arteries ( $180 \pm 17 \mu\text{m ID}$ ) were pre-treated with an acidic sphingomyelinase inhibitor desipramine at a concentration (10  $\mu\text{M}$ ) similar to that used previously to selectively block acidic sphingomyelinase (1, 15, 30). Desipramine had no effect on either basal tone or vasodilator responses to bradykinin and A-23187. In the presence of desipramine, however, the inhibitory effects of TNF- $\alpha$  on the vasorelaxation to bradykinin and A-23187 were significantly attenuated (Fig. 6). Consistent with these results, desipramine also significantly reversed the inhibitory effect of TNF- $\alpha$  on bradykinin-induced increase in endothelial [NO]. The maximal bradykinin-induced NO fluorescence response in the TNF- $\alpha$  + desipramine group was restored by  $>80\%$  (data not shown).

**Effect of TNF- $\alpha$  on intracellular ceramide concentrations in BCAECs.** To provide direct evidence that TNF- $\alpha$  stimulates production of ceramide in endothelial cells, intracellular ceramide concentrations were measured in cultured BCAECs. In these experiments, cells were treated with vehicle, TNF- $\alpha$  (1 ng/ml), or TNF- $\alpha$  + desipramine (10  $\mu\text{M}$ ) for the indicated time



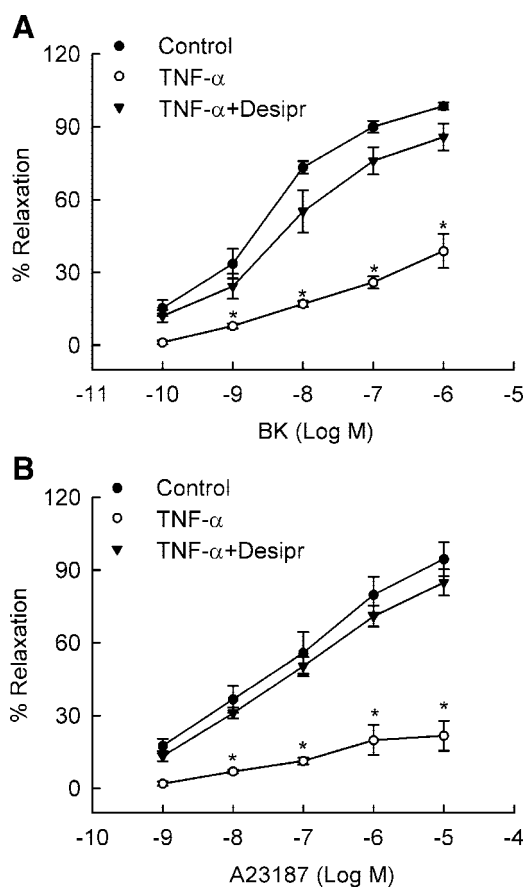


Fig. 6. Effect of desipramine, an acidic sphingomyelinase inhibitor, on TNF- $\alpha$ -induced impairment of vasodilator responses to BK (A) and A-23187 (B). The arteries were preincubated with desipramine (10  $\mu$ M), followed by perfusion with TNF- $\alpha$  (1 ng/ml) into the lumen of arteries and incubation for another 60 min. Values are means  $\pm$  SE;  $n = 6$  arteries. \* $P < 0.05$  vs. control.

periods. Endogenous ceramide concentrations were then determined with DG kinase assay, followed by separation and visualization of phospholipids by TLC. A representative TLC autoradiography is presented in Fig. 7A. In agreement with previous reports (8, 58), in addition to phosphatidic acid, two major radiolabeled phospholipid bands were detected in these reaction mixtures, including a phosphorylated endogenous ceramides and dihydroceramides from BCAECs (Cer-1-P), which comigrated with the type III ceramide-1-phosphate, and a phosphorylated C6:0 ceramide internal standard (C<sub>6</sub>Cer-1-P). Basal ceramide levels can be detected in control BCAECs. TNF- $\alpha$  treatment produced a rapid increase in endogenous ceramides. Pretreatment of cells with desipramine significantly blocked this increase in ceramides (Desipr + TNF- $\alpha$ ). The observed increases in ceramide-1-phosphate was not due to changes in the activity of the DG kinase but rather changes in ceramide concentrations within the assay because the phosphorylation of C6:0 ceramide as an internal standard did not differ significantly in different groups. Figure 7B shows the summarized data for intracellular ceramide concentrations in BCAECs. The basal ceramide concentrations in these

cells were 5.32 nmol/mg protein. TNF- $\alpha$  treatment led to a rapid increase in ceramides within 2 min (by  $\sim 25\%$ ), which gradually returned to basal concentrations  $\sim 60$  min after stimulation. This increase was markedly inhibited by desipramine.

*Effect of TNF- $\alpha$  on sphingomyelinase activity in BCAECs.* Because desipramine, an acidic sphingomyelinase inhibitor, blocked TNF- $\alpha$ -induced ceramide production, we examined the effects of TNF- $\alpha$  on acidic sphingomyelinase activity in endothelial cells to confirm that TNF- $\alpha$  stimulates ceramide production via this enzyme. As shown in Fig. 8, the basal activity of acidic sphingomyelinase was  $1.31 \pm 0.08$  nmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup> in cultured BCAECs. TNF- $\alpha$  rapidly induced a 2.7-fold increase in acidic sphingomyelinase activity within 2 min. Pretreatment of cells with desi-

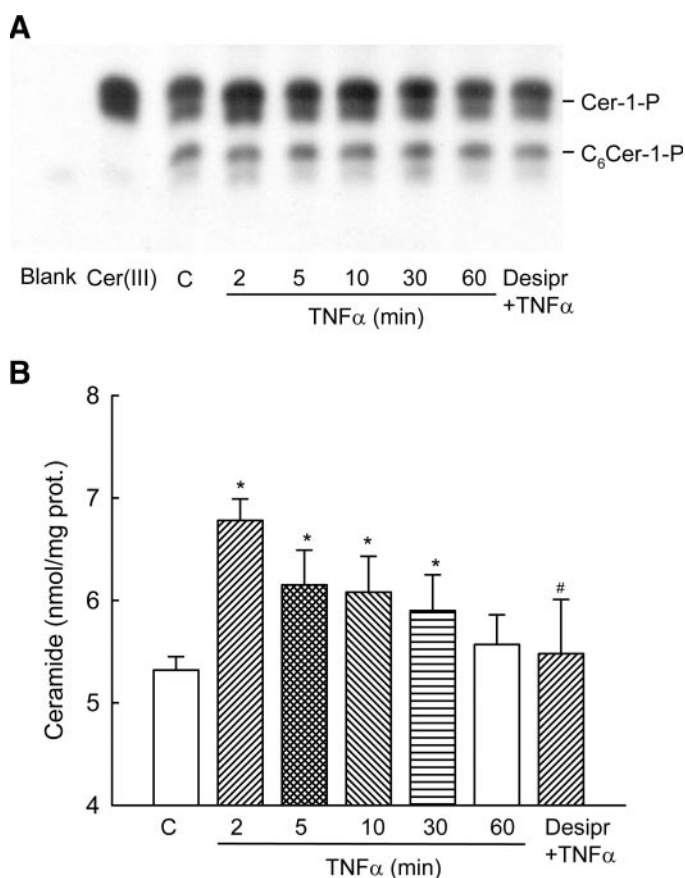


Fig. 7. Effect of TNF- $\alpha$  on intracellular ceramide (Cer) concentrations in bovine coronary arterial endothelial cells (BCAECs). The cells were treated with vehicle, TNF- $\alpha$  (1 ng/ml) for 2, 5, 10, 30, and 60 min, or desipramine (Desipr; 10  $\mu$ M), followed by TNF- $\alpha$  for 2 min. A: representative autoradiograph of phospholipids fractionated by thin-layer chromatography (TLC) after DG kinase assay. The assay was performed without lipid extracts (blank) or in the presence of type III ceramides as an external standard (Cer III) or the lipid extracts from the cells. The bands shown represented ceramide-1-phosphate (Cer-1-P), which were from endogenous ceramide, and C6:0 ceramide-1-phosphate (C<sub>6</sub>Cer-1-P) from internal standard, respectively. B: summarized quantitative changes in ceramide-1-phosphate. The Cer-1-P and C<sub>6</sub>Cer-1-P bands were scraped, and the radioactivity was counted using a liquid scintillation counter. Values are means  $\pm$  SE from five separate experiments. \* $P < 0.05$  vs. control; # $P < 0.05$  vs. TNF- $\alpha$  for 2 min.

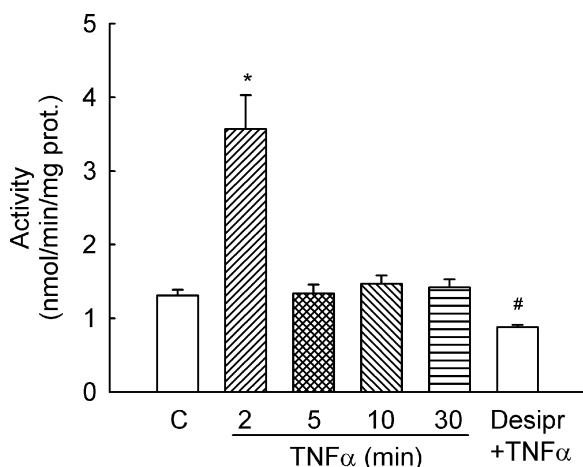


Fig. 8. Effect of TNF- $\alpha$  on acidic sphingomyelinase activity in BCAECs. The cells were treated with vehicle, TNF- $\alpha$  (1 ng/ml) for 2, 5, 10, 30 min, or desipramine (10  $\mu$ M), followed by TNF- $\alpha$  for 2 min. Values are means  $\pm$  SE from five separate experiments. \* $P$  < 0.05 vs. control; # $P$  < 0.05 vs. TNF- $\alpha$  for 2 min.

pramine markedly blocked these increases in the activity of acidic sphingomyelinase.

## DISCUSSION

The present study demonstrated that TNF- $\alpha$  inhibited bradykinin- and A-23187-induced vasorelaxation in small coronary arteries. In the presence of L-NAME, TNF- $\alpha$  had no further inhibitory effect on the responses to these endothelium-dependent vasodilators, suggesting that the effect of TNF- $\alpha$  is NO dependent. In contrast, TNF- $\alpha$  did not alter the vasorelaxation induced by the endothelium-independent vasodilator DETA NONOate. These results indicate that TNF- $\alpha$  has a detrimental effect on NO-mediated endothelium-dependent vasorelaxation in coronary microcirculation and thereby may lead to endothelial dysfunction.

It has been suggested that TNF- $\alpha$ , a pleiotropic cytokine involved in the regulation of multiple cellular functions, is an important mediator of circulatory changes associated with the development of various cardiovascular diseases, such as atherosclerosis, sepsis-associated cardiovascular dysfunction, and myocardial ischemia-reperfusion injuries (10, 13, 21, 35, 51). With respect to its action in myocardial ischemia-reperfusion, previous studies (13, 51) have shown that increased TNF- $\alpha$  may contribute to decreased coronary vascular tone at a late phase of myocardial ischemia and reperfusion. However, these actions of TNF- $\alpha$  on vascular tone are often attributable to the induction of inducible NOS in macrophages and cardiomyocytes, and usually occur with a lag time of 4–6 h after ischemia-reperfusion. To our knowledge, nevertheless, the acute effect of TNF- $\alpha$  on coronary vascular tone and related mechanism remain poorly understood. In a previous study (41), TNF- $\alpha$  (2-h incubation) was found to selectively impair acetylcholine-induced relaxation in feline left anterior descending coronary artery rings. The present study demonstrated that short-term incubation (1 h) with TNF- $\alpha$  attenuated

L-NAME-sensitive, bradykinin- and A-23187-induced vasorelaxation in coronary resistant arteries. This provides the direct evidence that TNF- $\alpha$  also impairs NO-mediated endothelium-dependent vasorelaxation in coronary microcirculation. These findings are in agreement with those of previous studies (2, 5, 46, 55, 61, 63), showing that TNF- $\alpha$  and other cytokines inhibit the release of endothelial NO and endothelium-dependent vasorelaxation in the feline carotid artery, rat aorta, rat and bovine pulmonary artery, and human forearm resistance artery and vein. It should be noted that TNF- $\alpha$  had no effect on the basal vascular tone in coronary small arteries, which is consistent with a previous report (63) showing that TNF- $\alpha$  inhibits bradykinin-induced vasorelaxation but not basal vascular tone in isolated pulmonary arteries. However, previous studies (20, 38) have shown that TNF- $\alpha$  caused acute constriction of rat coronary vessels *in vivo* or in isolated and perfused heart, as indicated by an increase in coronary perfusion pressure. The reason for this discrepancy is not clear at present. It is possible that the action of TNF- $\alpha$  on vascular tone varies with species. In addition, we used the isolated arterial preparation to study the effect of TNF- $\alpha$ , which may avoid the possible indirect effects of TNF- $\alpha$  via other tissues surrounding the vessels *in vivo* or in perfused heart used in previous studies (20, 38).

It has been proposed that decrease in the bioavailability of NO plays a central role in endothelial dysfunction or impairment of endothelium-dependent vasorelaxation (12, 25, 29). We wondered whether the action of TNF- $\alpha$  is associated with a decrease in endothelial NO levels. Therefore, we examined the effect of TNF- $\alpha$  on intracellular [NO] in the endothelium with the use of isolated small coronary arteries. As expected, bradykinin was found to induce a marked and time-dependent increase in [NO] in the endothelium of these freshly dissected arteries. In the presence of TNF- $\alpha$ , bradykinin-induced [NO] increase was significantly attenuated. It appears that the inhibitory effect of TNF- $\alpha$  on endothelium-dependent vasorelaxation in small coronary arteries is mediated by decreasing [NO] in the endothelium. In previous studies (11, 31), TNF- $\alpha$  has been found to increase NO production by inducing inducible NOS from macrophages, vascular smooth muscles or endothelial cells, which usually occurs several hours after TNF- $\alpha$  stimulation. In contrast, a short-term TNF- $\alpha$  treatment inhibits NO production by endothelial NOS (9, 36, 63). The results of the present study further support the view that TNF- $\alpha$  produces a rapid inhibitory action on NOS in the endothelium.

To answer the question of how TNF- $\alpha$  reduces endothelial [NO] and consequently inhibits endothelium-dependent vasorelaxation in coronary arteries, we addressed the possibility of the interaction between NO and O $_2^{\cdot-}$ . Recent studies (12, 25) have indicated that O $_2^{\cdot-}$  can interact with NO and thereby decrease [NO] and produce endothelial dysfunction. Therefore, TNF- $\alpha$ -stimulated O $_2^{\cdot-}$  production may be one of the important mechanisms in decreasing NO bioavailability and



consequently resulting in the impairment of endothelium-dependent vasorelaxation. In the present study, we found that tiron, a cell-permeable  $O_2^-$  scavenger (32), and PEG-SOD prevented TNF- $\alpha$ -induced impairment of endothelium-dependent vasorelaxation. Therefore, the TNF- $\alpha$ -induced NO decrease and endothelial dysfunction are associated with enhanced  $O_2^-$  production.

The mechanisms mediating TNF- $\alpha$ -induced  $O_2^-$  production have yet to be determined. Recent studies (6, 7, 15, 18, 24, 28, 43, 67) from our and other laboratories have indicated that ceramide and/or other sphingolipids may stimulate the production of  $O_2^-$  in vascular endothelial or other cells. The present study determined whether TNF- $\alpha$  stimulates ceramide production and consequently enhances  $O_2^-$  production, leading to a decrease in [NO] and impairment of endothelium-dependent vasorelaxation. In isolated coronary arterial preparations, pretreatment of the arteries with desipramine, an acidic sphingomyelinase inhibitor, was found to prevent TNF- $\alpha$ -induced impairment of endothelium-dependent vasorelaxation in small coronary arteries, indicating that the production of endogenous ceramide may be involved in the action of TNF- $\alpha$  on endothelium-dependent vasorelaxation. To further confirm the involvement of ceramide in TNF- $\alpha$  effect, we determined intracellular ceramide concentrations in response to TNF- $\alpha$  stimulation in coronary endothelial cells. In agreement with the results from pharmacological experiments in isolated coronary arteries, these biochemical assays demonstrated that TNF- $\alpha$  induced a rapid increase in endogenous ceramide in endothelial cells, which could be blocked by desipramine. Taken together, our results supported the view that the TNF- $\alpha$ -induced NO decrease and endothelial dysfunction are associated with endogenous ceramide production.

Furthermore, we examined the effects of TNF- $\alpha$  on sphingomyelinase activity in endothelial cells. Several isoforms of sphingomyelinase have been identified in mammalian cells and tissues, and have been implicated in the hydrolysis of membrane sphingomyelin to ceramide in response to a variety of cytokines or hormones (19, 27, 33, 37, 49, 57). As discussed above, the acidic sphingomyelinase inhibitor desipramine inhibited TNF- $\alpha$ -induced production of ceramide and impairment of endothelium-dependent vasorelaxation. It is possible that TNF- $\alpha$  can activate acidic sphingomyelinase and thereby stimulate  $O_2^-$  production. This study demonstrated that treatment of endothelial cells with TNF- $\alpha$  produced a rapid activation of acidic sphingomyelinase, suggesting that TNF- $\alpha$ -induced increase in intracellular ceramide concentrations is due to activation of acidic sphingomyelinase. This TNF- $\alpha$ -induced activation of acidic sphingomyelinase has also been found in other types of cells, such as the U397 monocyte and human leukemic T Jurkat cells (40, 59, 62). However, it should be noted that the specific mechanism utilized by cells to generate ceramide might be time dependent. In previous studies, ceramide generation was reported to occur without apparent stimula-

tion of sphingomyelinase in cerebral endothelial cells after long-term treatment of TNF- $\alpha$  (16 h), followed by cycloheximide (6 h), and this increase in ceramide has been attributed to de novo ceramide biosynthesis via ceramide synthase (64).

The present study did not attempt to determine the pathogenic significance of TNF- $\alpha$ -induced endothelial dysfunction in coronary circulation in vivo. Previous studies (13, 16, 22, 26, 51, 52) have shown that the levels of cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , markedly increased in the myocardial or coronary arterial tissues during ischemia-reperfusion, endotoxemia, or atherosclerosis. On the basis of our findings, the overproduction of these cytokines, specifically TNF- $\alpha$ , would induce a rapid production of ceramide in endothelial cells, thereby producing endothelial dysfunction, resulting in the development of various pathological processes such as depressed cardiac contractility in ischemia-reperfusion and atherosclerosis (51, 65). In this context, ceramide-stimulated  $O_2^-$  production in response to increased TNF- $\alpha$  may represent an important signaling pathway mediating endothelial dysfunction in coronary circulation under different pathological conditions.

In summary, the present study demonstrated that TNF- $\alpha$  inhibited NO-mediated, endothelium-dependent vasorelaxation in small coronary arteries by activating sphingomyelinase and enhancing  $O_2^-$  generation in coronary endothelial cells. These results led to a hypothesis that a sphingomyelinase/ceramide signaling pathway mediates the actions of cytokines such as TNF- $\alpha$  and thereby contributes to vascular endothelial dysfunction in coronary circulation under different pathological conditions with increased cytokines.

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