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Pravastatin suppresses the interleukin-8 production induced by thrombin in human aortic endothelial cells cultured with high glucose by inhibiting the p44/42 mitogen activated protein kinase

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1 3-Hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors (statins) prevent the progression of atherosclerosis by lowering cholesterol. However, the effect of statins on the synthesis of pro-inflammatory cytokines from endothelial cells has not yet been fully investigated. Here, we examined the effect of pravastatin, one of the statins, on IL-8 synthesis induced by thrombin in human aortic endothelial cells (AoEC) cultured with high glucose concentrations.

2 Pravastatin significantly decreased the IL-8 synthesis induced by thrombin.

3 Pravastatin inhibited the p44/42 MAP kinase activity induced by thrombin, but did not inhibit the p38 MAP kinase activity.

4 Translocation of *ras* protein from the cytosol to plasma membrane was inhibited by pravastatin.

5 Pravastatin inhibit the activator protein-1 activity, but did not inhibit the activation of IκB-α.

6 Dominant negative *ras* inhibited the p44/42 MAP kinase activity induced by PMA.

7 Our results suggest that pravastatin inhibits IL-8 synthesis by blocking the *ras*-MAP (p44/42) kinase pathway rather than nuclear factor-κB. Pravastatin may prevent atherosclerosis not only by lowering cholesterol levels, but also by suppressing IL-8 synthesis in AoEC through the inhibition of p44/42 MAP kinase, and this may be more beneficial in diabetic patients than in non-diabetics.
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Keywords: Pravastatin; interleukin-8; endothelium; signal transduction; thrombin; MAP kinase; diabetic macroangiopathy

Abbreviations: ANOVA, analysis of variance; AoEC, aortic endothelial cells; DIG, digoxigenin; ECL, enhanced chemiluminescence; ELISA, enzyme linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; FCS, foetal calf serum; FPP, farnesylpyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IκB-α, inhibitory factor κB-α; IL-8, interleukin-8; LDL, low density lipoprotein; MAPK, mitogen activated protein kinase; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-κB; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor-α;

Introduction

The development of atherosclerosis is a complicated process starting from endothelial dysfunction and leading to migration of smooth muscle cells into the intima. Several causes of endothelial dysfunction include lipid disorder, diabetes mellitus, hypertension, and cigarette smoking. Following endothelial dysfunction, the migration of leukocytes into the subendothelial space and migration of medial smooth muscle cells into intimal layer of arteries occur (Ross, 1999). Such cellular responses are regulated by growth factors and cytokines such as fibroblast growth factor, platelet derived growth factor, tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8 and monocyte chemoattractant protein (MCP)-1. We previously reported that in aortic endothelial cells (AoEC) high concentrations of glucose stimulated the IL-8 production, but did not stimulate IL-1, IL-6 and TNF-α production (Urakaze *et al*., 1996; Temaru *et al*., 1997). Therefore, we postulated that IL-8 plays some role in the pathogenesis of diabetic angiopathy. Furthermore, IL-8 has been implicated in the pathogenesis of atherosclerosis because the migration of aortic smooth muscle cells is induced by IL-8 (Yue *et al*., 1993, 1994) and also IL-8 is expressed in human atheroma (Koch *et al*., 1993; Wang *et al*., 1996; Rus *et al*., 1996). Recently, Boisvert *et al*. (1998) have reported that mice lacking IL-8 receptors are less susceptible to atherosclerosis. These data suggest that IL-8 may have an important role in the formation of human atherosclerotic lesions by inducing the accumulation of both leukocytes and smooth muscle cells.

On the other hand, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors are generally used as therapeutic agents for the treatment of hypercholesterolaemia. These drugs have an inhibitory effect on cholesterol synthesis mainly in the liver by inhibiting the conversion of HMG-CoA to mevalonate, the rate-limiting step of *in vivo* cholesterol synthesis (Goldstein & Brown, 1990). As a result, the plasma levels of low density lipoprotein (LDL) cholesterol is decreased, which leads to a longer survival rate with less incidence of the vascular events due to atherosclerosis (Scandinavian Simvastatin Survival Study Group, 1994;

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Shepherd *et al*., 1995; Sacks *et al*., 1996; The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group, 1998; Plehn *et al*., 1999). However, in the West of Scotland Coronary Prevention Study pravastatin, an HMG-CoA reductase inhibitor, showed greater benefits with a lower risk of cardiovascular events, even although the pravastatin treated patients showed a similar level of LDL cholesterol compared with the placebo subjects (West of Scotland Coronary Prevention Study Group, 1998). These data suggest that lipid changes may not fully account for the clinical benefits of pravastatin, and that it may have another beneficial effects. Indeed, other reports showed that HMG-CoA reductase inhibitors improved the endothelial dysfunction present in atherosclerosis, enhanced endothelial nitric oxide synthase (eNOS) mRNA expression (Hernandez-Perera *et al*., 1998; Endres *et al*., 1998), suppressed plasminogen activator inhibitor-1 mRNA expression in endothelial cells (Essig *et al*., 1998), and inhibited the proliferation of smooth muscle cells (Munro *et al*., 1994). However, it has not yet been fully investigated as to whether pravastatin may affect the production of pro-inflammatory cytokines especially IL-8 in vascular endothelial cells.

In this paper, we attempted to clarify the effect of pravastatin on the regulation of IL-8 synthesis induced by thrombin in human aortic endothelial cells cultured with high concentrations of glucose. We report here that pravastatin significantly inhibits the synthesis of IL-8 induced by thrombin at both the protein and mRNA levels *via* the inhibition of *ras-raf*-MAP (p44/42) kinase and transcription factor, activating protein (AP)-1. Our results suggest that pravastatin may prevent atherosclerosis by suppressing IL-8 synthesis in AoEC through the inhibition of p44/42 MAP kinase. Therefore, pravastatin may not only represent a cholesterol-lowering agent, but it could also contribute to improve the endothelial dysfunction present in diabetic macroangiopathy.

Methods

Cell culture

Human aortic endothelial cells (AoEC, from Kurabou, Japan) at passages 5–10 were cultured in 10 cm culture dishes coated with type-I collagen (Iwaki, Japan) in MCDB 131 (Sigma) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 20 mM HEPES, 60 μg ml^{-1} heparin, 20 μg ml^{-1} bovine pituitary extracts (Kyokutou), 1.25 μg ml^{-1} fungizone, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37°C at 95% air and 5% CO_2 atmosphere. For the experiments, AoEC were cultured with media containing 5.5, 10, 24 or 42.5 mM glucose in the presence or absence of pravastatin for 14 days. The medium was replaced every other day.

Measurement of IL-8 in the media

AoEC were cultured with media containing 5.5 mM glucose in the presence of various concentrations of pravastatin in 24 well culture plates coated with type-I collagen. For the measurement of IL-8 release from the AoEC in the medium, the media were collected after stimulation with thrombin. The concentrations of IL-8 in the media were measured by enzyme linked immunosorbent assay (ELISA) as previously reported (Urakaze *et al*., 1996).

Northern blot analysis

Northern blot analysis was performed according to the method as previously described (Temaru *et al*., 1997). Briefly, AoEC were cultured with media containing 5.5 mM glucose in the presence or absence of pravastatin (10 μM) in 6 cm culture dishes coated with type-I collagen, and then cells were serum-starved by the media containing 0.5% FCS for 16 h. After stimulation with thrombin, RNA from the AoEC was extracted by ISOGEN (Nippon Gene, Japan). A 20 μg aliquot of the total RNA was electrophoresed on a 1% agarose gel containing 6.6% formaldehyde at 45 V, and then blotted onto a nylon filter membrane (Amersham). The membrane was then prehybridized, hybridized, washed and analysed by the method recommended by the manufacturer. A digoxygenin-labelled probe for human IL-8 cDNA with dUTP by the random priming method (Roche Diagnostics Co.) was used for the hybridization. The intensity of the bands was analysed by NIH image.

Western blot analysis

The phosphorylation of MAP kinases and IκB-α was analysed by a non-radioactive method using a commercial kit (New England Biolabs, MA, U.S.A.). Briefly, cell lysates were prepared using lysis buffer (mM: Tris 30, NaCl 150, EDTA 10, 1% Triton X-100, sodium deoxycholic acid 12, sodium orthovanadate 1, sodium fluoride 160, 150 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, and phenylmethylsulphonyl fluoride (PMSF) 1, pH 7.5). The cell lysates were then loaded on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred onto a membrane (Millipore) at 100 V for 1 h. The membranes were washed with TBST (1X TBS, 0.1% Tween-20), and then blocked with blocking buffer (1X TBS, 0.1% Tween-20, 5% w v^{-1} non-fat dry milk) for 1 h at room temperature. The blots were then incubated with primary antibodies diluted in blocking buffer (1X TBS, 0.1% Tween-20, 5% w v^{-1} non-fat dry milk or 5% BSA) for 16 h at 4°C. They were then rinsed three times for 10 min with TBST at room temperature and then incubated in the appropriate secondary antibody diluted in blocking buffer for 1 h at room temperature. The blots were rinsed another three times for 10 min with TBST before detection by ECL Western blotting detection reagents. The following antibodies were used (New England Biolabs, MA, U.S.A.) : phospho-specific p44/42 MAP kinase (Thr202 and Tyr204) antibody, p44/42 MAP kinase antibody, phospho-p38 MAP kinase (Thr180 and Tyr182) antibody, p38 MAP kinase antibody, phospho-IκB-α (Ser32) antibody, IκB-α antibody, and an anti-rabbit secondary antibody conjugated to horseradish peroxidase.

For the analysis of p21 *ras* processing, the cells were lysed in detergent free lysis buffer (mM: NaCl 150, $MgCl_2$ 15, PMSF 1, Na_2HPO_4 1, dithiothreitol 1, sodium orthovanadate 1, 10 μg ml^{-1} aprotinin, and 10 μg ml^{-1} leupeptin, pH 7.5), sonicated for 30 s at 4°C and then fractionated by ultracentrifugation at 100,000 $\times g$ for 30 min at 4°C. The pellets

were resuspended in lysis buffer with 1% Triton X-100 and 0.05% SDS. p21*ras* proteins were detected in both cytosolic (supernatant) and plasma membrane (pellet) fractions by Western blot analysis using an Ha-*ras* antibody (Transduction Laboratories) as the primary antibody and anti-mouse IgG, horseradish peroxidase-linked whole antibody (Amersham) as a secondary antibody. The intensity of the bands was analysed by NIH image.

*Transfection of a mutant of p21*ras

Transient transfection of a mutant of p21*ras*, a dominant negative mutant of H-*ras* cDNA (serine 17 to asparagine, S17N, Upstate Biotechnology), was carried out using Lipofectin Reagent (Life Technologies). Sub-confluent AoEC were cultured in 35 mm type-I collagen coated dishes with serum-free medium containing Lipofectin Reagent mixed with a dominant negative mutant of H-*ras* cDNA (1.5 μg) for 5 h at 37°C. After remove the DNA-containing medium, the AoEC were further incubated with medium containing 10% FCS for 48 h, and then serum-starved by the media containing 0.5% FCS for 16 h. After stimulation with thrombin or phorbol 12-myristate 13-acetate (PMA), the cell lysates were prepared and Western blot analysis was performed.

Gel mobility shift assay

Nuclear extracts were isolated from AoEC and used for gel mobility shift assay. A double-stranded DNA fragment containing the sequence for the binding site of transcription factor AP-1 (5′-CGC TTG ATG AGT CAG CCG GAA-3′, purchased from Geneka) was 3′-end-labelled with digoxigenin-in-11-ddUTP by terminal transferase using a DIG gel shift kit (Roche Molecular Biochemicals). Nuclear proteins were incubated with the DIG-labelled DNA fragment in a buffer containing in mM: HEPES 20, pH 7.9, KCl 50, $MgCl_2$ 4, EDTA 1, 12% glycerol and 0.0625 μg μl^{-1} Poly (dI-dC) for 20 min at 4°C. The reaction mixture was loaded onto a 5% polyacrylamide gel and then transferred onto a membrane at 4°C. The bands of the DNA-nuclear protein complex were detected by an anti-digoxigenin antibody (Roche Molecular Biochemicals). The intensity of the bands was analysed by NIH image.

Data analysis

Data are presented as the mean ± s.d. Statistical analysis was performed by ANOVA with subsequent Scheffe's *t*-test. A value of $P<0.05$ was considered to be significant.

Results

Effect of thrombin on IL-8 synthesis and IL-8 mRNA expression in AoEC

First, we examined whether thrombin could stimulate the IL-8 production in AoEC. As shown in Figure 1A, thrombin increased the IL-8 concentration in the culture media in a time and dose dependent manner, which is consistent with other previous reports (Ueno *et al*., 1996; Kaplanski *et al*., 1997; Johnson *et al*., 1998; Anrather *et al*., 1997). Therefore, we used thrombin as a stimulator for IL-8 synthesis from endothelial cells. Next, we examined the expression of IL-8 mRNA induced by thrombin using Northern blot analysis. As shown in Figure 1B, the expression of IL-8 mRNA was increased after stimulation with thrombin. These data indicate that thrombin stimulates the IL-8 production at both the protein and mRNA levels in AoEC.

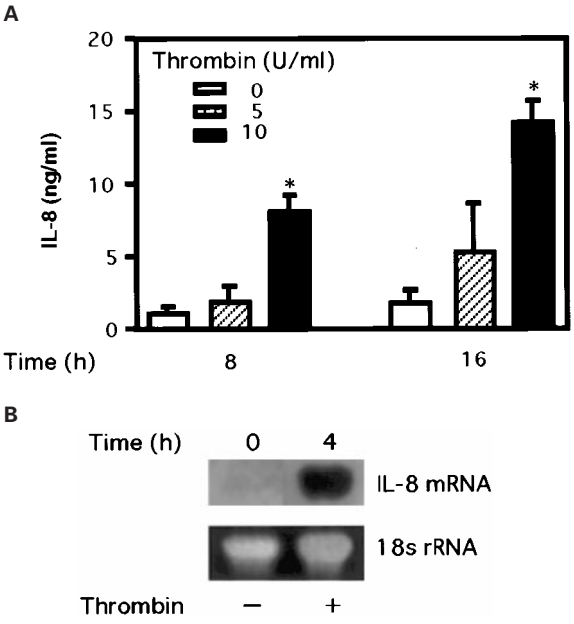

Figure 1 Effect of thrombin on IL-8 synthesis and mRNA expression in human aortic endothelial cells (AoEC). (A) AoEC at passages 5–10 were cultured with medium containing 5.5 mM of glucose in 24-well collagen-coated plates. The cells were then stimulated with the indicated concentration of thrombin for 8 and 16 h. The concentration of IL-8 in the medium was measured by ELISA. (B) AoEC were cultured with medium containing 5.5 mM of glucose in 6 cm collagen-coated dishes. The cells were then serum-starved with medium containing 0.5% FCS for 16 h. After stimulation with thrombin (10 U ml^{-1}) for 4 h, the expression of IL-8 mRNA was analysed by Northern blot analysis. The bands were analysed by NIH image. The data are representative of three different experiments (means ± s.d.). *$P<0.001$ vs control (white bar).

Effect of pravastatin on IL-8 synthesis and IL-8 mRNA expression induced by thrombin in AoEC

Next, we examined the effect of pravastatin on the IL-8 production induced by thrombin in AoEC. As shown in Figure 2A, the production of IL-8 induced by thrombin was significantly inhibited by all concentrations of pravastatin used. To confirm that pravastatin has no cytotoxic effects the viability of AoEC cultured with pravastatin for 14 days was evaluated by measuring LDH in the media. There were no significant differences in the levels of LDH among any of the samples (data not shown). Therefore, we used 10 μM pravastatin for the following experiments to examine the effect of the drug. We next examined the effect of pravastatin on the expression of IL-8 mRNA induced by thrombin using Northern blot analysis. The expression of IL-8 mRNA induced by thrombin was significantly diminished by the incubation with pravastatin (Figure 2B).

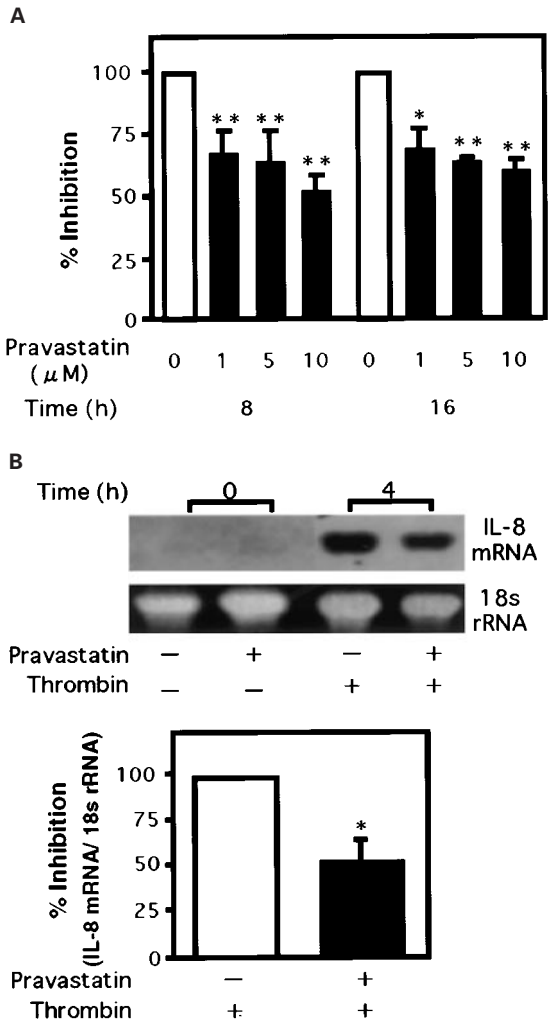

Figure 2 Effect of pravastatin on the IL-8 production (A) and the IL-8 mRNA expression (B) induced by thrombin in human aortic endothelial cells (AoEC). (A) AoEC were cultured with media containing 5.5 mM of glucose in 24-well collagen-coated plates with various concentrations of pravastatin for 14 days. The cells were then stimulated with thrombin for 8 and 16 h. The concentration of IL-8 in the medium was measured by ELISA. (B) AoEC were cultured in 6 cm collagen-coated dishes with or without pravastatin (10 μM) for 14 days, and the cells were then serum-starved with medium containing 0.5% FCS for 16 h. After stimulation with thrombin (10 U ml^{-1}) for 4 h, the expression of IL-8 mRNA was analysed by Northern blot analysis. The bands were analysed by NIH image, and the intensity of the IL-8 mRNA band was corrected with that of the 18s rRNA band. The data are representative of three different experiments (means$\pm$s.d.). *$P<0.01$ vs control (white bar), **$P<0.001$ vs control (white bar).

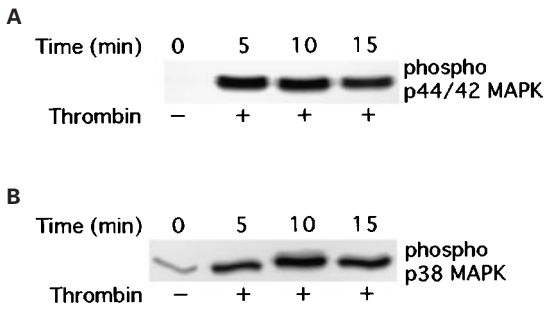

Figure 3 Thrombin activates the p44/42 MAP kinase and the p38 MAP kinase in human aortic endothelial cells (AoEC). AoEC were cultured with medium containing 5.5 mM glucose in 6 cm collagen-coated dishes before being serum-starved for 16 h with medium containing 0.5% FCS and then stimulated with thrombin (10 U ml^{-1}) for 5, 10 and 15 min. Cell lysates were separated by 10% SDS–PAGE, transferred onto membranes, and blotted with phospho-specific p44/42 MAP kinase (Thr202 and Tyr204) antibody (A) or a phospho-p38 MAP kinase (Thr180 and Tyr182) antibody (B).

Effect of pravastatin on the activity of MAP kinases induced by thrombin in AoEC

Since it has been reported that the expression of IL-8 mRNA is regulated by both p44/42 and p38 MAP kinases (Sano *et al*., 1998; Gon *et al*., 1998), we examined the effect of pravastatin on the activity of MAP kinases in AoEC. We observed that thrombin activated both p44/42 MAP kinase and p38 MAP kinase (Figure 3). As shown in Figure 4, both PD 98059, an inhibitor of p44/42 MAP kinase kinase (MEK), and SB 203580, an inhibitor of p38 MAP kinase, inhibited the expression of IL-8 mRNA induced by thrombin, which is consistent with previous reports (Kaplanski *et al*., 1997). These data suggest that the synthesis of IL-8 induced by thrombin is regulated by both p44/42 MAP kinase and p38 MAP kinase in AoEC. Interestingly, as shown in Figure 5, pravastatin inhibited the activation of p44/42 MAP kinase, but did not inhibit p38 MAP kinase induced by thrombin in AoEC. Taken together, these data indicate that thrombin stimulates the synthesis of IL-8 through both p44/42 MAP kinase and p38 MAP kinase pathways in AoEC, and that pravastatin reduces the synthesis of IL-8 by specifically inhibiting the p44/42 MAP kinase pathway, but not the p38 MAP kinase pathway.

Effect of mevalonate and farnesylpyrophosphate (FPP) on MAP kinase activity induced by thrombin in AoEC

We then examined the direct effect of pravastatin on MAP kinase activity induced by thrombin. As shown in Figure 6A, the inhibitory effect of pravastatin on the activation of p44/42 MAP kinase was abrogated by the co-incubation with mevalonate, suggesting that the action of pravastatin is done apparently through the mevalonate pathway. Next, we examined the effect of FPP on p44/42 MAP kinase induced by thrombin. As shown in Figure 6B, the inhibitory effect of pravastatin on the activation of p44/42 MAP kinase was also abolished by the co-incubation with FPP.

Effect of pravastatin on the processing of ras *protein in AoEC*

The effect of pravastatin on the processing of *ras* protein by Western blot analysis was examined. As shown in Figure 7, following treatment with pravastatin, the amount of *ras* protein in cytosol fraction was increased, while in plasma membrane fraction it diminished. These findings indicate that pravastatin inhibits the activation of p44/42 MAP kinases by preventing *ras* protein isoprenylation and its translocation from the cytosol to the plasma membrane.

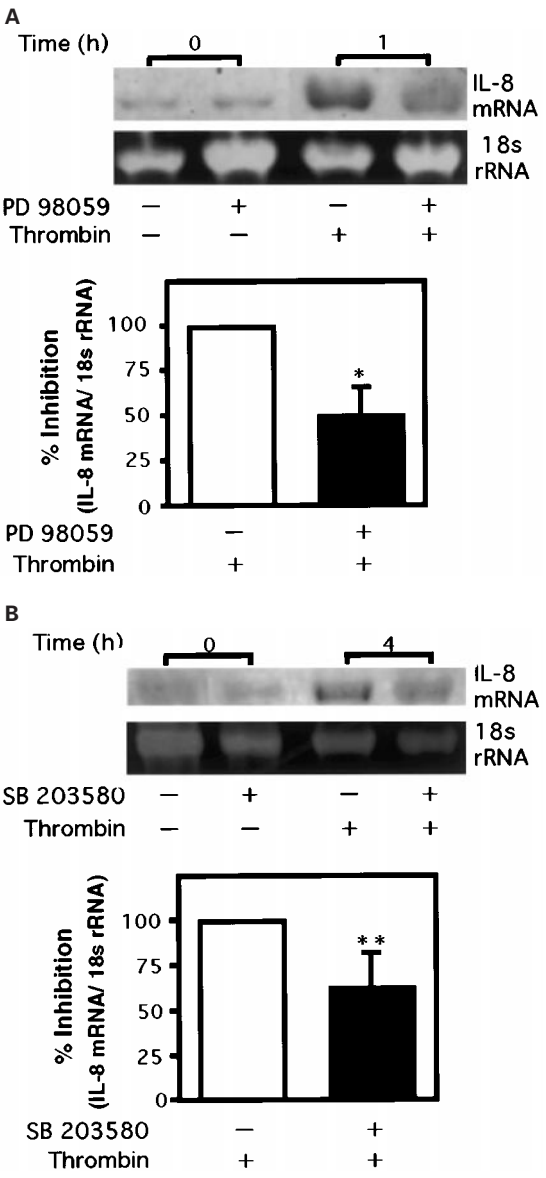

Figure 4 Effect of PD 98059 (A) or SB 203580 (B) on the IL-8 mRNA expression induced by thrombin in human aortic endothelial cells (AoEC). AoEC cultured with media containing 5.5 mM glucose were serum-starved with medium containing 0.5% FCS for 16 h. The cells were then treated for 1 h with PD 98059 (20 μM) (A), a specific inhibitor of MEK, or SB 203580 (20 μM) (B), an inhibitor of p38 MAP kinase. Next, they were stimulated with thrombin (10 U ml^{-1}), and then the RNA was extracted. The expression of IL-8 mRNA was analysed by Northern blot analysis. The bands were analysed by NIH image, and the intensity of the IL-8 mRNA band was corrected with that of the 18s rRNA band. The data are representative of three different experiments (means ± s.d.). *$P<0.01$ vs control (white bar), **$P<0.05$ vs control (white bar).

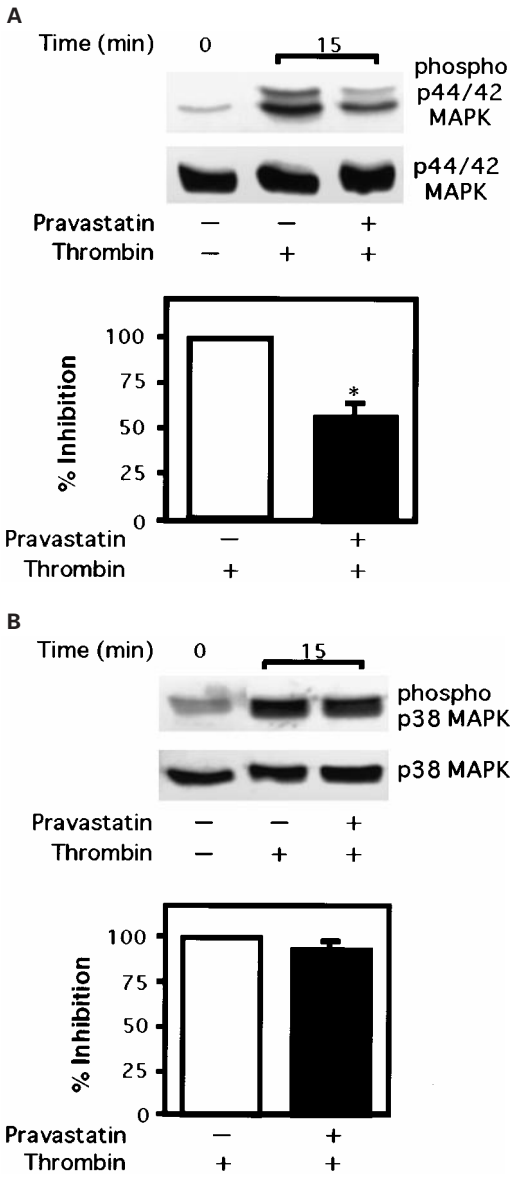

Figure 5 Effect of pravastatin on the activity of MAP kinases induced by thrombin in human aortic endothelial cells (AoEC). AoEC were cultured with or without pravastatin (10 μM) for 14 days before being serum-starved with medium containing 0.5% FCS for 16 h in 6 cm collagen-coated dishes and then stimulated with thrombin (10 U ml^{-1}) for 15 min. Cell lysates were separated by 10% SDS–PAGE, transferred onto membranes, and blotted with phospho-specific p44/42 MAP kinase (Thr202 and Tyr204) antibody (A) or a phospho-p38 MAP kinase (Thr180 and Tyr182) antibody (B). The bands were analysed by NIH image, and the intensity of each phospho-MAP kinase band was corrected with that of the respective MAP kinase band. The data are representative of three different experiments (means ± s.d.). *$P<0.001$ vs control (white bar).

Role of ras *protein on MAP kinase activity induced by thrombin in AoEC*

Next, we examined whether *ras* protein is involved in the signal transduction of the thrombin-induced p44/42 MAP kinase activation using the transfection of a dominant negative H-*ras* mutant (S17N). The activation of p44/42 MAP kinase induced by thrombin in AoEC was inhibited by the transfection of H-*ras* (S17N) (Figure 8), indicating that the *ras* protein is involved in the signal transduction of the thrombin-induced p44/42 MAP kinase activation.

Effect of pravastatin on the phosphorylation of IκB-α induced by thrombin in AoEC

Next we examined whether pravastatin could affect the activity of NF-κB. As the phosphorylation of IκB-α is

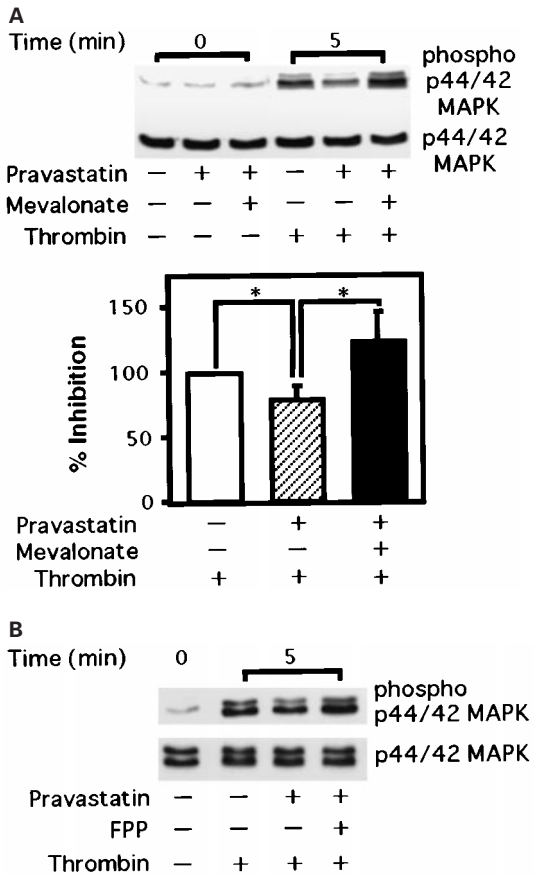

Figure 6 Effect of mevalonate (A) and FPP (B) on the activity of p44/42 MAP kinase induced by thrombin in human aortic endothelial cells (AoEC). AoEC were cultured with or without pravastatin (10 μM) or with pravastatin (10 μM) combined with mevalonate (400 μM) (A) or FPP (5 μM) (B), serum-starved by the media containing 0.5% FCS for 16 h in 6 cm collagen-coated dishes and then stimulated with thrombin (10 U ml^{-1}) for 5 min. Cell lysates were separated by 10% SDS–PAGE, transferred onto membranes, and blotted with phospho-specific p44/42 MAP kinase (Thr202 and Tyr204) antibody. The bands were analysed by NIH image, and the intensity of each phospho-MAP kinase band was corrected with that of the respective MAP kinase band. The data are representative of three different experiments (means$\pm$s.d.). *$P<0.05$.

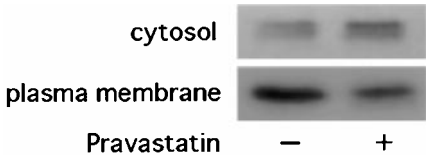

Figure 7 Effect of pravastatin on the processing of *ras* protein in human aortic endothelial cells (AoEC). AoEC were cultured with medium containing 5.5 mM of glucose in the presence or absence of pravastatin (10 μM) for 14 days. The cells were lysed with lysis buffer, and the cell lysates were fractionated by ultra-centrifugation. Cytosolic (supernatant) and plasma membrane (pellet) fractions were separated by 12% SDS–PAGE, transferred onto membranes and blotted with an Ha-ras antibody.

required for the activation of NF-κB activity, the effect of pravastatin on the phosphorylation of IκB-α induced by thrombin was examined by Western blot analysis. As shown in Figure 9, thrombin activated the phosphorylation of IκB-α,

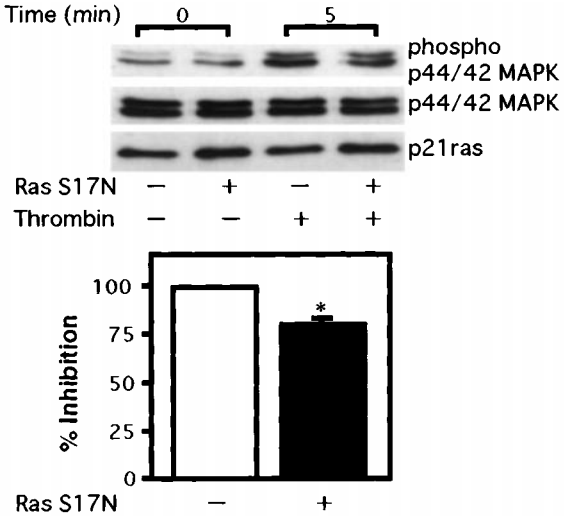

Figure 8 Effect of dominant negative *ras* protein on p44/42 MAP kinase activity induced by thrombin in human aortic endothelial cells (AoEC). A dominant negative H-*ras* (S17N) was transfected to AoEC using Lipofectin. After transfection, the AoEC were cultured with medium containing 10% FCS for 48 h, serum-starved by the media containing 0.5% FCS for 16 h, and then stimulated with thrombin (10 U ml^{-1}) for 5 min. Cell lysates were separated by 10% SDS–PAGE, transferred onto membranes and blotted with phospho-specific p44/42 MAP kinase (Thr202 and Tyr204) antibody and an Ha-*ras* antibody. The bands were analysed by NIH image, and the intensity of each phospho-MAP kinase band was corrected with that of the respective MAP kinase band. The data are the representative of three different experiments (mean$\pm$s.d.). *$P<0.001$ vs control (white bar).

and this activation was inhibited by Calphostin C, which is one of PKC inhibitors. In contrast, neither PD 98059 nor pravastatin inhibited the phosphorylation of IκB-α induced by thrombin. These data suggest that PKC is at the upstream of NF-κB in the signal transduction of the IL-8 synthesis by thrombin, and that pravastatin inhibits the activation of p44/42 MAP kinase pathway but not the activation of NF-κB. We also observed that Calphostin C inhibited the p44/42 MAP kinase activity induced by thrombin in AoEC (data not shown). Taken together, these data suggest that thrombin stimulates IL-8 production through at least two pathways including PKC to NF-κB and PKC to p44/42 MAP kinase pathways, and that pravastatin inhibits the IL-8 production through the inhibition of p44/42 MAP kinase pathway but not the PKC to NF-κB pathway.

Effect of pravastatin on the AP-1 activity induced by thrombin in AoEC

We examined the effect of pravastatin on AP-1 activity by gel mobility shift assay. As shown in Figure 10, thrombin enhanced the AP-1 activity, and pravastatin inhibited it suggesting that pravastatin may inhibit the IL-8 mRNA expression at the transcription level.

Effect of pravastatin on p44/42 MAP kinase activity induced by high glucose in AoEC

Next, we examined the effect of high glucose on the activity of p44/42 MAP kinase in AoEC. As shown in Figure 11, high glucose enhanced the activity of p44/42 MAP kinase and this

effect was inhibited by Calphostin C. These data indicate that the enhanced p44/42 MAP kinase activity by high glucose is dependent on PKC activation. The effect of pravastatin on the activity of p44/42 MAP kinase induced by high glucose was then examined. As shown in Figure 12, pravastatin inhibited the activity of p44/42 MAP kinase induced by high glucose. Furthermore, thrombin and high glucose had an additive effect on the activity of p44/42 MAP kinase but pravastatin was able to inhibit it. In addition, the activation of p44/42 MAP kinase induced by PMA, which activates PKC directly, was also inhibited by the transfection of the dominant negative H-*ras* mutant (S17N) in AoEC (Figure 13).

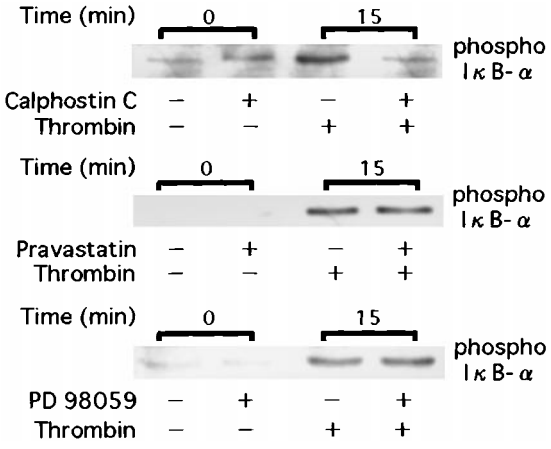

Figure 9 Changes in the thrombin induced-phosphorylation of IκB-α in human aortic endothelial cells (AoEC) treated with pravastatin, Calphostin C, or PD 98059. AoEC cultured with medium containing 5.5 mM glucose were treated with pravastatin (10 μM) for 14 days, or treated by PD 98059 (20 μM) for 1 h, or treated with Calphostin C (100 nM) for 1 h. Cells were stimulated with thrombin (10 U ml^{-1}) for 15 min, respectively. Cell lysates were separated by 10% SDS–PAGE, transferred to membranes and blotted with a phospho-IκB-α (Ser32) antibody. The data are representative of three different experiments.

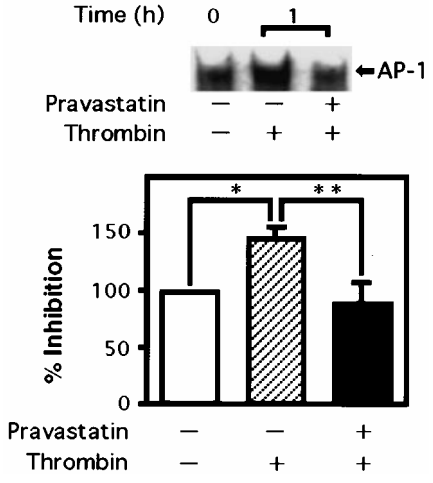

Figure 10 Effect of pravastatin on the binding of specific DNA sequences to AP-1 transcription factor complexes induced by thrombin in human aortic endothelial cells (AoEC). AoEC were cultured with medium containing 5.5 mM glucose in the presence or absence of pravastatin (10 μM) for 14 days, serum-starved by the media containing 0.5% FCS for 16 h in 6 cm collagen-coated dishes, and then stimulated with thrombin (10 U ml^{-1}) for 1 h. Nuclear extracts were isolated, and used for gel mobility shift assay. A double-stranded DNA fragments containing the sequence of the binding site for the transcription factor AP-1 was labelled with digoxigenin-11-ddUTP by terminal transferase using a DIG gel shift kit (Roche). Nuclear proteins were then incubated with the DIG-labelled DNA fragment. The reaction mixture was loaded onto a 5% polyacrylamide gel and transferred onto a membrane. The bands of DNA-nuclear protein complex were detected with an anti-digoxigenin antibody (Roche). The bands were analysed by NIH image. The data are representative of three different experiments (means ± s.d.). $*P<0.001$, $**P<0.01$.

Discussion

Our results show that pravastatin, one of HMG-CoA reductase inhibitors (statins), decreases the IL-8 mRNA

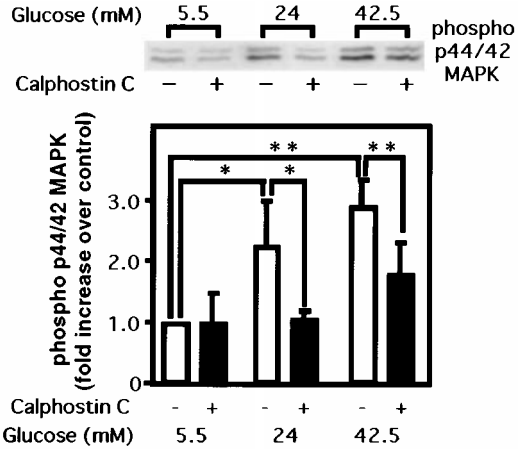

Figure 11 Effect of high glucose and Calphostin C on p44/42 MAP kinase activity in human aortic endothelial cells (AoEC). AoEC were cultured with medium containing 5.5, 24 or 42.5 mM glucose for 14 days, serum-starved with medium containing 0.5% FCS for 16 h, and then treated with Calphostin C (100 nM) for 1 h. Cell lysates were separated by 10% SDS–PAGE, transferred to membranes and blotted with phospho-specific p44/42 MAP kinase (Thr202 and Tyr204) antibody. The bands were analysed by NIH image, and the intensity of each phospho-MAP kinase band was corrected with that of the respective MAP kinase band. The data are representative of three different experiments (mean ± s.d.). $*P<0.05$, $**P<0.01$.

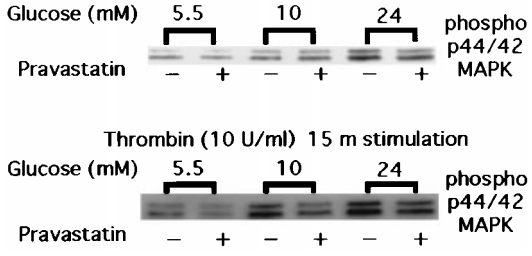

Figure 12 Effect of pravastatin on p44/42 MAP kinase activity induced by high glucose in human aortic endothelial cells (AoEC). AoEC were cultured with medium containing 5.5, 10 or 24 mM glucose in the presence or absence of pravastatin (10 μM) for 14 days, serum-starved by the medium containing 0.5% FCS for 16 h, and then stimulated with thrombin (10 U ml^{-1}) for 15 min. Cell lysates were separated by 10% SDS–PAGE, transferred onto membranes and blotted with phospho-specific p44/42 MAP kinase (Thr202 and Tyr204) antibody. The data are representative of three different experiments (mean ± s.d.).

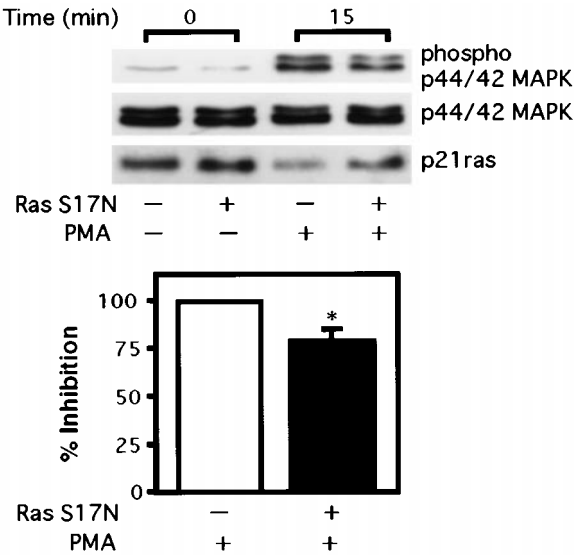

Figure 13 Effect of dominant negative *ras* protein on p44/42 MAP kinase activity induced by PMA in human aortic endothelial cells (AoEC). A dominant negative H-*ras* (S17N) was transfected into AoEC using Lipofectin. After transfection, the AoEC were cultured with medium containing 10% FCS for 48 h. The cells were then serum-starved with the media containing 0.5% FCS for 16 h before being stimulated with PMA (100 nM) for 15 min. Cell lysates were separated by 10% SDS–PAGE, transferred onto membranes and blotted with phospho-specific p44/42 MAP kinase (Thr202 and Tyr204) antibody and an Ha-*ras* antibody. The bands were analysed by NIH image, and the intensity of each phospho-MAP kinase band was corrected with that of the respective MAP kinase band. The data are the representative of three different experiments (mean ± s.d.). $^*P<0.001$ vs control (white bar).

expression induced by thrombin in endothelial cells through the inhibition of p44/42 MAP kinase activation. This is the first report showing that pravastatin may prevent the progression of atherosclerosis by regulating the inflammatory response and cytokine production from vascular endothelial cells.

IL-8 was originally reported as a chemotactic factor for the neutrophils. However, recent reports have revealed that IL-8 has also chemotactic effects on monocytes (Gerszten *et al*., 1999), T cells (Bacon *et al*., 1989), and aortic smooth muscle cells (Yue *et al*., 1993; 1994), suggesting that IL-8 may have an important role in the progression of atherosclerosis. It has been reported that thrombin stimulates the production of IL-8 from endothelial cells (Ueno *et al*., 1996; Kaplanski *et al*., 1997; Johnson *et al*., 1998; Anrather *et al*., 1997). We also reported here that thrombin enhanced the production of IL-8 in AoEC at both the protein and mRNA levels (Figure 1), suggesting that thrombin may accelerate atherosclerosis by increasing the inflammatory responses including IL-8 production. Besides, several investigators reported that both the generation and activation of thrombin were increased in diabetic patients compared to that in non diabetic subjects (Jones, 1985; Ceriello *et al*., 1992), which suggests that thrombin may play an important role in the progression of atherosclerosis in diabetic patients. The action of thrombin on endothelial cells is mediated through interactions with a specific thrombin receptor, which belongs to the G-protein coupled receptor family. The thrombin receptor is abundantly expressed on endothelial cells. Thrombin has been shown to activate a variety of signalling pathways in endothelial cells including NF-κB (Anrather *et al*., 1997), PKC (Haller *et al*., 1996), and MAPKK and MAPK (Wheeler-Jones *et al*., 1996). The transcription of the IL-8 gene is regulated by these signalling pathways. In our study, PD 98059, a potent inhibitor of p44/42 MAP kinase kinases (MEK), inhibited the thrombin-induced IL-8 mRNA in AoEC. However, as shown in Figure 9, PD 98059 did not inhibit the thrombin-induced phosphorylation of IκB-α, which is consistent with the report that PD 98059 failed to block the thrombin-induced NF-κB activation in porcine aortic endothelial cells (Anrather *et al*., 1997), suggesting that the p44/42 MAP kinase pathway does not transmit the signals responsible for thrombin activation of NF-κB. Calphostin C, an inhibitor of PKC, was found to inhibit the thrombin-induced phosphorylation of both p44/42 MAP kinase (data not shown) and IκB-α (Figure 9). Taken together, our results suggest that the MAP kinase pathway is involved in the signal transduction of thrombin-induced IL-8 mRNA expression, and that PKC exists upstream of NF-κB in the thrombin-induced signal transduction pathway for IL-8 synthesis. p44/42 MAP kinase phosphorylates Elk-1 and activates a serum response element, leading to c-*fos* induction, a component of AP-1. The AP-1 binding element is present in the promotor region of the IL-8 gene, and the AP-1, a complex of c-*fos* and c-*jun* or *jun* B, plays an important role in the transcriptional regulation of IL-8 mRNA expression (Matsushima *et al*., 1992). As shown in Figure 10, thrombin activated the AP-1 binding activity, and pravastatin inhibited the AP-1 activity, suggesting that pravastatin inhibits the expression of IL-8 mRNA at the level of transcription.

The large epidemiological studies show that HMG-CoA reductase inhibitors are useful for preventing the progression of atherosclerosis in patients with hypercholesterolaemia by reducing their serum cholesterol level (Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al*., 1995; Sacks *et al*., 1996; The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group, 1998; Plehn *et al*., 1999). However, the direct effect of HMG-CoA reductase inhibitors on the endothelial cells beyond cholesterol-lowering effect has not been examined fully. HMG-CoA reductase inhibitors reduce not only cholesterol but also intracellular pools of farnesyl and geranylgeranyl pyrophosphates, which are metabolites of mevalonate (Stark *et al*., 1998; Rubins *et al*., 1998). These metabolites are necessary for post-translational isoprenylation of proteins (Hancock *et al*., 1989; Maltese, 1990). Small GTP-binding proteins such as *ras* require post-translational isoprenylation to be localized in the plasma membrane. *Ras* protein localized in the plasma membrane is a key factor for extracellular signal activation, suggesting that HMG-CoA reductase inhibitors affect those signal transduction pathways. Indeed, inhibition of the *ras*-MAP kinase pathway by HMG-CoA reductase inhibitors especially in aortic smooth muscle cells (Negre-Aminou *et al*., 1997) and cardiac myocytes (Kashiwagi *et al*., 1998; Oi *et al*., 1999) has been reported. Therefore, we postulate that pravastatin may inhibit the isoprenylation of *ras* which in turn would affect it ability to interact with the plasma membrane and this inhibition of the *ras-raf*-MAP kinase pathway would lead to a reduced IL-8 production in AoEC. As shown in Figure 2, pravastatin inhibited the IL-8 synthesis induced by thrombin

in AoEC both at the protein and mRNA levels. We also confirmed that the treatment of AoEC with pravastatin also inhibited the processing of *ras* protein (Figure 7), showing the inhibition of *ras* protein isoprenylation. Interestingly, contrary to our expectations, pravastatin inhibited neither the p38 MAP kinase (Figure 5B) nor NF-κB pathways (Figure 9). These data suggest that pravastatin specifically inhibits the PKC dependent *ras-raf*-MAP kinase pathway in AoEC. On the other hand, several investigators reported that statins inhibited the *rho* activity in endothelial cells (Essig *et al*., 1998; Takeuchi *et al*., 2000). *Rho* is associated with cytoskeletal functions, however, since we did not examine the *rho* activity in the present study, we can not rule out the possibility that it is involved in the effects seen with pravastatin. However, we speculate that pravastatin may not affect *rho* activity because pravastatin did not inhibit the activity of p38 MAP kinase induced by thrombin (Zhang *et al*., 1995). Our results demonstrate the involvement of the *ras*-MEK-MAP kinase pathway as the mechanism by which pravastatin exerts its effect. However, the role of other small G proteins such as *rho* in the effect of pravastatin needs to be investigated further.

The mechanism of diabetic angiopathy is not yet fully understood. It has been suggested that a persistent activation of PKC in vascular tissues or cultured vascular cells induced by hyperglycaemia and the diabetic state is a major factor in the pathogenesis of diabetic angiopathy (King *et al*., 1994). As it has been reported that IL-8 gene expression is regulated by a PKC dependent pathway (Matsushima *et al*., 1992), the persistent activation of PKC by high glucose may lead to an increase in the steady state levels of IL-8 mRNA in AoEC. We also observed that Calphostin C and pravastatin inhibited the high glucose-induced p44/42 MAP kinase phosphorylation in AoEC, suggesting that IL-8 synthesis in response to high glucose is also regulated by the PKC-MAP kinase dependent pathway. PKC activates p44/42 MAP kinase through a *ras-raf* interaction by the activation of guanine-exchanging factor (Montessuit & Thorburn, 1999). We also showed that the phosphorylation of p44/42 MAP kinase induced by PMA was inhibited by the transfection of a dominant negative *ras* (Figure 13), suggesting that the pathway from PKC to p44/42 MAP kinase is at least partly dependent on the activation of *ras* protein.

In conclusion, our results suggest that pravastatin may prevent the progression of atherosclerosis not only by lowering plasma LDL cholesterol levels, but also suppressing IL-8 synthesis in endothelial cells *via* the inhibition of p44/42 MAP kinase and AP-1. The administration of pravastatin may be useful for preventing the progression of atherosclerosis even in normolipidaemic patients for whom the increased production of IL-8 from endothelial cells appears to aggravate the atherosclerosis such as diabetic patients (Urakaze *et al*., 1996; Temaru *et al*., 1997).

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