ELEVATION OF PLASMA LOW-DENSITY LIPOPROTEIN (LDL) has been considered a strong risk factor for the development of coronary artery disease (40). Lipoprotein(a) [Lp(a)] is a structural homolog of LDL with a unique apolipoprotein (Apo), Apo(a). Results from epidemiological studies suggest that increased plasma Lp(a) is an independent lipoprotein risk factor for premature cardiovascular disease (2, 4). It has been widely accepted that oxidative modification increases atherogenicity of lipoproteins (33). Multiple lines of evidence suggest that oxidation also increases thrombogenicity of lipoproteins. For example, Cu²⁺ oxidation enhanced the effect of LDL on the expression of tissue factor in macrophages (39). Oxidized Lp(a) was stronger than its native form in terms of the inhibition on the binding of plasminogen to U937 monocytes (23). Those findings suggest that oxidative modification of LDL may superimpose disorders in the coagulation and fibrinolysis induced by these atherogenic lipoproteins.

Intravascular thrombosis is a consequence of an imbalance between coagulation and fibrinolysis. Vascular endothelial cells (EC) synthesize tissue plasminogen activator, urokinase plasminogen activator, and their major physiological inhibitor, plasminogen activator inhibitor-1 (PAI-1). The expression of PAI-1 was increased in thrombotic vascular wall (1). Elevated PAI-1 activity in plasma has been considered a risk factor for coronary artery disease (7). The production of PAI-1 from vascular EC was stimulated by thrombin (20), heparin, EC growth factor (17), activated protein C (38), interleukin-1, endotoxin (8), and plasma lipoproteins (5, 14, 18, 19, 26, 27, 33, 37). The release of PAI-1 from cultured vascular EC was increased by LDL in native or oxidized form (5, 18, 19, 37). Lp(a) is a stronger agonist than LDL on the stimulation of PAI-1 production in EC (9, 26). Previous studies by our group demonstrated that oxidation enhanced the effects of Lp(a) and LDL on PAI-1 production in vascular EC (26). Protein kinase C (PKC) mediates PAI-1 production in vascular EC induced by thrombin (11). The signal transduction mechanism for PAI-1 overproduction induced by lipoproteins or their oxidized forms remains unknown. The present study examined the involvement of PKC and its isofrom in PAI-1 production in cultured endothelial cells.
1.063) was isolated from lipoprotein fractions eluted from the affinity column by sequential floating ultracentrifugation. For oxidative modification, lipoproteins were thoroughly dialyzed against EDTA-free buffer and then were treated with 5 µM CuSO4 for 24 h at 22°C. The reaction was terminated by the addition of EDTA to the final concentration of 0.6 mM (22). The extent of lipid peroxidation was verified by measurement of the levels of thiobarbituric acid reactive substance. Tetramethoxypropane was applied as a standard, as previously described (25). Lipoproteins were stored in sealed tubes filled with nitrogen and kept in the dark at 4°C to prevent oxidation during storage. Endotoxin in lipoprotein preparations was undetectable with a Limulus Amebocyte Lysate assay kit (Sigma, St. Louis, MO) with a low detection limit of 0.05 ng endotoxin/ml. Protein concentrations of the lipoproteins were estimated by a modified Lowry method (10). Molar concentrations of LDL and Lp(a) were determined from their apolipoproteins.

The concentration of LDL and Lp(a) was determined from their apolipoproteins. Heparin (100 µg/ml) and leupeptin (25 µg/ml) were added to the medium supplemented with 10% fetal bovine serum, 100 nM EGTA, 2 mM sodium pyrophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 25 µg/ml leupeptin, 100 µM CaCl2, 320 µg/ml phosphatidylserine, 30 µg/ml dactinomycin, 100 µM \( \gamma^\cdot \)ATP (1,000 cpm/mmol), and 5 µM neuregulin (CalBiochem, La Jolla, CA) to inhibit PKC.

The activity of PKC was presented in picomoles per minute per milligram of cellular proteins.

Detection of translocation of PKC isoform by Western blotting analysis. After experimental treatment, HUVEC were harvested by a rubber policeman and homogenized in a glass homogenizer. Cytosolic and membrane fractions of the cells were separated by TLX-100 ultracentrifugation (Beckman Canada, Ontario, Canada) at 100,000 g at 4°C for 1 h. Proteins in cytosol and membrane pellets were analyzed on 10% SDS-PAGE and then blotted to nitrocellulose membrane. PKC isoforms in the subcellular fractions were detected by polyclonal antibodies against human PKC-β1 or -β2 (Santa Cruz, CA) in combination with appropriate second antibodies conjugated with alkaline phosphatase.

Statistical analysis. Values were expressed as means ± SD from quadruplicate wells. Student's t-test was used to compare probability between two groups. For multiple groups, ANOVA was performed by one-way ANOVA followed by Duncan's test. A P value of <0.05 was considered to be statistically significant.

RESULTS

Effects of LDL, Lp(a), and their oxidized forms on generation of PAI-1 from EC. Previous studies in this laboratory demonstrated that the increases in PAI-1 release from HUVEC reached plateau after 48 h of incubation with ≥10 nM native or oxidized Lp(a). Native LDL at the 10 nM level did not significantly increase PAI-1 generation from EC (26). Initial experiments in the present study examined the effects of 100 nM native and oxidized LDL for 48 h on PAI-1 generation from HUVEC compared with 10 nM LDL or Lp(a) in native or oxidized form (Fig. 1). The levels of PAI-1 antigen in the EC medium were significantly elevated after treatment with 100 nM native LDL (P < 0.01) but not with 10 nM native LDL. Native Lp(a) at the level of 10 nM stimulated PAI-1 release (P < 0.01), as previ-
ously described (23). Cu²⁺ oxidation enhanced PAI-1 generation in EC induced by LDL or Lp(a) under all tested conditions (P < 0.05 or 0.01).

Changes of PKC activity in lipoprotein-treated EC. The levels of PKC activity in cell lysates of HUVEC were monitored during the incubation with LDL, Lp(a), or their oxidized forms. Cells were treated with 10 nM Lp(a) or 100 nM LDL in native or oxidized form for a time period of from 5 min to 24 h. The levels of PKC activity in cell lysates were transiently increased at ~15 min and 5.5 h after the addition of Lp(a), LDL, or their oxidized forms and quickly returned to the basal level. The elevations of PKC activity at 15 min and at 5.5 h in cells treated with oxidized Lp(a) or LDL were significantly greater than those in cells treated with native Lp(a) or LDL under equivalent conditions (Fig. 2).

Characterization of activation of PKC associated with PAI-1 production induced by lipoproteins. Treatments of EC with 1 µM calphostin C, a PKC inhibitor (15), begun at the start (0 h) of lipoprotein stimulation, blocked the overproduction of PAI-1 induced by LDL (100 nM), Lp(a) (10 nM), or their oxidized forms in HUVEC (Fig. 3). Addition of calphostin C at 5 h (Fig. 3), which was only 30 min before the second PKC peak but hours after the first one (Fig. 1), prevented the generation of PAI-1 induced by native and oxidized LDL or Lp(a). Interestingly, the addition of calphostin C at 9 h or at 16 h after the initiation of lipoprotein treatment did not significantly alter LDL-, Lp(a)-, or their oxidized form-induced PAI-1 generation from EC (Fig. 3).

Translocation of PKC-β1 in EC induced by lipoprotein treatment. PKC-β1, but not -β2, was detected in subcellular fractions of HUVEC. At basal condition, PKC-β1 was predominantly found in cytosol but was undetectable in membrane fractions. After treatment with 100 nM LDL, 10 nM Lp(a), or their oxidized forms for 5.5 h, substantial amounts of PKC-β1 were detected in membrane fractions of EC (Fig. 4, A and B).

Involvement of PKC-β in lipoprotein-induced PKC activation and PAI-1 overproduction. 379196 is one of...
the PKC-β-specific inhibitors recently developed at Eli Lilly (J. Gillig and co-workers, unpublished observations). Treatment with 60 nM 379196 blocked the elevation of PKC activity in HUVEC at 5.5 h after the addition of 100 nM LDL, 10 nM Lp(a), or their oxidized forms (Fig. 5A). An identical dose of the PKC-β inhibitor effectively prevented generation of PAI-1 induced by LDL, Lp(a), or their oxidized forms from EC (Fig. 5B). Treatment with 379196 alone did not significantly affect PKC activity or PAI-1 production in HUVEC (Fig. 5).

Involvement of PKC-β in PAI-1 production induced by LDL, Lp(a), and their oxidized forms in arterial EC. Because atherosclerosis occurs predominantly in middle- to large-size arteries, the effects of the lipoproteins on PAI-1 production and the involvement of PKC-β were verified in EC isolated from human coronary artery. The basal level of PAI-1 antigen in the medium of HCAEC after 48 h of incubation was 2 to 3 times greater than that of HUVEC. Treatments with native and oxidized LDL (100 nM) or Lp(a) (10 nM) for 48 h significantly increased PAI-1 release from HCAEC (P < 0.01 or 0.001). Oxidation significantly enhanced the effects of LDL and Lp(a) on PAI-1 generation from HCAEC (P < 0.05). Increases in PAI-1 generation induced by LDL, Lp(a), or their oxidized forms from HCAEC (+23 to +6%) were less extensive than those from HUVEC (+52 to +113%). PKC-β-specific inhibitor, 379196 (60 nM), effectively prevented PAI-1 overproduction induced by LDL, Lp(a), and their oxidized forms in HCAEC (Fig. 6).

**DISCUSSION**

The results of the present study demonstrate for the first time that PKC mediates the regulation of PAI-1 generation induced by atherogenic lipoproteins in vascular EC. Previous studies indicated that PAI-1 production induced by oxidized LDL in EC may be mimicked by the addition of lysophosphatidylcholine (lyso-PC), a peroxidation product of phosphatidylcholine (7). The content of lyso-PC was increased in oxidized LDL compared with native LDL (22). PKC activation is involved in lyso-PC-induced production of monocyte chemoattractant protein-1 in HUVEC (34). The present study indicates that physiological levels of LDL and Lp(a) elevate PKC activity and stimulate PAI-1 production, and the effects of both types of lipoproteins are enhanced by Cu²⁺ oxidation. The involvement of PKC in this process was verified by the effectiveness of PKC-specific inhibitors on PKC activation and PAI-1 generation induced by the atherogenic lipoproteins. Lipid peroxidation products, including lyso-PC, potentially contribute to PKC activation and PAI-1 production induced by oxidized lipoproteins in EC. LDL and Lp(a) share extensive structural homology with the major difference in the presence of Apo(a) in Lp(a) but not in LDL. Lp(a) particles were more susceptible to oxidation than LDL (24). LDL particles may be oxida-
The additions of PKC inhibitor at 30 min before the first PKC peak still effectively prevented lipoprotein-induced PAI-1 production in EC, on the basis of the fact that the addition of calphostin C at 5 h after the first PKC peak induced PAI-1 production. The second PKC peak occurred between 5 and 9 h after the initiation of lipoprotein stimulation. Those findings suggest that PKC-β mediates PAI-1 overproduction induced by native and oxidized LDL and Lp(a) in EC may be mediated via a PKC-β-specific pathway. Therefore, cellular response inhibited by < 200 nM 379196 may be considered PKC-β-specific activity (J. Gillig and co-workers, unpublished observations). The results of the present study demonstrate that 60 nM 379196 prevented PKC activation and PAI-1 overproduction in EC induced by LDL, Lp(a), or their oxidized forms. This suggests that PKC-1 overproduction induced by native and oxidized LDL and Lp(a) in EC may be mediated via a PKC-β-specific pathway. Previous studies indicated that LY-333531 prevented the development of vascular complications in diabetic animals (13). Hyperglycemia promotes the glycation of plasma proteins, including lipoproteins. Glycation augments the susceptibility of lipoproteins to oxidation (16). Our studies indicated that glycation enhanced LDL-induced PAI-1 production in vascular EC (42). PKC-β inhibitors may prevent vascular complications in diabetic animals partially by inhibiting the generation of PAI-1 induced by glycoxidized lipoproteins.

The results of the present study demonstrate that LDL, Lp(a), and their oxidized forms increase PAI-1 levels in HUVEC at basal or stimulated conditions; however, the possibility of the involvement of PKC-β2 in lipoprotein-induced PAI-1 production has not been completely excluded by the data of this study.

The PKC-β inhibitor 379196, as well as LY-333531 (3), is a dimethylamine analog and specifically inhibits the activity of PKC-β1 and -β2. The E50 of 379196 for PKC-β1 (50 nM) and -β2 (30 nM) was 12- to 23-fold lower than that for PKC-α (0.6 μM), PKC-γ (0.6 μM), and PKC-δ (0.7 μM) assessed with histone H1. Therefore, cellular response inhibited by < 200 nM 379196 may be considered PKC-β-specific activity (J. Gillig and co-workers, unpublished observations). The results of the present study demonstrate that 60 nM 379196 prevented PKC activation and PAI-1 overproduction in EC induced by LDL, Lp(a), or their oxidized forms. This suggests that PKC-1 overproduction induced by native and oxidized LDL and Lp(a) in EC may be mediated via a PKC-β-specific pathway. Previous studies indicated that LY-333531 prevented the development of vascular complications in diabetic animals (13). Hyperglycemia promotes the glycation of plasma proteins, including lipoproteins. Glycation augments the susceptibility of lipoproteins to oxidation (16). Our studies indicated that glycation enhanced LDL-induced PAI-1 production in vascular EC (42). PKC-β inhibitors may prevent vascular complications in diabetic animals partially by inhibiting the generation of PAI-1 induced by glycoxidized lipoproteins.

The results of the present study demonstrate that LDL, Lp(a), and their oxidized forms increase PAI-1 generation not only in HUVEC but also in HCAEC. The production of PAI-1 induced by the lipoproteins in both HUVEC and HCAEC was effectively inhibited by a PKC-β-specific inhibitor. Those findings suggest that PKC-β mediates PAI-1 overproduction induced by native and oxidized LDL or Lp(a) in both venous and arterial EC. In an unstimulated condition, HCAEC produced greater amounts of PAI-1 than HUVEC did. The relative increases in PAI-1 generation induced by LDL, Lp(a), or their oxidized forms from HCAEC were generally less than those from HUVEC; however, differences in the types of media and passage numbers between HCAEC and HUVEC potentially affect the PAI-1 generation at basal level or their responses to the lipoproteins. Besides, fibrinolytic activity is known to be affected by plasminogen activators and other components of the fibrinolytic system, including the receptors and binding proteins of plasminogen activators (28), which are beyond the scope of the present study.

Impaired fibrinolytic activity characterized by elevated levels of PAI-1 with or without a reduction in tissue plasminogen activator has been found frequently in patients with coronary artery disease (7, 12). Increases in PAI-1 protein and mRNA were detected in atherosclerotic and thrombotic lesions in arteries and
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veins (1, 41). Elevation of PAI-1 generation from vascular EC may attenuate fibrinolytic activity and promote the development of thrombosis with and without the presence of atherosclerotic lesions. The results of the present study provide original evidence that PKC-β may be involved in atherogenic lipoprotein-induced overproduction of PAI-1 in coronary arterial EC. PKC-β-specific inhibitors may also be considered a type of candidate for the prevention of ischemic events in patients with coronary artery disease.

In summary, the present study indicates that a delayed activation of PKC-β mediates PAI-1 overproduction induced by LDL, Lp(a), and their oxidized forms in vascular EC. Treatment with PKC-β inhibitor effectively prevented the generation of PAI-1 induced by the atherogenic lipoproteins in cultured venous and arterial EC. The findings of this study provide a rationale to investigate the effect of PKC-β inhibitors on the prevention of thrombotic events in vivo.

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Address for reprint requests and other correspondence: G.X. Shen, Departments of Internal Medicine and Physiology, University of Manitoba, BS4 730 William Ave, Winnipeg, Manitoba, Canada R3E 0W3 (E-mail: gshen@rs.umanitoba.ca).

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