Fas Signaling Induces Akt Activation and Upregulation of Endothelial Nitric Oxide Synthase Expression

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Abstract—A growing body of evidence has shown that Fas, a death receptor, mediates apoptosis-unrelated biological effects. Here, we report that Fas engagement with Fas ligand induced activation of Akt and upregulation of endothelial nitric oxide synthase expression without induction of apoptosis. In the presence of the phosphatidylinositol 3-kinase inhibitor wortmannin, Fas ligand, however, induced apoptosis instead of upregulation of endothelial nitric oxide synthase expression. In vivo, systolic blood pressure was slightly higher in mutant mice with decreased cell surface Fas expression (lpr mice) compared with wild-type mice. In addition, chronic inhibition of nitric oxide synthesis by Nω-nitro-l-arginine induced a progressive increase in the levels of blood pressure in wild-type mice, whereas no further increase in the levels of blood pressure was observed in lpr mice. Furthermore, acetylcholine caused a lesser endothelium-dependent relaxation of the strips from lpr mice compared with wild-type mice, although the vasoconstrictor potency of phenylephrine was not different between the two groups. These findings indicate that Fas signaling may have a role in the regulation of endothelial function and blood pressure through modulating endothelial nitric oxide synthase expression in the Akt signal-dependent manner. (Hypertension. 2004;43:880-884.)

Key Words: endothelial growth factors ■ nitric oxide synthase ■ hypertension

Fas is a type I membrane receptor belonging to a member of the tumor necrosis factor (TNF) receptor superfamily. Activation of Fas by Fas ligand (FasL) induces apoptosis via activation of the caspase cascade.1 Fas is expressed almost ubiquitously in a variety of cells, including endothelial cells (ECs). However, FasL is mainly expressed in immune cells such as natural killer cells, activated T cells, and macrophages, and also at some immune privileged sites, for example, eye and testis, where it is believed to protect those tissues by inducing apoptosis in Fas-bearing immune cells.2,3 FasL, a member of the TNF family, is rapidly cleaved off by a metalloproteinase from the membrane to become a soluble form (sFasL) as well as TNF-α.4 In human studies, sFasL has been detected in the serum of patients with several cardiovascular diseases, for example, congestive heart failure and acute coronary syndromes.5,6 Importantly, FasL is also expressed at low levels on the vascular endothelium. TNF-α, an inflammatory cytokine, downregulates FasL expression in ECs, and overexpression of FasL on the endothelium attenuates leukocyte extravasations induced by TNF-α.7 We also reported that sFasL released from ECs protect from hypoxia-induced apoptosis in ECs.8 Finally, EC overexpression of FasL on the endothelium attenuates ischemia-reperfusion injury in the heart.9 Collectively, these data suggest that FasL may play important roles in the maintenance of endothelial function.

Because many atherogenic factors can induce apoptosis in cultured ECs, Fas-mediated apoptosis leading to EC loss is thought to contribute to the pathogenesis of endothelial dysfunction and atherosclerosis.10 However, it has been well established that ECs are highly resistant to Fas-mediated apoptosis.11 Interestingly, recent evidence has shown that Fas signaling can have apoptosis-unrelated biological effects, such as induction of neurite growth through activation of the extracellular signal-regulated kinase.12 Fas engagement is also reported to induce cardiomyocyte hypertrophy instead of apoptotic cell death.13 However, it is not clear whether Fas signaling can induce apoptosis-unrelated effects in ECs.

Here, we show that Fas engagement induces an Akt-dependent upregulation of endothelial nitric oxide synthase (eNOS) expression in ECs, and that mutant mice with decreased cell surface Fas expression have hypertension with endothelial dysfunction.

Methods

Cell Culture and Reagents

Human umbilical vein ECs were purchased from Sanko Junyaku (Tokyo, Japan) and cultured in EBM-2 (Sanko Junyaku) supplemented with 2% FCS and antibiotics. Medium was replaced with fresh medium, with or without serum, typically at the time reagents were added. Recombinant FasL and the agonistic antibody to Fas
(Jo2) were generously provided by Dr. Shigekazu Nagata (Osaka University). Wortmannin, \( \text{N}^\text{-nitro-l-arginine methyl ester (l-NAME)} \), phenylephrine, and acetylcholine were purchased from Sigma. Anti-phospho-Akt and anti-\( \alpha \)-tubulin antibodies were from Cell Signaling and Carbiochem, respectively.

**Western Blot Analysis**

Cells were washed with phosphate-buffered saline (PBS) twice and harvested by scraping. Cell lysates were prepared in cell lysates buffer (50 mmol/L Tris-HCl, pH 8.0, 20 mmol/L EDTA, 1% SDS, 100 mmol/L NaCl). Protein concentration was determined using the BioRad protein assay kit (Bio-Rad); 20 \( \mu \)g of protein extract was fractionated on SDS-polyacrylamide electrophoresis gel and transferred to a polyvinylidine difluoride membrane (Immobilon-P, Millipore). The membrane was blocked with T-PBS (1X PBS, 0.3% Tween 20) containing 3% dry milk and incubated with a primary antibody overnight at 4°C. After 3 washes with T-PBS, the membrane was incubated with a secondary antibody (anti-mouse or anti-goat IgG HRP conjugate [Promega]) for 1 hour, then washed with 0.05% Tween 20 in PBS. The immune complexes were detected by chemiluminescence methods (ECL, Amersham International).

**Detection of Apoptosis by Annexin V Staining**

Fluorescein isothiocyanate-labeled Annexin V (Fluos, Boehringer Mannheim) was used to bind exposed phosphatidylserine on cells undergoing the early stages of apoptosis. In brief, 10^5 cells/mL were incubated with 1 mL Annexin V–fluorescein isothiocyanate of the provided solution for 30 minutes at room temperature and subsequently analyzed by FACS (Becton Dickinson). Electronic compensation was used to eliminate spreading into adjacent fluorescence channels. Data analysis was performed with Cell Quest software.

**Animal Experiments**

Male MRL/MpJ\( ^{pr} \) mice and MRL/MpJ\( ^{+/+} \) mice were purchased from breeding colonies at Japan SLC (Shizuoka, Japan). Animal experiments were performed in 8-week-old to 10-week-old mice in accordance with the Ethics Committee on Animal Experiments and Care of the Osaka University.

**Organ Culture**

Thoracic aortas were collected from mice and were cultured in Dulbecco modified eagle medium (DMEM) with 10% FBS in the presence or absence of the agonistic antibody to Fas (Jo2) at a concentration of 1 \( \mu \)g/mL for 60 minutes at 37°C, 5% CO2. The aortas were then washed in PBS and snap-frozen in OCT compound (Sakura, Tokyo). Serial 10-\( \mu \)m-thick cryostat sections were collected on poly-L-lysine-coated slides and fixed in acetone. Sections were immunostained using anti-CD31 monoclonal antibody (BD Bioscience) and anti-phospho-Akt antibody (Cell Signaling), respectively, followed by ABC method (Dako, Carpinteria, Calif).

**Vascular Reactivity Studies**

An aortic strip was prepared and placed in oxygenated modified Krebs-Henseleit solution. The strip was equilibrated for 60 minutes under resting tension of 1.0 g, which is optimal for inducing the maximal contraction in all vessels used. Relaxation response to acetylcholine was expressed as a percentage of decreased tension in contractile force induced by phenylephrine (3 mmol/L).

**Measurement of Blood Pressure**

Blood pressure was measured directly and indirectly. For direct measurements, we anesthetized mice with an intraperitoneal injection of pentobarbital sodium at 0.08 mg/g body weight. A femoral artery was cannulated with polyethylene tubing, after which the mouse was allowed to recover. To record blood pressure, the cannula was connected to a pressure transducer; arterial pressure was recorded continuously with mice conscious and unrestrained. For indirect measurements, blood pressure was measured without anesthesia using a programmable sphygmanometer connected with a cuff probe for mouse (MCP-1, Softron). L-NAME was administrated in the drinking water at 1 mg/mL, whereas control animals (lpr and wild-type) received unmodified drinking water.

**Statistical Analysis**

Statistical analysis was performed by 1-way ANOVA after a post hoc test. Results are expressed as mean±SEM. A value of \( P<0.05 \) was considered significant.

**Results**

**Fas Ligand Induces Upregulation of eNOS but not Apoptosis in ECs**

As shown in the previous studies, treatment with recombinant FasL induced apoptosis in Jurkat T lymphocytes and Fas-sensitive cells, but not in ECs or Fas-resistant cells (Figure 1A). FasL treatment, however, resulted in upregulation of eNOS expression in ECs after 24 hours without apoptosis induction (Figure 1B, 1C). Importantly, in the presence of the PI3 kinase...
inhibitor wortmannin, FasL induced apoptosis instead of upregulation of eNOS expression in ECs, indicating that the functional system of this apoptotic program in ECs was intact and that Fas-mediated eNOS expression may be Akt-dependent.

FasL Induces Akt Phosphorylation in ECs

We next examined whether Fas engagement induces Akt activation in ECs. Incubation with FasL induced a transient increase in the levels of Akt phosphorylation, as did insulin in ECs (Figure 2A, B), whereas it did not induce an increase in the levels of Akt phosphorylation in Jurkat T lymphocytes (data not shown), indicating that the downstream signals of Fas may be different between these two cell types. Activation of the Akt signaling in ECs was observed from 5 minutes to 60 minutes after the stimulation with FasL. In addition, Fas engagement with the agonistic antibody Jo2 also induced Akt activation in aortic segments, suggesting that this activation can be induced in vivo (Figure 2C).

Hypertension Develops in Mutant lpr Mice but Progressive Hypertension Fails to Develop in Response to Long-Term NOS Inhibition

Because nitric oxide derived from eNOS is an important regulator of blood pressure, we next compared the blood pressure of lpr mice, which express decreased cell surface Fas, with wild-type mice. We found that systolic and diastolic blood pressures of lpr mice were significantly higher than those of wild-type mice, indicating that lpr mice are hypertensive (Figure 3A). To determine whether this elevation of blood pressure was related to diminished eNOS-derived nitric oxide production, we next examined the effect of L-NAME, an NOS inhibitor, on blood pressure in these mice. As shown...
in Figure 3B, chronic inhibition of NOS by l-NAME induced a progressive increase in blood pressure in wild-type mice but not in lpr mice.

**lpr Mice Have Impaired Endothelial Function**

To determine the mechanism underlying systemic hypertension in Fas-lacking mice, we next examined the vascular responsiveness to phenylephrine and acetylcholine in Fas-lacking mice compared with wild-type mice. As shown in Figure 4A, the responsiveness to vasoconstrictor phenylephrine was different between these two mice. In contrast, the endothelium-dependent relaxation induced by acetylcholine was significantly impaired in Fas-lacking mice compared with wild-type mice (Figure 4B). However, the endothelium-independent relaxation by a nitric oxide donor was not different between the groups (data not shown).

**Discussion**

In the present study, we have shown that Fas activation induces Akt-dependent upregulation of eNOS expression in ECs. Reduced expression of Fas in vivo results in a development of hypertension. In addition, administration of the NOS inhibitor l-NAME fails to induce a further increase in the levels of blood pressure in mutant lpr mice with impairment of endothelial function. These findings imply that Fas signaling may have an important role in the pathogenesis of hypertension.

Recent evidence has shown that Fas signaling can mediate apoptosis-unrelated biological effects. For example, Fas activation induces T cell proliferation and activation of the prohypertrophic transcription factor AP-1 in cardiomyocytes rather than inducing apoptosis. In the present study, we have demonstrated that Fas engagement with FasL induces upregulation of eNOS expression instead of apoptosis in ECs. This is a first demonstration that Fas signaling can mediate an apoptosis-unrelated effect on ECs. Importantly, this effect is dependent on the survival signal Akt. In the presence of wortmannin, FasL did not induce upregulation of eNOS expression, but apoptosis in ECs and Fas signaling induced activation of Akt in ECs. These results are consistent with the recent report that Fas receptor signaling inhibits glycogen synthase kinase 3β via phosphorylation of the upstream kinase Akt in cardiomyocytes. Notably, the PI3K/Akt signaling pathway is important in maintaining the expression of FLIP, an inhibitor of Fas-mediated death signaling. In addition, this signaling pathway can mediate activation of eNOS, which induces upregulation of NO release from ECs. Nitric oxide then activates the Akt signaling in ECs. Thus, the level of Akt activity appears to be critical to determine the directions of Fas signaling in terms of apoptosis-unrelated effects in ECs.

FasL, a member of TNF family, is shed by a metalloprotease from the membrane to become sFasL as well as TNF-α. We previously reported that FasL expression of circulating mononuclear cells is upregulated in patients with acute myocardial infarction, and that hypoxia stimulates the release of sFasL from these cells. The levels of sFasL released from ECs are also upregulated under hypoxia. In addition, sFasL released from peripheral blood mononuclear cells can induce apoptosis in keratinocytes, indicating that functional sFasL may be released from these FasL expressing cells, including ECs themselves. Although the levels of sFasL concentration in the circulation are lower compared with those in our studies, we assume that sFasL would mainly act locally on ECs as a paracrine factor, where its concentrations seem to be much higher compared with those in the circulation. Previously, it is believed that sFasL can bind to Fas but cannot activate its downstream signaling events. This activity, however, could be restored if two sFasL are physically linked at the contact site with the target cell. Consistently, it has been recently shown that sFasL is able to induce cardiomyocyte hypertrophy but not apoptosis through inactivation of glycogen synthase 3β. In addition, we found that sFasL induces Akt activation in cultured ECs and that Fas engagement with the agonistic antibody Jo2 also induced Akt activation in aortic segments (Figure 2C), suggesting that Fas engagement with the membrane-bound form of FasL and sFasL can induce Akt activation in ECs.

Consistent with a possible role for Fas signaling to mediate eNOS expression in ECs in vivo, lpr mice, which express decreased cell surface Fas, had impairment of endothelium-
dependent vascular relaxation and slightly higher systolic blood pressure compared with wild-type mice. In addition, chronic inhibition of NOS with L-NAME failed to exhibit further increases in blood pressure in lpr mice, whereas systolic blood pressure progressively increased in the L-NAME-treated wild-type mice, suggesting that diminished nitric oxide synthesis is partially responsible for the elevated blood pressure in lpr mice. Thus, Fas signaling might be important in the maintenance of endothelial function and the regulation of blood pressure in the cardiovascular system. Consistently, it has been shown that hypertension develops in eNOS-deficient mice. However, recently reported that chronic inhibition of NOS by L-NAME induces perivascular fibrosis and upregulation of local angiotensin-converting enzyme expression in coronary microvessels with decreased blood pressure in eNOS-deficient mice, and suggested that chronic inhibition of NOS by L-NAME are not mediated by simple inhibition of eNOS. In this regard, it is notable that Fas receptor signaling is suggested to be a novel signal necessary for cardiac hypertrophy in response to the biomechanical stress of pressure overload and angiotensin II. In addition, it has been shown that chronic NOS inhibition induces cardiac hypertrophy via increased local angiotensin-converting enzyme expression, suggesting that the failure of cardiac hypertrophy might also be involved in the mechanism underlying unresponsiveness of lpr mice to induce hypertrophy after L-NAME administration. However, it is possible that some unknown compensatory mechanisms could be additionally involved in these mechanisms. Thus, further studies are necessary to clarify the exact mechanism by which long-term NOS inhibition does not induce a further increase in the levels of blood pressure in lpr mice.

In conclusion, we demonstrated for the first time to our knowledge that Fas signaling may mediate upregulation of endothelial eNOS expression in the Akt signal-dependent manner and that loss of function of the Fas mutation may cause hypertension via impairment of endothelium-dependent nitric oxide generation.

Perspectives
Fas signaling regulates endothelium-derived nitric oxide generation in the survival signal Akt-dependent manner. Thus, Fas could be a new therapeutic target for nitric oxide-related pathological conditions such as hypertension, atherosclerosis, and angiogenesis.

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References
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