Diabetes Mellitus Enhances Vascular Matrix Metalloproteinase Activity
Role of Oxidative Stress

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Abstract—Diabetes mellitus (DM) is a primary risk factor for cardiovascular disease. Although recent studies have demonstrated an important role for extracellular matrix metalloproteinases (MMPs) in atherosclerosis, little is known about the effects of hyperglycemia on MMP regulation in vascular cells. Gelatin zymography and Western blot analysis revealed that the activity and expression of 92-kDa (MMP-9) gelatinase, but not of 72 kDa (MMP-2) gelatinase, were significantly increased in vascular tissue and plasma of two distinct rodent models of DM. Bovine aortic endothelial cells (BAECs) grown in culture did not express MMP-9 constitutively; however, chronic (2-week) incubation with high glucose medium induced MMP-9 promoter activity, mRNA and protein expression, and gelatinase activity in BAECs. On the other hand, high glucose culture did not change MMP-9 activity from vascular smooth muscle cells or macrophages. Electron paramagnetic resonance studies indicate that BAECs chronically grown in high glucose conditions produce 70% more ROS than do control cells. Enhanced MMP-9 activity was significantly reduced by treatment with the antioxidants polyethylene glycol–superoxide dismutase and N-acetyl-L-cysteine but not by inhibitors of protein kinase C. In conclusion, vascular MMP-9 activity is increased in DM, in part because of enhanced elaboration from vascular endothelial cells, and oxidative stress plays an important role. This novel mechanism of redox-sensitive MMP-9 expression by hyperglycemia may provide a rationale for antioxidant therapy to modulate diabetic vascular complications. (Circ Res. 2001;88:1291-1298.)

Key Words: endothelium • atherosclerosis • gelatinase • oxidative stress • remodeling

Cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes mellitus (DM). Because the onset and progression of complications are delayed in patients with good glycemic control, hyperglycemia is thought to be an important regulator of vascular lesion development. Recent studies indicate that elevated glucose concentrations can induce dysfunction of several intracellular signal transduction cascades, including modulation of protein kinase C (PKC), activation of mitogen-activated protein kinase, generation of reactive oxygen species (ROS), and accumulation of advanced glycation end products (AGEs). However, the underlying mechanisms between hyperglycemia and vascular disease remain unclear.

Matrix metalloproteinases (MMPs) are members of a family of Zn$^{2+}$- and Ca$^{2+}$-dependent endopeptidases, which are essential for cellular migration and tissue remodeling in both physiological and pathological conditions. MMPs are secreted by many types of cells as proenzymes. On activation by proteolytic cleavage, activated enzymes are capable of degrading many extracellular matrix components. Because MMPs appear to be involved in monocyte invasion and vascular smooth muscle cell migration, derangement of MMP regulation is considered to be a critical factor in the development of vascular lesions. In situ zymography and immunohistochemical studies have demonstrated that MMPs, especially MMP-2 (72-kDa gelatinase A) and MMP-9 (92-kDa gelatinase B), are actively synthesized in atheromatous plaque and are particularly prevalent in rupture-prone shoulder regions. Both MMP-2 and MMP-9 are activated by ROS, and their expression seems to be regulated by oxidant stress. Furthermore, MMP activity has been correlated with clinical manifestations of unstable angina, plaque rupture, and the development of abdominal aortic aneurysms. Because the prevalence of acute coronary syndromes is significantly greater in diabetic patients than in nondiabetic subjects, we hypothesized that MMPs may be preferentially activated in the setting of diabetes. We first studied the gelatinolytic activity of vascular tissue and plasma in two established rodent models of DM. In addition, we investigated the possible cellular origins of enhanced gelatinolytic activity in vascular cells exposed to high glucose conditions. Our findings indicate that the activity of MMP-9, but not MMP-2,
is preferentially enhanced in vascular endothelial cells by hyperglycemia. This effect is partly due to increased transcrip-
tion of MMP-9 via a redox-sensitive mechanism.

**Materials and Methods**

**Reagents**

DMEM, FBS, insulin-transferrin supplement, Trizol, and antibiotics for cell culture experiments were obtained from Life Technologies Inc (GIBCO-BRL). Unless otherwise stated, all other chemicals were purchased from Sigma Chemical Co. Antibodies against human MMP-9 were purchased from Oncogene Research Products, and peroxidase-labeled goat anti-mouse IgG was from Kirkegaard and Perry Laboratories.

**Animal Models of DM**

Male Sprague-Dawley rats (Harlan Sprague Dawley Inc, Indianapolis, Ind), 8 weeks of age and weighing ~170 g, were used for all studies. Animals were housed in a room with a 12-hour light/12-hour dark cycle and an ambient temperature of 22°C. Each rat was assigned to one of the following four groups: normal chow (controls to type 1 DM [control 1]), normal chow and streptozotocin (STZ 55 mg/kg) (type 1 DM), high fat diet (60% fat, Harlan Teklad) (control to type 2 DM [control 2]), or high fat and STZ (35 mg/kg) (type 2 DM).13,14 STZ was injected via the tail vein. At the end of the fourth week, rats were euthanized after a 6-hour fast, blood was collected, and the plasma levels of glucose and insulin were measured. We have previously shown that high dose (55 mg/kg) STZ induces an insulinopenic hyperglycemic state, whereas the low dose (35 mg/kg) results in hyperglycemia with insulin resistance (as measured by the insulin suppression test) and modest decreases in insulin levels after high fat challenge. These protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University and were performed in accordance with the recommendation of the American Association for the Accreditation of Laboratory Animal Care.

**Cell Culture**

Bovine aortic endothelial cells (BAECs), rat vascular smooth muscle cells, and a mouse macrophage line (RAW264) were used. For experiments, cells were grown in control (low glucose) DMEM (LG, 5.5 mmol/L glucose), mannosone-added DMEM (MN, 5.5 mmol/L glucose and 20 mmol/L D-mannose), or high glucose DMEM (HG, 25.5 mmol/L glucose). After 24-hour (acute) or 14-day (chronic) exposure to each medium, cells were washed extensively to remove serum and cultured in each medium (LG, HG, or MN) supplemented with insulin and transferrin for the final 24 hours. Phorbol 12-myristate 13-acetate (PMA, 10 nmol/L) was used as a positive control for MMP-9 induction as previously described.15 Inhibition studies were performed with BAECs chronically cultured in HG media in which antioxidants or PKC inhibitors were added for the last 24 hours. Experiments were performed by using cultured cells at passages 8 to 15.

**Gelatin Zymography**

Gelatinolytic activities of aortic tissue homogenates, rat plasma, and conditioned media of cultured cells were analyzed by electrophoresis in the presence of 6.0% SDS-polyacrylamide gels containing 1 mg/mL gelatin.16 Samples were applied to the gel in a sample buffer containing 2.5% SDS but lacking β-mercaptoethanol. After electrophoresis, the gels were incubated overnight at 37°C in 50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L CaCl₂, and 0.05% Brij solution. Subsequently, gels were stained with Coomassie brilliant blue R-250, and the zone of enzyme activity was quantified by using NIH Image 1.62.

**Western Blotting**

MMP-9 protein in rat tissue homogenates and conditioned cell culture medium were analyzed by Western blotting with use of a monoclonal antibody raised against human MMP-9 as previously described.17

**Northern Blotting**

Total RNA was isolated from confluent BAECs grown in the conditions described above. Aliquots of 20 μg RNA were denatured and electrophoresed on 1% agarose gels containing 3.4% formaldehyde. Membranes containing transferred RNA were subsequently probed for bovine MMP-9 and cyclophilin as previously described.18 A 308-bp cDNA fragment of the bovine MMP-9 gene was produced by reverse transcription–polymerase chain reaction (PCR) of PMA-stimulated BAECs by use of the following oligonucleotide sequences: forward 5'-GGGATTAGGAAACCGCTTGCA-3' and reverse 5'-TGAAACAGCACCCCTTACCTT-3'.

**Measurement of ROS Accumulation**

ROS accumulation was measured by using conditioned medium supplemented with a spin-trapping agent, 4-amino-2,2,6,6-tetramethylpiperidino-1-oxyl (Tempamine [TA]). Electron paramagnetic resonance (EPR) spectra were obtained by using a Bruker EPS 300 or a Bruker EMX spectrometer. Quantification of the EPR signal intensity was determined by comparing the double integration of the recorded first-derivative EPR peak of each sample with a standard TA spin solution.

**Luciferase Promoter Assay**

A 1808-bp DNA fragment of the human MMP-9 promoter (−1879 to −12 from the transcription start site) was isolated by PCR by using a modification of a previously described method.19 BglII and Mlu sites were added in the oligonucleotide primers (forward 5-AATCCAGGACCTCGTA and reverse 5-CAAAACCCCCGAAATTCCTC, respectively), and the resultant PCR fragment was subcloned into the pGEM-T Easy Vector (Promega). After verification of the sequence, the promoter fragment was then subcloned upstream from the luciferase gene in the pGL-basic vector (Promega).

For transient transfections, BAECs grown in HG or LG conditions were seeded at 50% to 70% confluence and transfected by using LipofectAMINE (Life Technologies, Inc) for 6 hours. MMP-9 promoter–luciferase constructs were cotransfected with a constitutive β-galactosidase reporter plasmid driven by the cytomegalovirus promoter at a ratio of 2:1 (LipofectAMINE:DNA). Luciferase and β-galactosidase activity were then measured by a Dual-Light Kit (Tropix) according to the manufacturer’s instructions. All results are reported as luciferase activity (relative light units) normalized to cotransfected β-galactosidase activity.

**Results**

**Biochemical Parameters of Diabetic Rats**

In models of both type 1 and type 2 DM, blood glucose levels were significantly higher than those of control animals. Furthermore, blood glucose levels of type 1 rats were significantly higher than those of type 2 rats (Figure 1A) (control, 152±13 mg/dL; type 1 DM, 550±16 mg/dL; and type 2 DM, 458±22 mg/dL). Plasma insulin of type 1 and type 2 rats was significantly decreased, and there was a significant difference between type 1 and type 2 (Figure 1B) (control, 33.9±5.4 μU/mL; type 1 DM, 4.8±0.6 μU/mL; and type 2 DM, 14.5±2.6 μU/mL).
Gelatinolytic Activity of Aortic Tissue and Plasma in Rat Models of DM

Striking differences in vascular 92-kDa gelatinolytic activity were observed between diabetic rats and control rats (Figure 2A). Independent experiments using recombinant human MMP-9 and Western blotting with anti-MMP-9 antibody demonstrated that this increase in 92-kDa gelatinolytic activity of aortic homogenates was due to increases in MMP-9 protein expression. Densitometric analysis revealed that aortic homogenates from both type 1 and type 2 models of DM had significantly enhanced gelatinolytic activity compared with each control (3.4 ± 0.3-fold in type 1 DM and 2.3 ± 0.2-fold in type 2 DM) (Figure 2B). The effects appeared to be specific for the latent 92-kDa form of MMP-9, inasmuch as no measurable differences in gelatinolytic activity were observed in the “active” 83-kDa form. Similar results were also obtained when plasma was used as a substrate for gelatin zymography (Figures 2C and 2D). Contrary to 92-kDa gelatinase, activity of MMP-2 (either the latent 72-kDa or the 62-kDa active form) was not different between DM and control rats.

Chronic HG Culture Conditions Increase MMP-9 Activity of Endothelial Cells

To evaluate the possible origin of enhanced MMP-9 in diabetic vascular tissue and plasma, we assessed the acute and chronic effects of HG culture on the MMP-9 activity of endothelial cells, vascular smooth muscle cells, and macrophages. Under LG conditions, both endothelial cells and smooth muscle cells expressed 72-kDa gelatinase (MMP-2) constitutively but demonstrated relatively little 92-kDa gelatinase (MMP-9) activity. On the other hand, RAW264 macrophages constitutively express MMP-9 but not MMP-2 (data not shown). As previously reported,19 stimulation with PMA markedly enhanced MMP-9 activity in endothelial cells but not in vascular smooth muscle cells or RAW264 macrophages.

Acute (24-hour) incubation with HG medium had no effect on MMP-9 activity in BAECs (Figure 3A) or any of the cell types tested. On the other hand, chronic (2-week) incubation with HG medium dramatically increased MMP-9 gelatinolytic activity in conditioned medium of endothelial cells (Figure 3B) but not of vascular smooth muscle cells or macrophages. No changes in gelatinolytic activity were observed during acute or chronic culture with MN medium. Western blot analysis indicated that chronic culture of BAECs with HG resulted in greater elaboration of MMP-9 protein into the conditioned medium (Figure 3C). To confirm the identity of the 92-kDa bovine protein responsible for glucose-enhanced gelatinolytic activity, conditioned medium from BAECs chronically cultured with HG was incubated with serial dilution of anti-human MMP-9 antibody (IgG). Although it did not have blocking effects on MMP-9 activity, incubation with the antibody clearly shifted the gelatinolytic activity to a higher molecular weight (Figure 3D). This finding confirms the cross-reactivity of the human MMP-9 antibody to bovine MMP-9 and demonstrates that it recognizes a site separate from the enzymatic portion of the protein. In addition,
increases in MMP-9 protein and activity were associated with elevated mRNA levels in endothelial cells exposed to chronic HG conditions (Figure 4).

Role of ROS

To document the participation of oxidative stress in this setting, oxygen-derived free radical production from endothelial cells was analyzed by EPR with the use of TA. As noted in Figure 5A, HG culture resulted in a reduction of the TA radical signal, indicating a significant increase in ROS by endothelial cells. The results of six separate experiments indicate that endothelial cells chronically grown in HG conditions produce 70% more ROS than do control cells (LG, 24 ± 6 fmol/cell for 24 hours; HG, 41 ± 5 fmol/cell for 24 hours; \( P < 0.05 \)). As shown in Figure 5B, the increase in ROS production by HG conditions was significantly reduced by polyethylene glycol (PEG)–superoxide dismutase (SOD) treatment (HG+PEG-SOD, 28 ± 3 fmol/cell for 24 hours; \( P < 0.05 \))

**Effects of PKC Inhibition and Antioxidants on MMP-9 Activity**

Because PKC is thought to participate in the regulation of MMP-9 expression in several cell types and because hyperglycemia increases vascular PKC activity, we tested the effects of PKC inhibition on HG-stimulated MMP-9 activity in cultured endothelial cells. Contrary to our hypothesis, calphostin C did not reduce the MMP-9 activity of endothelial cells. Furthermore, the nonspecific PKC antagonist staurosporine produced a paradoxical increase of 92-kDa gelatinolytic activity (Figure 6A). To test whether oxygen-derived free radicals contribute to the regulation of MMP-9 expression, we treated endothelial cells with the antioxidants PEG-SOD or N-acetylcysteine (NAC). We found that coincubation with NAC for the final 24 hours of HG culture significantly reduced gelatinolytic activity in a dose-dependent fashion (Figures 6B and 6C); similar inhibitory effects were found with PEG-SOD. As expected, stimulation of endothelial cells with PMA in LG conditions potently induced MMP-9 activity. Interestingly, coincubation with NAC also dose-
and plasma from rat models of diabetes is significantly increased compared with that from control rats; (2) chronic culture in HG conditions enhances MMP-9 expression and activity in endothelial cells but not in vascular smooth muscle cells or macrophages; and (3) glucose-induced expression of MMP-9 in cultured endothelial cells is mediated by a ROS-sensitive pathway but not by the activation of PKC.

Although recent studies have demonstrated that hyperglycemia can modulate various intracellular signaling events, a possible link between these alterations and vascular disease remains unclear. Because accumulating evidence supports the role of enhanced MMP-9 activity in atherogenesis, we evaluated the vascular and plasma gelatinase activity in two distinct animal models of DM. Both models use STZ as a pancreatic islet toxin. However, the model of type 2 DM has significantly higher circulating levels of insulin, and the animals become hyperglycemic only when these levels are no longer able to compensate for the degree of insulin resistance produced by the high fat diet. In the present study, MMP-9 was equally induced in vascular tissue of both type 1 and 2 DM models but not in control groups fed either normal or high fat chow, implicating an essential role for hyperglycemia.

Elevated levels of circulating glucose in vivo may affect MMP activity in several different cell types. Thus, we next sought to determine the possible cellular origin of enhanced MMP-9 activity in vascular tissue. Although acute (24-hour) HG incubation in vitro did not alter gelatinolytic activity, chronic elevations in glucose concentrations resulted in enhanced MMP-9 activity in cultured endothelial cells but had little effect in vascular smooth muscle cells or macrophages. The effect was not due to the increased osmolality of HG conditions, because no change in MMP-9 was observed with equimolar concentrations of mannose. Taken together, our results indicate that the increased activity of MMP-9 in vascular tissue in vivo appears to be due to enhanced elaboration from the endothelium. The elevation in vascular 92-kDa gelatinase activity was mirrored by changes in the plasma of diabetic animals (both type 1 and type 2). Although endothelial production of MMP-9 could partially account for these findings, there are several other potential sources of metalloproteinases in peripheral blood, including platelets and leukocytes other than monocyte/macrophages. Therefore, with the increasing evidence for its role in atherogenesis, plasma MMP-9 levels may be a good index of the severity and stability of atherosclerotic plaques. Indeed, Kai et al. have demonstrated that plasma levels of MMP-9 are elevated 2- and 3-fold in patients on presentation with acute myocardial infarctions or unstable angina, respectively.

In addition to the implicated role in plaque rupture, the observed increase in MMP-9 activity may have important consequences for the development of vascular complications associated with diabetes. For instance, Ebihara et al. demonstrated that elevated plasma MMP-9 levels predicted eventual microalbuminuria in diabetic individuals. Recent studies have also established that MMP activity is required for angiogenesis. Therefore, enhanced...
MMP-9 activity may be critical for microvascular complications, such as retinal neovascularization associated with proliferative diabetic retinopathy. Moreover, angiogenesis and neovascularization is recognized as a necessary component for the continuous growth of atherosclerotic lesions. Increased metalloproteinase activity has also been shown to play an important role in vascular remodeling after angioplasty. Thus, elevated expression of MMP-9 observed in the present study may offer one explanation for the accelerated restenosis seen after angioplasty and stent deployment in diabetic patients. It is interesting to note that although vascular tissue and plasma were not investigated, similar changes in MMP-9, but not MMP-2 activity, were found in gingiva and skin extracts derived from a rodent model of diabetic periodontitis, further implicating a role for hyperglycemia and metalloproteinase activity in several diabetic complications.

Accumulating evidence indicates that oxidative stress may play an important role in the pathogenesis of atherosclerosis. Addition of the antioxidant NAC inhibits platelet-derived growth factor–induced smooth muscle proliferation. Furthermore, oxidative stress regulates the expression of several genes associated with atherogenesis, including vascular cell adhesion molecule-1, monocyte chemotactic protein-1, and monocyte-colony stimulating factor. In the present study, we demonstrate that treatment with the antioxidants PEG-SOD and NAC reduced MMP-9 activity of endothelial cells chronically incubated with high glucose, suggesting that oxidative stress is involved in MMP-9 induction by hyperglycemia.

Several biochemical pathways associated with hyperglycemia seem to increase the production of free radicals. For example, enhanced activity of the polyol pathway results in the oxidation of sorbitol to fructose coupled to the reduction of NADH. The increased ratio of NADH/NAD+ can support free radical production by several pathways, including increased activation of xanthine oxidase, auto-oxidation of NADH, and inactivation of CuZn-SOD. In addition, recent data from Hink et al indicate that endothelial NO synthase dysfunction, coupled with activation of an NAD(P)H-dependent oxidase, is largely responsible for enhanced superoxide production in vascular tissue derived from type 1 DM animals. Indeed, the finding that MMP-9 is activated only after chronic exposure of cells to HG conditions may depend on initial dysregulation of endothelial redox balance.

Oxidative stress associated with hyperglycemia may also be due to increased concentrations of AGEs. AGEs have been demonstrated to interact with specific receptors and induce oxidative stress, enhance vascular cell adhesion molecule-1 expression, and increase endothelial adhesiveness for monocytes. The relatively short time course used in both our in vitro and in vivo experiments has not been demonstrated to result in elevated concentrations of AGEs. However, because we have not directly measured AGEs in the present study, their role in the observed effects on MMP-9 activation cannot be excluded.

MMP regulation occurs at the level of gene transcription and on activation of pro-MMPs. Various stimuli, including growth factors, cytokines, chemical agents (phorbol esters), and mechanical stress, induce MMP gene expression. The MMP-9 promoter region contains nuclear factor-κB, activator protein-1, stimulatory protein-1, and phorbol ester-responsive elements. Previous findings indicating that nuclear factor-κB and activator protein-1 are redox sensitive offer a potential mechanism by which glucose-induced oxidative stress may regulate MMP-9 transcription and activity.

The proteolytic activities of MMPs are tightly controlled during activation from their proenzymes to active forms by the combination of endogenous activators (eg, membrane-type MMPs and urokinase plasminogen activators) and inhibitors (eg, α2-macroglobulins and tissue inhibitors of metalloproteinases). Another possible mechanism for the activation of MMP activity is posttranslational modification by ROS. Although activation of MMPs by ROS may occur in the setting of DM, we do not believe that this is the major mechanism for the present findings, inasmuch as alterations in the active forms of MMP-9 (83 kDa) and MMP-2 (62 kDa) were not observed in either our in vivo or in vitro models. However, the elevated vascular expression of MMP-9 in diabetes may set the stage for enhanced activation due to acute stimuli, such as inflammatory mediators or vascular injury.

A central role for PKC in the activation of MMP-9 has been assumed because PMA increases its expression in various cell types, including vascular endothelial cells. In addition, hyperglycemia activates various PKC isoforms in endothelial and vascular smooth muscle cells. Thus, our finding that glucose-induced MMP-9 activity was not altered by antagonists of PKC was quite surprising. Interestingly, the paradoxical effect of staurosporine on MMP-9 activity that we observed has previously been reported in human B lymphocytes. Recent data demonstrating the interaction of the ROS and PKC signaling pathways may offer some explanation. For instance, PMA has been demonstrated to activate specific NAD(P)H oxidase subunits and to regulate subsequent superoxide anion production. Moreover, free radical scavengers can reduce PMA- and cytokine-stimulated PKC activity in fibroblasts and vascular smooth muscle cells. Similarly, in the present study, PEG-SOD and NAC inhibited PMA- and glucose-induced MMP-9 activity and expression. Thus, the ability of PMA to induce MMP-9 may be due to its effects on free radical generation in vascular cells rather than simply its action on PKC.

In summary, we have demonstrated that MMP-9 expression and activity in endothelial cells are upregulated during hyperglycemia, indicating a novel mechanism by which hyperglycemia could adversely affect the development of atherosclerotic lesions. Furthermore, oxidative stress appears to play a primary role in glucose-induced MMP-9 activity, suggesting a possible beneficial effect of antioxidant therapy in the vascular complications of DM.

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References


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