3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors Increase Fibrinolytic Activity in Rat Aortic Endothelial Cells

Role of Geranylgeranylation and Rho Proteins

Marie Essig, Geneviève Nguyen, Dominique Prié, Brigitte Escoubet, Jean-Daniel Sraer, Gérard Friedlander

Abstract—3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (HRIs) have been recently shown to prevent atherosclerosis progression. Clinical benefit results from combined actions on various components of the atherosclerotic lesion. This study was designed to identify the effects of HRI on one of these components, the endothelial fibrinolytic system. Aortas isolated from rats treated for 2 days with lovastatin (4 mg/kg body wt per day) showed a 3-fold increase in tissue plasminogen activator (tPA) activity. In a rat aortic endothelial cell line (SVARECs) and in human nontransformed endothelial cells (HUVECs), HRI induced an increase in tPA activity and antigen in a time- and concentration-dependent manner. In SVARECs, the maximal response was observed when cells were incubated for 48 hours with 50 \( \mu \text{mol/L} \) HRI. An increase of tPA mRNA was also in evidence. In contrast, HRI inhibited plasminogen activator inhibitor-1 activity and mRNA. The effects of HRI were reversed by mevalonate and geranylgeranyl pyrophosphate, but not by LDL cholesterol and farnesyl pyrophosphate, and were not induced by \( \alpha \)-hydroxyfarnesyl phosphonic acid, an inhibitor of protein farnesyl transferase. C3 exoenzyme, an inhibitor of the geranylgeranylated-activated Rho protein, reproduced the effect of lovastatin on tPA and plasminogen activator inhibitor-1 activity and blocked its reversal by geranylgeranyl pyrophosphate. The effect of HRI was associated with a disruption of cellular actin filaments without modification of microtubules. A disrupter of actin filaments, cytochalasin D, induced the same effect as lovastatin on tPA, whereas a disrupter of microtubules, nocodazole, did not. In conclusion, HRI can modify the fibrinolytic potential of endothelial cells, likely via inhibition of geranylgeranylated Rho protein and disruption of the actin filaments. The resulting increase of fibrinolytic activity of endothelial cells may contribute to the beneficial effects of HRI in the progression of atherosclerosis. (Circ Res. 1998;83:683-690.)

Key Words: atherosclerosis, plasminogen activator, isoprenoid, Rho protein, endothelium

Recent clinical trials have demonstrated that the major function of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (HRIs) is to prevent cardiovascular diseases. HRIs were shown to reduce cardiovascular events in patients suffering from coronary artery diseases, in patients with hypercholesterolemia but no artery disease, and in patients without hypercholesterolemia. HRIs also prevented the progression of atherosclerosis in coronary artery bypass grafts. HRIs were designed to inhibit the endogenous synthesis of cholesterol, thereby decreasing LDL synthesis in liver, thus leading to a reduction of lipid deposition in arteries and to a delayed growth of atherosclerotic plaques. However, some studies raised the question of the exclusive involvement of the decrease in plasma LDL concentration on the effects of HRIs. Indeed, HRIs induced clinical benefits before any regression in atherosclerotic plaques could be detected, they diminished cardiovascular mortality even in normocholesterolemic or hypocholesterolemic patients, and their effects were different from those observed after reduction of plasma cholesterol levels by surgical therapy. All these results suggest that the clinical benefit of HRI administration may result from multiple effects on the components of the atherosclerotic lesions. Atherosclerosis and vascular thrombosis result from complex changes and interactions between blood vessels and blood constituents, of which, fibrin deposition and fibrin lysis are major factors. Fibrinolysis results from the cleavage of fibrin by plasmin, a serine protease that is activated by the plasminogen activators (PAs). Two physiological PAs have been described, tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). In turn, tPA and uPA activity are tightly regulated by a specific inhibitor, plasminogen activator inhibitor-1 (PAI-1). Abnormalities such as high fibrinogen levels or high PAI-1 and low tPA levels have been described in patients with atherosclerosis.
with hyperlipoproteinemia. Lipoprotein(a), which reduces tPA-mediated clot lysis, and PAI-1 have also been shown to be an independent risk factor for myocardial infarction. In vivo studies of the effect of HRIs on fibrinolysis, in animals or in humans, led to conflicting results on tPA activity, and these discrepant results may be explained by a rapid clearance of PAs and PA/PAl-1 complexes by receptor-mediated endocytosis in the liver. Thus, analysis of plasma tPA and/or PAI-1 activity may not reflect the local modification of the fibrinolytic system, which could regulate the atherosclerotic process.

Since the local fibrinolytic activity in blood vessels is determined to a large extent by the endothelial cell production of tPA and PAI-1, we investigated the effect of HRI on the balance between tPA and PAI-1. We show that HRIs induce an increase of tPA synthesis and release and a decrease in PAI-1 activity. The resulting increase in local fibrinolytic activity may explain the beneficial effects observed with HRIs on atherosclerosis progression.

Materials and Methods

Materials

Lovastatin and simvastatin were kindly provided by Merck Sharp & Dohme-Chibret (Rahway, NJ). Mevastatin (compactin), mevalonic acid lactone, LDL cholesterol, farnesyl pyrophosphate (FPP), gera- nylnleranyl pyrophosphate (GGPP), cytochalasin D, nocardazole, mouse monoclonal antibody anti-α-tubulin, FITC-conjugated secondary antibody, TRITC-phalloidin, bovine fibrinogen, and bovine thrombin were purchased from Sigma Chemical Co. α-Hydroxyfarnesyl phosphonic acid (HFPA) was from Calbiochem. Agarose was from Eurbio. Anti-human uterine tPA and anti-human PAI-1 were from Biopool. SDS and 30% acrylamide/Bis (37:1 and 5:1) solutions were from Bio-Rad. Mevalonic acid lactone was incubated in 0.1N NaOH (2 hours, 50°C, 5:1) solutions were from Bio-Rad. Murine tPA cDNA was a gift from Dr J.-B. Michel (INSERM U 460, Paris, France). Culture media and reagents were from Gibco-BRL. Plasticware was from Costar.

The molecule of lovastatin was converted to its active form as described by Kita et al. To produce the mevalonate salt, 13 mg of mevalonic acid lactone was incubated in 0.1N NaOH (2 hours, 50°C, pH 7.4); the 0.1 mol/L stock solution was stored at -20°C.

Animal Experiments

Male Wistar rats (Ifa Credo) (160 to 180 g) received once-daily subcutaneous injections of either lovastatin or subcutaneous injections of either lovastatin at a dose of 4 mg/kg body wt or the propylene glycol/ethanol (vol/vol) vehicle. The proteins were transferred to a nitrocellulose membrane (Bio-Rad). Murine tPA cDNA was a gift from R. Lijnen (Leuven, Belgium). Human PAI-1 cDNA was from D.J. Loskutoff (La Jolla, Calif). Recombinant C3 exoenzyme was a kind gift from M.R. Popoff (Institut Pasteur, Paris, France). Culture media and reagents were from Gibco-BRL. Plasticware was from Costar.

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0.1× SSC and 0.1% SDS at 60°C. Autoradiography was performed by standard procedures using X-AR5 films (Kodak) and intensifying screens at −80°C. Radioactivity was quantified using an InstantImager (Packard).

Immunofluorescence

Cells, grown on glass coverslips, were fixed and stained after 24-hour incubation with cytochalasin D, nocodazole, lovastatin, GGPP, or C3 exoenzyme. Cells were washed with PBS, fixed in 4% formaldehyde/PBS for 10 minutes, and permeabilized in Triton X-100 (0.1% in PBS) for 5 minutes at room temperature. Cells were incubated either with TRITC-phalloidin (1:1000 in PBS) for 30 minutes at 37°C or with the monoclonal antibody anti-human α-tubulin for 1 hour at 37°C (1:2000 in 1% PBS/BSA) revealed by an FITC-conjugated secondary antibody. After 2 washes in PBS, coverslips were mounted using the Dako Faramount mounting medium. Stained cells were stored in the dark at 4°C until analysis with a Nikon microscope.

Results

In Vivo Effects of Lovastatin on tPA Activity

Rats treated for 2 days with lovastatin (4 mg/kg per day) showed a slight but nonsignificant decrease in the plasma cholesterol level compared with that of vehicle-treated rats (2.42±0.12 versus 2.18±0.2 mmol/L, \(P<0.002\)). Moreover, a previous study in the laboratory showed that a similar treatment of lovastatin for 15 days induced a significant decrease in plasma cholesterol values. These 2 results suggested that the treatment with lovastatin was effective. Isolated aortas showed a 3-fold increase in tPA activity compared with aortas of vehicle-treated rats (0.35±0.05 versus 1.05±0.8 IU/mg protein, \(P<0.002\)) (Figure 1). In contrast to tPA, no uPA activity could be detected by zymography or by immunoblot.

Characterization of the Effects of Lovastatin on the Fibrinolytic System of Endothelial Cells

To better characterize the effects of lovastatin on the fibrinolytic activity of endothelial cells, we next performed experiments on a rat aortic endothelial cell line (SVARECs) and on human endothelial cells (HUVECs) as controlled nontransformed cells. SVARECs, as nontransformed endothelial cells, synthesized and released some PAs to promote clot lysis. In SVAREC supernatants, only tPA was observed. No uPA activity could be detected by zymography or by immunoblot.

Figure 1. Stimulation of tPA activity in vivo. Aortas were isolated from rats treated or not treated with lovastatin (4 mg/kg per day) and lysed as described in Materials and Methods. tPA activity was quantified by a chromogenic assay and related to protein content of aortic lysates. Results are expressed as percentage of vehicle value. **\(P<0.002\).

Figure 2. Concentration-dependent stimulation of tPA activity by HRLs. Increasing concentrations of lovastatin, simvastatin, or compactin were added to confluent cultures of SVARECs or HUVECs for 48 hours. tPA activity was evaluated by a chromogenic assay. A, tPA activity (IU) of SVAREC supernatants. B, tPA activity related to protein content of HUVEC cell lysates. Results are expressed as percentage of control values. Values represent the mean±SE of 3 or 4 independent experiments. *\(P<0.05\) vs control value.

The same pattern of activity was observed in HUVEC supernatants. However, tPA activity was higher in SVAREC supernatant than in HUVEC supernatant (2.88±0.22 versus 0.11±0.006 IU).

As described in other systems, tPA synthesis by SVARECs and HUVECs was affected by the presence of FCS in the medium. Since the effect of FCS could have masked the effects of the HRL, we performed all subsequent experiments on SVARECs after 12 hours of FCS deprivation, and the cells were incubated in medium without FCS. For HUVECs, which could not be serum-starved, we performed the experiments with 1% FCS, the lowest FCS concentration capable of maintaining the viability of HUVECs for 36 hours.

Lovastatin, added to the medium for 48 hours, induced a dramatic increase in tPA activity that was detectable in SVAREC supernatants (control, 2.88±0.22 IU; 25 μmol/L lovastatin, 8.75±0.66 IU; \(P<0.001\)). This effect on tPA secretion was observed in the 1- to 50-μmol/L range, with a maximum at 50 μmol/L (Figure 2A), and was reproduced by
Lovastatin Increases Endothelial Fibrinolytic Activity

Figure 3. Time course showing stimulation of tPA activity by lovastatin. Confluent SVARECs were incubated in the absence or presence of lovastatin (25 μmol/L). Supernatants were collected after various incubation times and analyzed by zymography.

2 other HRIs, simvastatin and compactin, in the same range of concentrations (Figure 2A).

Studies performed on HUVECs showed that lovastatin similarly increased tPA activity (control, 0.457±0.018 IU/mg protein; 5 μmol/L lovastatin, 1.29±0.117 IU/mg protein; P<0.001). Furthermore, the effects were observed for 10-fold lower concentrations of lovastatin in HUVECs compared with SVARECs (Figure 2B).

Time-course studies showed that the increase of tPA due to lovastatin was already detectable after a short incubation time (3 hours) and increased in magnitude with the incubation time, reaching a maximum after 48 hours of incubation (Figure 3).

The increase in tPA activity induced by lovastatin was attributed to an increase in protein expression and not to an increase of tPA-specific activity, since it was accompanied by an increase of tPA antigen visualized by immunoblot: a single band of 68 kDa was detected by immunoblot analysis in control cells and increased in the presence of lovastatin (Figure 4A). This increase was likely the consequence of increased protein synthesis, inasmuch as tPA mRNA content was induced 2.5-fold after 60 minutes of incubation with lovastatin (25 μmol/L) (Figure 4B).

PAI-1 secretion was also affected by lovastatin, which induced a decrease in PAI-1 activity (Figure 5A) and mRNA (Figure 5B). Because of the poor sensitivity of the method, we could not detect PAI-1 antigen by immunoblot. The decrease in PAI-1 activity occurred for the same concentration range of lovastatin. The concomitant increase in tPA and decrease in PAI-1 activities is likely to be synergistic with respect to the stimulation of the fibrinolytic system by lovastatin.

Reversal of the Effects of Lovastatin by Isoprenoids

The effects of lovastatin on tPA activity and protein expression were completely reversed by mevalonate (500 μmol/L), the synthesis of which is catalyzed by HMG CoA reductase, whereas LDL cholesterol (50 μg/mL) did not (control, 3.49±0.06 IU; lovastatin, 12.76±0.782 IU; lovastatin+mevalonate, 3.53±0.53 IU; and lovastatin+LDL cholesterol, 13.99±0.53 IU; P<0.001). These results suggested that the effects of lovastatin involved a modulation of the mevalonate pathway but that end derivatives of the mevalonate pathway, such as cholesterol, were not involved in tPA modulation.

Among the early derivatives synthesized along the mevalonate pathway, the isoprenoids FPP and GGPP are involved in protein regulation by a posttranslational modification leading to the adjunction of the isoprenoids to the COOH terminal part of the protein. To determine whether the effects of lovastatin were mediated by the isoprenylation of a protein, we incubated cells in the presence of lovastatin and of the 2 isoprenoids FPP and GGPP (Figure 6). FPP and GGPP alone did not modify tPA and PAI-1 activity (data not shown). FPP (15 μmol/L) did not abolish tPA increase, and HFPP (15 μmol/L), a competitive inhibitor of the farnesyltransferase, could not reproduce the effects of lovastatin, suggesting that modulation of a farnesylated protein was not involved in tPA regulation. In contrast with FPP, GGPP (15 μmol/L) completely inhibited the augmentation of tPA and the decrease of PAI-1 activity observed with lovastatin (Figure 6A). tPA protein increase was also prevented by
GGPP, as shown by the immunoblot analysis (Figure 6C), suggesting the involvement of a geranylgeranylated protein in tPA regulation.

Interaction With Intracellular Signaling Pathways
Among the isoprenylated small GTP proteins, the Rho and the Rab proteins are known to be mostly geranylgeranylated. To determine whether the effects of lovastatin on tPA activity may result from the inhibition of Rho proteins, we incubated cells in the presence of C3 exoenzyme, a specific inhibitor of the Rho proteins (26) (Figure 7). C3 exoenzyme (5 μg/mL) induced the same effect as lovastatin on tPA and PAI-1 activity, had no synergistic effects with FPP and lovastatin, and blocked the reversal of the effects of lovastatin by GGPP, suggesting the involvement of Rho proteins. The involvement of other geranylgeranylated proteins different from Rho proteins seemed highly improbable, since C3 exoenzyme completely prevented the effects of GGPP, and GGPP did not modify the effects of C3 exoenzyme on tPA and PAI-1 synthesis.

Modification of the Cytoskeleton
Since Rho proteins are known to regulate the cytoskeletal proteins, we evaluated whether the modulation of tPA activity could be accounted for by a modification of the cytoskeleton. Cytochalasin D, a well-known disrupter of the actin filaments, dose-dependently (20 nmol/L to 20 μmol/L) induced an increase in tPA activity. This effect was correlated with the progressive disappearance of the cellular actin stress fibers (Figure 8A). In contrast, nocodazole, which disrupts microtubules, had no effect on tPA activity and actin stress fibers (Figure 8B). Lovastatin (25 μmol/L) induced a disruption of the cellular actin stress fibers compared with control cells, without any modification of the microtubule network (Figure 9). The correlation between the organization of actin filaments and the level of tPA was also reproduced with the GGPP, FPP, and C3 exoenzyme (data not shown). GGPP completely prevented the disappearance of actin stress fibers induced by lovastatin, whereas FPP did not. C3 exoenzyme induced the same disruption of the cellular actin filaments as lovastatin and blocked the reversal of the effect by GGPP. These results may suggest that tPA activity was regulated by the cytoarchitecture of the cells and that the induction of tPA synthesis by lovastatin might result from modifications of the actin filaments.

Figure 6. Reversal of lovastatin-induced tPA stimulation and PAI-1 inhibition by isoprenoids. SVARECs were incubated with HFPA (15 μmol/L), GGPP (15 μmol/L), and FPP (15 μmol/L) in the presence or absence of lovastatin (25 μmol/L) for 48 hours before cell supernatants were collected for analysis. A, Detection of tPA and PAI-1 activity by zymography and reverse zymography, respectively. B, Quantification of tPA lysis zone (zymography) and PAI-1 lysis protection zone (reverse zymography). Results are the mean of 5 independent experiments. C, Immunoblot of tPA protein.

Figure 7. Effect of Rho inhibition on fibrinolysis and reversal of the effects of lovastatin by GGPP. SVARECs were incubated with C3 exoenzyme (5 μg/mL), GGPP (15 μmol/L), and FPP (15 μmol/L) in the presence or absence of lovastatin (25 μmol/L) for 48 hours before cell supernatants were collected for analysis. A, tPA and PAI-1 activity detected by zymography and reverse zymography, respectively. B, Quantification of tPA lysis zone (zymography). Results are the mean of 4 independent experiments.
Discussion

In the present study, we demonstrated for the first time a direct action of inhibitors of the HMG CoA reductase on the endothelial fibrinolytic system. Lovastatin and 2 other inhibitors, simvastatin and compactin, induced a dramatic increase in the local fibrinolytic system by stimulating tPA expression and activity, an effect that was potentiated by the inhibition of PAI-1. This effect was dependent on geranylgeranylated proteins, which could be the Rho proteins, and was associated with a disruption of the actin filaments.

Although the concentration of lovastatin used in the present study was higher than that observed in the plasma of patients treated with lovastatin (0.01 to 0.5 μmol/L),28 the relevance of the effects observed in this study for patients treated with HRI was supported by several facts. First, the results were observed in vivo with doses of lovastatin previously reported in the treatment of hyperlipidemia in different models of hyperlipidemic rats.29,30 Second, the concentration of lovastatin needed to induce tPA activity in nontransformed human endothelial cells (HUVECs) was in the same range as that which inhibits the proliferation of smooth muscle cells,31 glomerular mesangial cells,32 and fibroblasts.33 In SVARECs, higher concentrations of HRIs were necessary to observe the same effects. It is likely that these differences could be accounted for by the higher HMG CoA reductase activity present in simian virus 40–transformed cell lines, such as SVARECs, as demonstrated by Larsson.34

In contrast to the inhibitory effect of lovastatin on proliferation, which was observed after a long incubation period with the drug (24 and 48 hours), stimulation of fibrinolysis was detectable after treatment as short as 2 days and an incubation time of 3 hours.

The fact that simvastatin and compactin, 2 other HRIs, reproduced the effects of lovastatin indicated that the mechanism of the stimulation was related to the inhibition of the mevalonate pathway (which leads to the synthesis of cholesterol) and of a variety of isoprenoid metabolites: dolichol, ubiquinone, and the isoprenoids FPP and GGPP. The com-
complete reversal by mevalonate of the action of lovastatin confirmed this hypothesis.

Since interactions between cholesterol, LDL cholesterol, and fibrinolysis have been suspected for a long time by epidemiological studies or by immunohistological and in situ hybridization analysis of atherosclerotic lesions, we first explored the modulation of fibrinolysis exerted by the exogenous precursor of cholesterol, LDL cholesterol. In the present study, incubation of SVARECs with LDL cholesterol did not induce any modification of tPA. Furthermore, LDL cholesterol did not prevent the increase in tPA activity induced by lovastatin. As demonstrated by Steinbrecher et al. LDL cholesterol is oxidized by endothelial cells to form oxidized LDL cholesterol. Our results suggested that oxidized LDL did not modulate fibrinolysis in SVARECs and that lovastatin action did not result from an inhibition of oxidized LDL. These results are in agreement with those of Latron et al., who did not find any modulation of tPA synthesis by native LDL or oxidized LDL. Results obtained in SVARECs suggest that lovastatin-induced modulation of tPA did not result from a decrease in endogenous cholesterol synthesis and raise the possibility that HRIs could prevent atherosclerosis even in normcholesterolemic patients.

Regarding other metabolites of the mevalonate pathway, we determined that lovastatin action was blocked by GGPP, whereas FPP had no effect. Furthermore, HFPA, a potent inhibitor of farnesyl transferase, did not reproduce lovastatin stimulation of tPA synthesis. FPP and GGPP have been implicated in protein isoprenylation, in the regulation of cell proliferation, and in signal transduction. Prenylated proteins share characteristic C-terminal sequences that determine which of the 2 isoprenoids, FPP or GGPP, is covalently linked to the protein. Since C-terminal sequences of tPA and PAI-1 do not possess such sequences, it is unlikely that these proteins are directly regulated by isoprenylation. The absence of reversal by FPP and the lack of effect of HFPA suggested that p21 ras, the major farnesylated protein that is inhibited by HRIs, is not involved in lovastatin action. Indeed, previous studies have rather demonstrated that p21 ras stimulated fibrinolysis. In contrast, reversal of the action of lovastatin by GGPP occurred at low concentrations of this metabolite (15 μmol/L) compared with the effective concentration of mevalonate (500 μmol/L), thus suggesting a specific involvement of a geranylgeranylated protein in fibrinolysis modulation. To our knowledge, the present study is the first to demonstrate a direct effect of geranylgeranylated proteins in the regulation of fibrinolysis of endothelial cells.

Among the geranylgeranylated proteins, the Rho family is one of the most important being implicated in various cell functions, such as cell morphology, cell motility, or cyto- kinesis. We demonstrated that the effect of lovastatin on tPA and PAI-1 was reproduced by the C3 exoenzyme, which inhibits Rho proteins by ADP-ribosylation on asparagine 41 in the effector region of the GTPase. Furthermore, we determined that C3 exoenzyme blocked the reversal of the effects of lovastatin induced by GGPP, thus suggesting the involvement of the Rho protein family in the action of lovastatin on fibrinolysis.

The mechanism linking Rho proteins to fibrinolysis is not fully elucidated. An involvement of the cytoskeleton is suspected, since Rho proteins are known to regulate the organization of the cytoskeleton and the formation of actin filaments. Along this line, Fenton et al. and Bifulco et al. demonstrated that HRIs could modify the cytoskeleton organization and the polymerization of intracellular actin. We confirmed on SVARECs that HRIs induced a disruption of the actin filament network without affecting the microtubes. This effect, which was reversed by GGPP, seemed to depend on activated Rho proteins, since it was blocked by C3 exoenzyme. The effects of HRIs on fibrinolysis, on the one hand, and on the disruption of the actin filaments through the inhibition of Rho proteins, on the other hand, are likely to be linked. Indeed, cytochalasin D, which is known to disrupt actin filaments, induced the same effect as lovastatin and C3 exoenzyme, whereas nocodazole, which disrupts the microtubules, did not. Along the same line, Snyder et al. demonstrated a stimulation of tPA by dihydrocyclochalin B in F9 teratocarcinoma cells. Moreover, the extensively studied link between cytoskeleton and urokinase suggests that modulation of the cytoskeleton could be a potent regulator of tPA synthesis. However, although a link between cytoskeleton reorganization and upregulation of tPA by HRI is suspected, further studies are necessary to assess it definitely.

In conclusion, in the present study, we demonstrated a link between an enhancement of the endothelial fibrinolytic system and the inhibition of the mevalonate pathway by the well-known hypocholesterolemic agents lovastatin, simvastatin, and compactin. The action of the HRIs involved an inhibition of geranylgeranylated proteins, probably the Rho proteins, and was associated with a disruption of the cytoskeleton. These effects, which were observed also in vivo, suggest that HRIs, through the enhancement of local plasmin generation, might prevent fibrin or extracellular matrix deposition in arterial plaques and acute thrombosis in the injured vessel, which are 2 major determinants of the progression of atherosclerosis and cardiovascular mortality.

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