Induction of Plasminogen Activator Inhibitor Type 1 and Type 1 Collagen Expression in Rat Cardiac Microvascular Endothelial Cells by Interleukin-1 and Its Dependence on Oxygen-Centered Free Radicals

Hitoshi Okada, MD; Janet Woodcock-Mitchell, PhD; John Mitchell, PhD; Tomohiro Sakamoto, MD; Kousuke Marutsuka, MD; Burton E. Sobel, MD; Satoshi Fujii, MD, PhD

Background—Ischemia with or without reperfusion induces the release of diverse products from monocytes, including cytokines such as interleukin-1 (IL-1). To determine whether these phenomena modulate fibrinolysis and potentially exacerbate impairment of the macrocirculation, microcirculation, or both, we characterized the effects of IL-1 on the expression of fibrinolytic system and matrix proteins in rat cardiac microvascular endothelial cells (CMECs).

Methods and Results—Confluent CMECs were exposed to IL-1 in serum-free medium for 24 hours, and cell-conditioned medium was assayed for plasminogen activator inhibitor type 1 (PAI-1), the primary physiological inhibitor of plasminogen activators, and for type 1 collagen with Western blotting. IL-1 (2 ng/mL) specifically increased the accumulation of PAI-1 (4.4±0.6-fold; mean±SD; n=9) without affecting tissue plasminogen activator (t-PA) or urokinase plasminogen activator (u-PA) levels, which remained unchanged. IL-1 increased the accumulation of collagen in conditioned media by 3.5±0.7-fold (n=6). Conversely, the accumulation of both PAI-1 and collagen induced by IL-1 was inhibited with an IL-1 receptor antagonist (200 ng/mL; n=6) and with cycloheximide (10 μg/mL; n=6), implying that protein synthesis was a requirement for the effect. To determine whether the IL-1 effect was mediated by induction of oxygen-centered free radical production, known to be induced by IL-1, we exposed the cells to the hydroxyl radical scavenger tetramethylthiourea (10 mmol/L) and observed abolition of the IL-1–induced increase in the expression of PAI-1 and collagen (n=6). Conversely, superoxides (generated with 10 mU/mL xanthine oxidase plus 0.6 mmol/L hypoxanthine, and 100 μmol/L hydrogen peroxide) induced the accumulation of PAI-1 and collagen (n=6). IL-1 (1 μg/kg body wt) and lipopolysaccharide (50 μg/kg body wt) administered in vivo increased PAI-1 protein in rat hearts as detected with Western blotting and PAI-1 immunostaining of rat heart microvessels, indicating the effects delineated in vitro were paralleled by effects in vivo.

Conclusions—These results indicate that IL-1–induced oxygen-centered free radicals stimulate elaboration of PAI-1 and collagen by CMECs. Accordingly, microvascularly mediated inhibition of fibrinolysis may predispose to the persistence of microvascular thrombi, thereby contributing to impaired microcirculatory function, the no-reflow phenomenon, and cardiac dysfunction after ischemia and reperfusion. (Circulation. 1998;97:2175-2182.)

Key Words: endothelium ▪ coronary disease ▪ interleukins ▪ plasminogen activators

Modulation of activity of the fibrinolytic system has been implicated in the pathogenesis of thromboembolic phenomena associated with inflammation and in the pathogenesis of vasculopathy. Activity of this system is regulated highly at transcriptional, translational, and post-translational levels and is influenced by specific growth factors and cytokines. Because the secretion of specific cytokines associated with ischemia may play a role in coronary endothelial injury, activation of cellular elements on the vessel wall, and the progression of atherosclerosis, this study was performed to determine whether IL-1, a prototypic multifunctional cytokine elaborated by immune system cells, modulates expression of proteins participating in the intramural (proteo)-fibrinolytic system.

Endothelial cells represent a major site of elaboration of fibrinolytic system proteins that affect diverse cell types in concert with other cytokines and small molecules, including t-PA and u-PA and their primary physiological inhibitor, PAI-1, a 50-kD glycoprotein serine protease inhibitor. IL-1 modulates the expression of u-PA, PAI-1, PAI-2, and colla-

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From the Departments of Medicine (H.O., T.S., K.M., B.E.S., S.F.) and Molecular Physiology and Biophysics (J.W.-M., J.M.), The University of Vermont, College of Medicine (Burlington).
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Correspondence to Satoshi Fujii, MD, PhD, The University of Vermont, College of Medicine, B-227 Given Building, Burlington, VT 05405.
E-mail sfujii@zoo.uvm.edu
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gen in endothelial and smooth muscle cells,7–10 but the effects of IL-1 on synthesis of fibrinolytic system proteins in CMECs have not been elucidated. Because cytokines may predispose to thromboembolic phenomenon and vasculopathy associated with inflammation, we characterized the influence of IL-1 on elaboration of fibrinolytic system components in vitro in cultured rat CMECs and in vivo in rat hearts.

Methods

Materials

CMECs were obtained as described previously.11 Cell type was identified with the use of biochemical and immunofluorescence techniques as described previously.12 Penicillin-streptomycin solution, Dulbecco’s modified Eagle’s medium, Medium 199, and trypsin were purchased from Sigma Chemical Co. Fetal bovine serum (FBS) was obtained from Hyclone. NuSerum was obtained from Collaborative Research. Six-well culture plates were fromBD Bioscience. Fetal bovine serum (FBS) was obtained from Hyclone. NuSerum was obtained from Collaborative Research. Six-well culture plates were from BD Bioscience. Fetal bovine serum (FBS) was obtained from Hyclone. NuSerum was obtained from Collaborative Research. Six-well culture plates were from BD Bioscience. Fetal bovine serum (FBS) was obtained from Hyclone. NuSerum was obtained from Collaborative Research. Six-well culture plates were from BD Bioscience. Fetal bovine serum (FBS) was obtained from Hyclone.

Cell Culture Procedures

CMECs between the second and third passages were grown to confluence on six-well plates or in T-25 flasks with Dulbecco’s modified Eagle’s medium containing 20% FBS and 50 U/mL penicillin and 50 µg/mL streptomycin. Cells were then incubated in Dulbecco’s modified Eagle’s medium (without serum) supplemented with 50 µL/mL penicillin and 50 µg/mL streptomycin overnight before and throughout experiments in which stimulation by cytokines was assessed. Confluent CMECs incubated in six-well plates were exposed to fresh medium containing IL-1 (0.02 to 20 ng/mL) for 24 hours. Media were collected again and stored at 70°C. HUVECs were cultured as previously described.13 Confluent HUVECs in six-well plates were incubated in Medium 199 containing 2.5% NuSerum, 50 µM/mL penicillin and 50 µg/mL streptomycin overnight before and throughout experiments in which stimulation by cytokines was assessed.

Procedures in Animals

All animal procedures conformed to the “Position of the American Heart Association on Research Animal Use” of November 11, 1984, and were approved by the Institutional Animal Care and Use Committee of The University of Vermont. Recombinant human IL-1 (1 µg/kg body wt) or LPS (Escherichia coli serotype 0111:B4, 50 µg/kg; Sigma Chemical) was diluted in 500 µL of endotoxin free PBS containing BSA and injected intraperitoneally into adult male rats (Charles River) weighing 150 to 200 g. Control rats were administered equivalent amounts of saline (vehicle) alone. After 24 hours, the rats were anesthetized, the chest cavity was exposed, and the heart was perfused immediately with PBS in situ and removed rapidly. For Western blotting, tissues were minced and quick-frozen in liquid nitrogen and homogenized in 10 mmol/L sodium phosphate buffer (pH 7.0) containing 2% SDS and 2 mmol/L phenylmethylsulfonyl fluoride. Samples were heated at 95°C for 10 minutes and centrifuged at 12 000g for 5 minutes before loading on gels. For immunohistochemical analysis, tissues were fixed in ethanol for a minimum of 24 hours.

Assays for PAI-1 Activity, Antigen, u-PA Antigen, and Total Protein

PAI-1 activity in CMEC-conditioned media, PAI-1, and u-PA antigens in HUVEC-conditioned media were determined by specific ELISAs as described previously.13 Total protein in the conditioned media were assayed according to the Pierce protocol.

Assay for PAI-1, t-PA, u-PA, and Collagen

PAI-1, t-PA, u-PA, and collagen were assayed with Western blotting14,15 with the use of antibodies specific for the respective antigens. Western blotting was performed according to the protocols of Clontech Laboratories and measured according to the method of Huang and Amero.16 Briefly, equivalent amounts of protein from rat heart extracts (5 µg) and conditioned medium were diluted 1:1 with sample buffer (0.25 mol/L Tris · HCl, pH 6.8, 40% glycerol, 4% SDS), 2% β-mercaptoethanol, and 0.01% bromophenol blue; heated at 100°C for 5 minutes; cooled; and loaded onto a 10% polyacrylamide gel. Proteins were electrophoresed for 60 minutes at 100 V and transferred to polyvinilidene difluoride membranes, which were then blocked with 1% BSA and 0.1% Tween-20 in PBS, pH 7.4. The transfer of protein to the membrane was checked by the transfer of prestained molecular mass marker (BioRad). Membranes were washed with 0.5% BSA and 0.1% Tween-20 in PBS several times and incubated with 1% BSA and 0.1% Tween-20 in PBS with 0.02% sodium azide, containing 1 µg/mL rabbit anti-rat PAI-1 IgG for PAI-1 Western blotting, 2 µg/mL rabbit anti-mouse t-PA IgG and rabbit anti-rat u-PA IgG for t-PA and u-PA Western blotting, and 1 µg/mL mouse anti-human type 1 collagen IgM for collagen Western blotting. Membranes were washed with 0.5% BSA and 0.1% Tween-20 in PBS several times and incubated with 1% BSA and 0.1% Tween-20 in PBS with 0.02% sodium azide in which alkaline phosphatase-conjugated goat anti-rabbit IgG or anti-mouse IgM was diluted 1:5000. Membranes were washed with chemiluminescent enhancer and then with 0.25 mmol/L chemiluminescent substrate and exposed to X-ray film, which was then developed. Bands were quantified with the use of a densitometer (model GS-700; BioRad).

Immunohistochemistry

The ethanol-fixed rat heart tissues were embedded in paraffin blocks and sectioned at 5-µm thicknesses. Sections mounted on slides were deparaffinized with two washes in xylene and hydrated sequentially through 100%, 95%, 75%, and 50% ethanol and PBS. Slides were exposed to 3% hydrogen peroxide in methanol (methanol/3% H2O2, 3:1) to quench endogenous peroxidase, rinsed, and blocked against nonspecific binding with 3% BSA in PBS followed by 10% normal goat serum for 30 minutes each. Subsequently, sections were incubated with primary antibody (rabbit anti-rat PAI-1; 10 µg/mL) or normal rabbit IgG in a humidified chamber at 37°C for 30 minutes. After the slides were washed three times with PBS, secondary antibody from the DAKO Envision kit for either mouse or rabbit primary antibodies was applied to the sections. Slides were incubated for 30 minutes at 37°C, washed with 0.05 mol/L Tris, pH 7.6, and treated with DAB chromogen. Anti–smooth muscle α-actin antibody was used for identification of smooth muscle cells, and anti–factor VIII–related antigen was used for identification of endothelial cells.
Statistical Analysis
Data are mean±SD. Differences were assessed with ANOVA with Bonferroni’s least significant posthoc tests for comparisons within multiple groups. Significance was defined as a value of \( P<0.05 \).

Results

Effects of IL-1 on CMECs
Proinflammatory cytokine IL-1 increased CMEC PAI-1 activity in the conditioned media (Figure 1A) in a concentration-dependent fashion (1.6±0.5 AU/mL at baseline, 5.1±5.0 AU at 0.002 ng/mL, 15.7±3.4 AU at 0.02 ng/mL, 15.3±3.9 AU at 0.2 ng/mL, 17.9±1.2 AU at 2 ng/mL, and 10.1±0.7 AU at 20 ng/mL; \( n=4 \)). Peak effects were seen with 2 ng/mL, and the response was diminished somewhat with concentrations of 20 ng/mL. IL-1 increased PAI-1 protein accumulation in a concentration-dependent fashion (Figure 1B). Peak effects were seen at 2 ng/mL. Increased accumulation secondary to IL-1 (2 ng/mL) was evident by 6 hours (1.6±0.4-fold over control; \( n=6 \)) and at 15 hours (2.0±0.3-fold over control; \( n=6 \)), with further increases at 24 hours (4.4±0.6-fold over control; \( n=9 \)). Figure 1C is a representative Western blot showing the effect of IL-1 on PAI-1 in conditioned medium from CMECs. IL-1 did not augment accumulation of t-PA or u-PA (results not shown); thus, the effect of IL-1 was to increase net PAI-1 activity and accumulation. In addition, IL-1 increased accumulation of collagen elaborated by CMECs in a concentration-dependent manner with effects peaking at 2 ng/mL (3.5±0.7-fold over control at 24 hours; \( n=6 \)) (Figure 2A). The response was diminished somewhat at higher concentrations of IL-1. Total protein content in the conditioned media was not altered by IL-1 (results not shown). Figure 2B shows a representative Western blot in which the effect was examined of selected concentrations of IL-1 on collagen in conditioned medium. In HUVECs, IL-1 increased PAI-1 antigen moderately (384±56 ng \( \times 10^5 \) cells\(^{-1}\) \( \times 24\) h\(^{-1}\) at baseline, 601±71 ng \( \times 10^5 \) cells\(^{-1}\) \( \times 24\) h\(^{-1}\) at 2 ng/mL; \( n=3 \)) and u-PA markedly (<0.2 ng \( \times 10^5 \) cells\(^{-1}\) \( \times 24\) h\(^{-1}\) at baseline, 2.1±0.5 ng \( \times 10^5 \) cells\(^{-1}\) \( \times 24\) h\(^{-1}\) at 2 ng/mL; \( n=3 \)).

Figure 1. A, Effects of IL-1 on activity of PAI-1 in conditioned medium of CMECs. Confluent cells were serum starved overnight and then incubated with fresh serum-free medium containing IL-1 (0 to 20 ng/mL) for 24 hours. Conditioned medium was harvested and the activity of PAI-1 was assayed as described in “Methods.” Values are mean±SD (\( n=4 \)). #\( P<0.05 \), *\( P<0.01 \) compared with values in untreated control cells. B, Effects of IL-1 on the concentration of PAI-1 in conditioned media of CMECs. Confluent cells were serum starved overnight and then incubated with fresh serum-free medium containing IL-1 (0 to 20 ng/mL) for 24 hours. Conditioned medium was harvested, and concentrations of PAI-1 were assayed with Western blotting as described in “Methods.” Values are mean±SD (\( n=9 \)) of fold increase over control without IL-1. *\( P<0.01 \) compared with untreated, control cells. C, Western blots showing effects of IL-1 on concentrations of PAI-1 in conditioned media of CMECs. Confluent cells were serum starved overnight and then incubated with fresh serum-free medium containing IL-1 (lane 1, 0; lane 2, 0.02; lane 3, 0.2; lane 4, 2; lane 5, 5; and lane 6, 20 ng/mL) for 24 hours. Conditioned medium was harvested and concentrations of PAI-1 were assayed with Western blotting. Representative results are shown from one of six separate experiments. The molecular weight range (markers) is shown on the far left.
IL-1ra significantly diminished but did not totally abolish the accumulation of PAI-1 and collagen from CMECs exposed to IL-1 (Figure 3). At a concentration of 200 ng/mL, it suppressed the increase in PAI-1 accumulation induced by IL-1 (2 ng/mL; n=6) and suppressed the increase in collagen induced by IL-1 (n=6). Basal accumulations of PAI-1 and collagen were not affected by IL-1ra (results not shown). Cycloheximide (10 μg/mL) inhibited IL-1–induced PAI-1 accumulation by 96±10% and collagen accumulation by 84±9% (n=6).

Effects of Reactive Oxygen Species
The hydroxyl radical scavenger TMTU (10 mmol/L) almost completely inhibited the accumulation of PAI-1 protein in response to IL-1 by CMECs at 24 hours (Figure 4A) (n=6). Equimolar urea, used as a control, had no effect. In contrast to TMTU, DMSO inhibited IL-1–induced PAI-1 accumulation only partially. When the cells were incubated with hydrogen peroxide (100 μmol/L) or a superoxide-generating system (10 μl/mL xanthine oxidase plus 0.6 mmol/L hypoxanthine), PAI-1 accumulation increased (Figure 4B) (n=6). The extent to which reactive oxygen species induced PAI-1 was similar to the extent to which IL-1 induced PAI-1 (Figure 4B). TMTU inhibited accumulation of collagen in IL-1–stimulated CMEC (Figure 4C) (n=6). Equimolar urea had no effect. DMSO partially inhibited IL-1–induced accumulation of collagen. Hydrogen peroxide or xanthine oxidase plus hypoxanthine increased the accumulation of collagen (Figure 4D). The extent to which reactive oxygen species induced collagen corresponded to the IL-1–induced increase (Figure 4D).

Effects of IL-1 and LPS on PAI-1 in Rat Hearts
In Vivo
IL-1 and LPS increased PAI-1 protein in rat hearts in vivo with maximum inductions of 2.7±0.3- and 3.4±0.7-fold at 24 hours compared with those in animals infused with saline as determined with Western blotting (n=6). Immunohistochemical analysis indicated a consistent, relatively strong signal for PAI-1 antigen in the endothelial layer within the small arteries of the hearts from rats injected with IL-1 (Figure 5). PAI-1 antigen also was evident in microvessels in...
the myocardium. Endothelial cells of large epicardial vessels showed only a weak signal after stimulation with IL-1. This general pattern of PAI-1 antigen expression was relatively uniform throughout the vasculature in all animals studied. A similar pattern of PAI-1 antigen distribution was observed in rat heart tissues of animals injected with LPS. In contrast, the arteries from hearts of animals injected with saline showed little or no staining (results not shown). No immunohistochemical staining was apparent as judged from control animals with normal rabbit IgG.

**Discussion**

In the present study, IL-1 produced by immune system cells was shown to influence the production of (proteo)fibrinolytic system proteins in CMECs. Furthermore, IL-1 increased matrix protein type 1 collagen. Increased PAI-1 and collagen
induced by IL-1 was attenuated by cycloheximide, suggesting that PAI-1 and collagen expression induced by IL-1 requires new protein synthesis. IL-1ra at a concentration of 200 ng/mL diminished accumulation of PAI-1 and collagen from CMECs exposed to IL-1 (2 ng/mL). This verified, at least in part, the specificity of the IL-1 response. Thus, the effects appear to have been mediated through the IL-1 receptor. Because basal accumulations of PAI-1 and collagen were not affected by the IL-1ra, constitutive synthesis appears to be independent of IL-1. The effect of IL-1 on CMEC PAI-1 production is in marked contrast to those on endothelial cells from umbilical vein, in which IL-1 stimulated expression of u-PA, whereas the effect on PAI-1 was modest as reported previously.7 Because little is known about molecular differences between large and small vessel endothelium, the present observation contributes to a better understanding of functional differences between microvascular and macrovascular endothelium.

Factors participating in signal transduction associated with IL-1 have not yet been thoroughly elucidated. Reactive oxygen species are commonly produced by inflammatory cells during the course of inflammatory processes.17 In glomerulonephritis, glomerular cells can generate reactive oxygen intermediates, independently of infiltrating cells, that may play an autacoid role in glomerular injury.15 IL-1 can induce the production of both superoxide and hydrogen peroxide.19

To determine whether reactive oxygen species were involved in the increased expression of PAI-1 induced by IL-1, the effects were compared of radical scavengers TMTU and other antioxidants. The hydroxyl radical scavenger TMTU and, to a lesser extent, DMSO inhibited IL-1–induced PAI-1 expression. To determine whether reactive oxygen species could directly induce PAI-1 expression in CMECs, cells were incubated with either hydrogen peroxide or a superoxide-generating system. Both increased PAI-1 accumulation.

In many cell types, NADPH oxidase is involved in the generation of reactive oxygen species.20 Endothelial cells contain membrane-bound oxidase or oxidases that use NADH and NADPH as substrates for electron transfer to molecular oxygen and can produce reactive oxygen species.21,22 Our results show that NADPH oxidase–generated reactive oxygen may mediate production of PAI-1 by CMECs. Because IL-1 and reactive oxygen species had similar effects on the accumulation of collagen from CMECs, a common mechanism may be operating in the signal transduction pathways.
leading to induction of collagen synthesis by IL-1 in CMECs. Oxidative stress is suggested to play a role in the development of perivascular fibrosis, and fibrolitic responses may modulate the interstitial and perivascular fibrinosis of intramyocardial coronary arteries. Type 1 collagen, the predominant matrix protein deposited in myocardial disease in humans, may contribute. Furthermore, impairment of the microcirculation may induce myocytolytic necrosis and reperfusion injury. Because oxidant stress and cytokines appear to be involved in myocardial ischemia, reperfusion injury, and thrombosis, our results are consistent with the possibility that IL-1 influences cardiac remodeling of the extracellular matrix by inducing collagen synthesis and perivascular fibrinosis and that it may alter coronary microcirculation dynamics by altering microvascular fibrinolysis associated with activation of immunocompetent cells.

In the present study, CMECs were used to determine whether altered elaboration of specific fibrinolytic system proteins is likely to occur in cardiac microvasculature under the conditions of ischemia. CMECs have distinctive characteristics compared with endothelial cells isolated from large vessels. When we included IL-1 in the medium, the concentrations of matrix protein collagen increased, suggesting CMECs are capable of producing extracellular matrix proteins.

IL-1 is a prototypic multifunctional cytokine. Unlike lymphocyte and colony-stimulating growth factors, IL-1 affects diverse cell types, often in concert with other cytokines or small mediator molecules. Both IL-1 and LPS, a potent inducer of cytokines elaborated by immune system cells, administered in vivo increased PAI-1 in rat heart as determined from analysis of Western blots. PAI-1 immunostaining was evident in microvessels, showing that PAI-1 synthesis in coronary microvessels can occur in vivo and potentially contribute to high concentrations of PAI-1 in the coronary circulation.

Activation of blood coagulation and inadequate activation of endogenous fibrinolysis may contribute to a no-reflow phenomenon by inducing fibrin deposition and formation of microthrombi. Thus, the recently reported poor prognosis associated with Thrombolysis in Myocardial Infarction Trial 2 flow compared with Thrombolysis in Myocardial Infarction Trial 3 flow may be a reflection in part of microvascular damage and is therefore a predictor of a poor outcome in patients with myocardial infarction. The findings reported here suggest a potential efficacy for antioxidants in ameliorating microvascular fibrinolytic system dysfunction related to inflammatory disease or ischemia and reperfusion injury. Antioxidants may not only inhibit direct effects of cytokines but also suppress elaboration of PAI-1 and collagen and their potentially adverse consequences.

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References


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