

Statins inhibited erythropoietin-induced proliferation of rat vascular smooth muscle cells.

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Abstract

Erythropoietin (EPO) directly stimulates the proliferation of vascular smooth muscle cells, and this is believed to be one of the mechanisms of vascular access failure of hemodialysis patients. However, precise mechanisms of the EPO-induced proliferation of vascular smooth muscle cells are not certain. HMG-CoA reductase inhibitors (statins) are primarily used to reduce cholesterol levels, but also exert other effects, including reno-protective effects. We evaluated the effect of several statins with various hydrophilicities on the EPO-induced proliferation of primary cultured rat vascular smooth muscle cells (VSMCs) in vitro. EPO significantly and concentration-dependently increased DNA synthesis as assessed by [³H]thymidine incorporation, cell proliferation as assessed by WST-1 assay, and activation of the *p44/42MAPK* pathway. Therapeutic doses of statins (pravastatin, simvastatin, atorvastatin and fluvastatin) in patients with hypercholesterolemia almost completely suppressed all of the EPO-induced effects in a concentration-dependent manner. Co-addition of mevalonic acid almost completely reversed the effects of statins. Statin alone did not affect the basal proliferation capacity of the cells. The effects were almost similar among the statins. We concluded that statins inhibited EPO-induced proliferation in rat VSMCs at least partly through their inhibition of HMG-CoA reductase activity. In the future, statins might prove useful for the treatment of EPO-induced hyperplasia of vascular access. Because the statins all showed comparable effects irrespective of their hydrophilicities, these effects might be a class effect.

Keywords: erythropoietin, smooth muscle cell, proliferation, statin, vascular access failure, MAP kinase

1. Introduction

Erythropoietin (EPO) is the main glycoprotein growth factor that regulates the survival, proliferation, and differentiation of erythroid progenitor cells.(Krantz, 1991) Its receptor is expressed not only in these cells but also in vascular smooth muscle cells (VSMCs) and endothelial cells.(Ammarguella et al., 1996) as well as neuronal and glial cells (Ntaigos et al, 2008). EPO is important for the treatment of renal anemia, and correction of anemia by the drug improves the quality of life in patients with renal failure. However, EPO can contribute to stenosis and occlusion of vascular access for hemodialysis by promoting hyperplasia of vascular wall and thrombosis (Himmelfarb et al., 2005; Lee et al., 2009). Some studies in vivo have shown that long term EPO therapy may not increase the risk of stenosis of the arteriovenous fistula. (De Marchi et al.,1997) Vascular access failure is a major cause of morbidity and hospitalization in hemodialysis populations worldwide (Himmelfarb et al., 2005; Roy-Chaudhury et al., 2006). However, no pharmacological approach to avoid the EPO-related adverse reaction were developed until present time. EPO also causes hypertension in 20-30% of hemodialysis patients(Maschio, 1995) and the EPO-induced hyperplasia of blood vessel is believed as one of the mechanisms of the hypertension (Eggena et al., 1991; Hand et al., 1995).

The mechanism of EPO-induced hyperplasia of vascular wall is not fully understood. Intravenous administration of EPO results in a severe, but transient, increase in drug concentration within the vascular access. It has been reported that EPO acts directly on VSMCs in vitro, such as by increasing their cytoplasmic Ca⁺⁺ concentration, causing contraction(Akimoto et al., 2001) and

stimulating proliferation(Ito et al., 2002). In erythroid cells, EPO stimulates several signal transduction systems for cell proliferation, such as JAK-STAT, RAS-MAPkinase, inositol phosphate, and PI3K-AKT. Among these systems, the MAP kinase cascade is the most important for the cell proliferation stimulated by EPO.(Nishida and Gotoh, 1993) EPO also stimulates vasoconstriction by activation of the local renin angiotensin system,(Eggena et al., 1991) increased vasoconstrictor response to catecholamines,(Hand et al., 1995) and effects on prostaglandins(Bode-Böger et al., 1996), and actions on the endothelium, such as increased endothelin production(Nagai et al., 1995) and modification of endothelial function.(Ioka et al., 2009)

HMG-CoA reductase inhibitors (statins) are used for the treatment of hypercholesteremia, which they ameliorate in part by decreasing the generation of mevalonic acid from acetate in the liver.(Yusuf et al., 2009) The most important adverse reaction of these drugs is rhabdomyolysis although it is rare except in patients also receiving some agents such as cyclosporine, nicotinic acid, and gemfibrozil.(Yusuf et al., 2009) Statins are partly excreted into urine.(Lennernäs and Fager, 1997) Therefore, the prescription of statins to patients with renal failure patients needs to be handled with caution. On the other hand, statins possess pleiotropic effects, such as the improvement of endothelial function, anti-inflammatory effects, anti-oxidant effects and inhibition of cell proliferation.(Liao and Laufs, 2005) It has also been reported that statins protect against renal injury in both clinical and basic researches.(Navaneethan et al., 2009a; Navaneethan et al., 2009b) There are a number of negative

controlled trials, which have demonstrated that statins are ineffective in preventing cardiovascular disease in hemodialysis patients. (Fellström BC et al., 2009) However, their pleiotropic effects on other sites (such as VSMCs) may still be of benefit to the patient.

The purpose of this study was to evaluate the effect of statins on EPO-induced cell proliferation in rat VSMCs in vitro. Because the hydrophilicities of statins vary,(Lennernäs and Fager, 1997) we used several hydrophilic and lipophilic statins and compared their effects. We also assessed the pathway through which statins exhibit their effect using mevalonic acid.

2. MATERIALS AND METHODS

2.1 Materials

All materials were obtained from Sigma Chemical Co. (St. Louis, MO) except for the following. Recombinant human erythropoietin (epoetin-alfa, EPO) was a generous gift from Chugai Pharmacological (Tokyo, Japan). Pravastatin and fluvastatin were supplied from Daiichi-Sankyo Company Limited (Tokyo, Japan). Simvastatin was purchased from Wako Chemical (Osaka, Japan). The p44/42 MAPK assay kit and the anti-p44/42 MAPK and anti-phospho-p44/42 MAPK (Thr202/Try204) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). [3H]Thymidine was purchased from Perkin Elmer Life Sciences (Boston, MA, USA). Pravastatin and fluvastatin were dissolved with deionized water, while atorvastatin and simvastatin were dissolved with DMSO (0.005% at final concentration) and ethanol (0.005% at final concentration), respectively.

2.2 Cell culture

Primary culture of VSMCs were isolated from the aortas of male Sprague–Dawley rats (150–200 g; purchased from Japan SLC, Inc., Shizuoka, Japan) as previously described.(Ito et al., 2002)(Yamamoto et al., 2006)(Akimoto et al., 2001) In brief, primary culture of VSMCs were identified by their typical hill and valley morphology and by indirect immunofluorescent staining for α -smooth muscle actin (R&D Systems Inc., Minneapolis, MN, USA). The VSMCs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (ICN Biomedicals, Osaka,

Japan), 100 U/ml penicillin, and 100 mg/ml streptomycin (Life Technology Inc., Rockville, MD, USA) in a 5% CO₂ incubator at 37°C. The cells were used between passages 3 and 8. Mono-layered VSMCs at 70–80% confluence were growth-arrested by incubation in DMEM with 0.5% FBS for 48 h and served for the experiments. We measured endotoxin concentration in the medium by the limulus amoebocyte lysate method (Wako Pure Chemical Industries, Osaka, Japan) and found that it was under the detection limit, which strongly indicated the absence of lipopolysaccharide in the medium. All procedures were conducted in accordance with the Jichi Medical School guide for laboratory animals.

2.3 [³H]Thymidine incorporation (Yamamoto et al., 2006)

The cells were seeded on 24-well culture plates, allowed to grow to 70–80% confluence, and then growth-arrested by incubation in DMEM with 0.5% FBS for 48 h. The cells were incubated for 24 h with EPO (or 10% FBS as a positive control), and [³H]thymidine (1 mCi/ml; specific activity, 79 Ci/mmol) was added for the last 6 h of the incubation period. The cells were then washed three times with phosphate-buffered saline, treated with ice-cold 10% trichloroacetic acid at 4°C for 15 min, and washed with phosphate-buffered saline. The acid-insoluble material was dissolved in 0.5 ml of 0.3 N NaOH. The protein content was measured by a DC Protein Assay (Bio-Rad, Hercules, CA, USA), and radioactivity was determined by using a liquid scintillation counter (Aloka, Tokyo, Japan).

2.4 WST-1 assay for Cell proliferation(Yamamoto et al., 2006)

VSMC proliferation was assessed using a Cell Counting Kit.(Yamamoto et al., 2006) The cells were seeded at a density of 5×10^3 cells/well on 96-well culture plates in DMEM with 10% FBS for 72 h. After serum starvation for 48 h in DMEM with 0.5% FBS, the cells were stimulated by EPO (or 10% FBS as a positive control) for 24 h. For the final 4 h of incubation, 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] was added to each well and the absorbance was measured at 450 nm with a reference wavelength of 650 nm using a microplate spectrophotometer system (SOFTmax PRO; Molecular Devices Corporation, Sunnyvale, CA, USA). The difference of absorbance between 450 and 650 nm was regarded as the cell proliferation.

2.5 Western blot analysis (Yamamoto et al., 2006)

Growth-arrested cells cultured in 100-mm dishes were stimulated by EPO or 10% FBS as a positive control for the indicated times. The cells were washed with ice-cold phosphate-buffered saline and lysed in 400 μ l of lysis buffer (1% Triton, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA , 1 mM ethyleneglycol tetraacetate, 2.5 mM sodium pyrophosphate, 1 mM -glycerophosphate, 1 mM Na_3VO_4 , 1 mg/ml L-leupeptin, 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C. The cell lysates were centrifuged for 15 min at 15,000 g and the supernatants were collected. Equal amounts of protein (20 μ g) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrophoretically transferred to

polyvinylidene difluoride membranes (Invitrogen Corp., Carlsbad, CA, USA). The membranes were blocked for 1 h at room temperature with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% bovine serum albumin. After washing with TBS-T, the membranes were incubated overnight with phospho-p44/42 MAPK (Thr202/Tyr204) antibody (1:1000) or p44/42 MAPK antibody (1:1000) at 4°C with gentle shaking. The primary antibodies were detected using horseradish peroxidase-conjugated goat antirabbit IgG and visualized by enhanced chemiluminescence Western blotting reagents (Amersham Biosciences, Buckinghamshire, UK). Band intensity was analyzed using a lumino-image analyzer (LAS-3000 min; Fuji Film Company Limited, Tokyo, Japan).

2.6 Statistics

The results are expressed as the mean \pm S.E.M. Number of each experiment was 3 to 6. Data were analyzed by the unpaired Student's t-test or by one-way analysis of variance combined with Fisher's protected least significant difference using a personal computer with StatView version 5.0 software (SAS Institute, Cary, NC, USA). Values of $P < 0.05$ were considered to indicate statistical significance. Figures are each representative of three or more experiments.

3. Results

3.1 EPO increased [³H]thymidine incorporation and cell proliferation, and activated the p44/42MAPK pathway in rat VSMCs

First, we tried to confirm the previous finding about EPO-stimulated [³H]thymidine incorporation and cell proliferation. As shown in Fig.1, EPO increased [³H]thymidine incorporation in a concentration-dependent manner. The increase was significant at concentrations of 120 IU/ml or higher, and thus 120 IU/ml EPO was the concentration used in the following experiments. 10% FBS, which was used as a positive control, also increased the thymidine incorporation. VSMC proliferation determined by WST-1 assay was also increased by addition of EPO in a concentration-dependent manner, and the increase was significant at 120 IU/ml or more (Fig.2).

To identify further intracellular signaling events involved in the effects of EPO on the rat VSMC proliferation, we investigated the activation of p44/42 MAPK in VSMCs. We confirmed in a preliminary study that the phosphorylation of p44/42 MAPK was most prominent when EPO was added for 5 min (data not shown). As shown in Fig.3, EPO activated the p44/42MAPK pathway in a concentration-dependent manner, and the activation was significant at more than 30 IU/ml. Thus we confirmed the previous finding (Ito et al., 2002) that EPO increases [³H]thymidine incorporation and cell proliferation, and activates the p44/42MAPK pathway in rat VSMCs. According to these findings, we selected 120 IU/ml of EPO for the following experiments to determine the effect of statins on EPO-induced VSMC proliferation.

3.2 EPO-stimulated VSMC proliferation was prevented by pravastatin via inhibition of HMG-CoA reductase activity

As shown in Fig.4, pravastatin significantly inhibited EPO-stimulated [³H]thymidine incorporation. The inhibition was partial at 10 nM and was complete at more than 100 nM, and the latter concentration is compatible with the plasma maximum concentration in clinical use. Furthermore, 100 nM pravastatin alone did not affect the [³H]thymidine incorporation. These results indicated that the therapeutic dose of pravastatin did not affect basal DNA synthesis, but inhibited EPO-stimulated DNA synthesis in rat VSMCs.

In order to evaluate whether the effect of pravastatin was due to inhibition of HMG-CoA reductase by the drug, we added mevalonic acid with pravastatin. As shown in Fig.5A, co-addition of mevalonic acid (100 μM) partly recovered the pravastatin-inhibited [³H]thymidine incorporation stimulation. This result indicated that EPO-stimulated DNA synthesis was prevented by inhibition of HMG-CoA reductase by pravastatin.

A similar inhibitory effect of pravastatin via suppression of HMG-CoA reductase activity was observed in EPO-induced cell proliferation and activation of MAPK. As shown in Fig.5B, EPO-induced cell proliferation was inhibited by pravastatin, and this effect was reversed by co-addition of mevalonic acid. Figure 5C shows that phosphorylation of MAPK was activated by EPO and it was inhibited by addition of pravastatin. Furthermore, the inhibition by pravastatin was reversed by co-addition of mevalonic acid. Thus pravastatin suppressed VSMC

proliferation via the inhibition of HMG-CoA reductase activity.

3.3 Effects of simvastatin, atorvastatin, and fluvastatin on EPO-induced VSMC proliferation

Next, we evaluated the effects of other statins. Similar to pravastatin, simvastatin (up to 1000 nM) inhibited EPO-induced [³H]thymidine incorporation in a concentration-dependent manner, and the inhibition was significant when the dose was more than 100 nM, while simvastatin at 100 nM alone did not affect the [³H]thymidine incorporation (data not shown). As shown in Fig. 6A, the inhibition by 100 nM of simvastatin was reversed by co-addition of mevalonic acid (100 μM). The stimulation of cell proliferation by EPO was also decreased by simvastatin and also reversed by co-addition of mevalonic acid (Fig. 6B). Activation of p44/42MAPK by EPO was inhibited by simvastatin and also reversed by co-addition of mevalonic acid (Fig. 6C).

We also evaluated atorvastatin and fluvastatin. These two statins (up to 1000 nM) also inhibited EPO-induced [³H]thymidine incorporation in a concentration-dependent manner, and the inhibition was significant when the dose was more than 100 nM, while statins at 100 nM alone did not affect the [³H]thymidine incorporation (data not shown). The inhibition of [³H]thymidine incorporation by 100 nM of the drugs was reversed by co-addition of mevalonic acid (100 μM). The EPO-stimulated cell proliferation was also decreased by statins and also reversed by co-addition of mevalonic acid. Activation of p44/42MAPK by EPO was inhibited by the statins and also reversed by

co-addition of mevalonic acid (Table 1 and 2).

4. Discussion

Before we evaluated the effects of EPO on statins, we first tried to confirm the previous findings that EPO stimulated VSMC proliferation in vitro.(Ito et al., 2002) We found that EPO at a concentration of more than 120 IU/ml stimulated thymidine uptake and DNA synthesis, and subsequently increased cell proliferation. The concentration needed for the stimulation was also compatible with previous reports. We also found that EPO activates phosphorylation of MAPK. This is compatible with previous results that EPO activated Raf-1 and MEK, which are upstream of the MAPK cascade in VSMC.(Carroll et al., 1991; Ito et al., 2002)

After this confirmation, we demonstrated that statins inhibited the EPO-induced proliferation of VSMCs, which was the most important observation in this study. In addition to their effects on cell proliferation, we also found that statins prevented DNA synthesis and activation of MAPK by EPO. It has previously been reported that statins inhibited the VSMC proliferation induced by several growth-factors.(Rupérez et al., 2007; Sakamoto et al., 2005) However, an inhibitory effect of statins on EPO-induced proliferation has not been reported. EPO-induced hypertension is one of the most important adverse reactions of the drug(Maschio, 1995). EPO-induced VSMC proliferation in vitro has been reported as a possible model for use in investigating the mechanisms of EPO-induced hypertension. Therefore, our present finding that statins inhibited EPO-stimulated VSMC proliferation suggests a possible future application of statins for reversing EPO-induced hypertension in patients with renal anemia.

It has been reported that statins inhibit the growth factor-induced proliferation of

several types of cells, including VSMCs.(Rupérez et al., 2007; Sakamoto et al., 2005) But the doses used in these studies were on the micromolar order, which is larger than the therapeutic doses used for the treatment of hyperlipidemia.(Rupérez et al., 2007; Sakamoto et al., 2005) It has also been reported that the basal VSMC proliferation capacity without stimulants was inhibited by a pharmacological dose of statins.(Bellosta et al., 2004) In our study, the dose of statins required to inhibit the cell proliferations was about 100 nM, which is lower than those reported in the literature and close to the therapeutic dose required for the treatment of hyperlipidemia.(Lennernäs and Fager, 1997) Furthermore, the administration of 100 nM of statin alone did not affect the basal VSMC proliferation in this study. Thus, our findings indicated that lower or therapeutic doses of statins did not inhibit basal VSMC proliferation but prevented the EPO-stimulated proliferation..

Because statins primarily inhibit HMG-CoA reductase activity and generation of mevalonic acid from HMG-CoA in the liver,(Yusuf et al., 2009) we evaluated the effect of co-addition of mevalonic acid with statins in order to examine the mechanism of the preventive effect of statins on the EPO-induced cell proliferation. We found that co-addition of mevalonic acid with statins partly reversed the effect of statins. This result indicated that the effect of statins observed in this study was at least partly related to the inhibition of HMG-CoA reductase by the drug. It is uncertain how inhibition of HMG-CoA reductase activity leads to inhibition of VSMC. It has also been reported that statins have several pleiotropic effects that are not related to their inhibition of HMG-CoA reductase,(Liao and Laufs, 2005) and such effects may also have played a role

in the present findings.

We evaluated four statins in this study. Although all these drugs equally inhibit HMG-CoA reductase, their hydrophilicities vary. Pravastatin is the most hydrophilic and simvastatin is the most lipophilic among commercially available statins, while atorvastatin and fluvastatin are intermediate.(Yusuf et al., 2009)

The prevention of EPO-induced VSMC proliferation by the statins obtained in this study was not dependent on the hydrophilicity of the drug, and seems to be a class effect. On the other hand, it has also been reported that lipophilic statins inhibit cell proliferation induced by serum, while pravastatin, a hydrophilic statin, does not inhibit the cell proliferation in SV40-transfected smooth muscle cells in mice.(Sindermann et al., 2000) The authors of this previous study speculated that pravastatin is hydrophilic and less permeable to the cell membrane if specific transporters of the drug are not present, while other lipophilic statins can enter into the cell without the assistance of transporters. Pravastatin is a substrate of organic anion transporting polypeptide (OATP), and OATP is expressed in some muscle cells.(leiri et al., 2009) Therefore, although all statins inhibited EPO-induced VSMC proliferation in the present study, the mechanism by which the different statins entered into the VSMCs may have differed. It is also reported that some lipophilic statins induced apoptosis of VSMCs (Katsiki N, et al., 2009), which may affect to the phenomenon observed in this study.

In this study, we confirmed the previous finding that EPO stimulated VSMC proliferation, and the dose of EPO required for this stimulation was comparable with those reported previously.(Akimoto et al., 2001)(Ammarguella et al., 1996; Ito et al., 2002) In a clinical setting, the plasma concentration of EPO after

repeated dosing is reported to be about 10 times lower than the dose used in this study.(Krzyzanski et al., 2005) However, Intravenous administration of EPO which is often used in hemodialysis patients, results in a severe, but transient, increase in drug concentration within the vascular access. Therefore, concentration of EPO used in this vitro study is enough to consider the mechanism of EPO-induced vascular access failure in hemodialysis patients. We used rat VSMC in this study, which is a limitation of this study to consider mechanism of EPO-induced vascular access failure in hemodialysis patients. Future study is needed to solve the problem.

In conclusion, statins inhibited EPO-induced DNA synthesis, cell proliferation and activation of MAPK. The effect was comparable with all statins irrespective of their hydrophilicity, and thus may be a class effect. Although the treatment of renal failure patients with statins should be handled with caution, these drugs have been reported to have beneficial effects for the treatment of renal disease, and thus the opportunities to use statins for the treatment of renal failure patients will likely continue to increase. Our findings may be useful for the treatment of EPO-induced vascular access failure in renal failure patients.

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Table 1. Effect of atorvastatin (ATV) and mevalonic acid (MVA) on EPO-induced VSMC proliferation.

	[³ H]thymidine incorporation (fold increase)	Cell proliferation (fold increase)	phosphorylated p44/42 MAPK (fold increase)
vehicle	1.00 ± 0.02	1.00 ± 0.03	1.0 ± 0.4
EPO	1.21 ± 0.03 ^a	1.18 ± 0.06 ^a	5.2 ± 1.2 ^a
EPO+ATV	0.98 ± 0.04 ^b	1.03 ± 0.03 ^b	1.1 ± 0.3 ^b
EPO+ATV+MVA	1.11 ± 0.06 ^c	1.15 ± 0.05 ^c	3.2 ± 0.9 ^c
ATV	1.02 ± 0.03	0.99 ± 0.06	0.9 ± 0.3
10% FBS	1.33 ± 0.10 ^a	1.34 ± 0.09 ^a	NA

Concentrations of EPO, ATV and MVA were 120 IU/ml, 100nM and 100mM, respectively.

Values are the mean ± S.E.M. ^aP<0.05 compared with vehicle. ^bP<0.05 compared with EPO alone. ^cP<0.05 compared with EPO + ATV. NA: not available. n=3 to 6.

Table 2. Effect of fluvastatin (FLV) and mevalonic acid (MVA) on EPO-induced VSMC proliferation.

	[³ H]thymidine incorporation (fold increase)	Cell proliferation (fold increase)	phosphorylated p44/42 MAPK (fold increase)
vehicle	0.99 ± 0.04	1.00 ± 0.02	1.0 ± 0.6
EPO	1.29 ± 0.03 ^a	1.24 ± 0.07 ^a	3.3 ± 1.4 ^a
EPO+FLV	0.89 ± 0.08 ^c	0.99 ± 0.01 ^c	1.4 ± 0.3 ^c
EPO+FLV+MVA	1.02 ± 0.03 ^d	1.17 ± 0.05 ^d	4.1 ± 1.2 ^d
FLV	0.97 ± 0.03	1.04 ± 0.03	1.8 ± 0.8
10% FBS	5.04 ± 0.11 ^b	2.58 ± 0.13 ^b	NA

Concentrations of EPO, FLV and MVA were 120 IU/ml, 100nM and 100mM, respectively.

Values are the mean ± S.E.M. ^aP<0.05, ^bP<0.01 compared with vehicle. ^cP<0.05 compared with EPO alone. ^dP<0.05 compared with EPO + FLV. NA; not available. n=3 to 6.

Figure legends

Fig. 1 Effect of EPO on the proliferation of VSMCs as assessed by DNA synthesis.

Growth-arrested VSMCs were stimulated by EPO and FBS for 24 h. [³H]Thymidine was added for the last 4 h. 10% FBS was used as a positive control. Values are the mean \pm S.E.M.(n=3). *P<0.05 compared with vehicle (10% PBS).

Fig. 2 Effect of EPO on the proliferation of VSMCs as assessed by WST-1

Growth-arrested VSMCs were stimulated by EPO and FBS for 24 h. WST-1 was added for last 4 h. 10% FBS was used as a positive control. Absorbance was measured by using a microplate spectrophotometer. Values are the mean \pm S.E.M.(n=6). *P<0.05,**P<0.01 compared with the vehicle.

Fig. 3 Effect of EPO on p44/42MAPK phosphorylation.

Growth-arrested VSMCs were stimulated by EPO for 5 min. (a) Representative immunoblots are shown with antibodies that recognize phosphorylated p44/42 MAPK and total p44/42 MAPK. (b) Densitometric analysis of phosphorylated p44/42 MAPK. Values are the mean \pm S.E.M. (n=4). *P<0.05 compared with the vehicle.

Fig. 4 Effect of pravastatin (PRV) on the EPO-induced increase of DNA synthesis in VSMCs.

Growth-arrested VSMCs were stimulated by EPO, PRV and FBS for 24 h. Values are the mean \pm S.E.M. (n=3-6). *P<0.05, **P<0.01 compared with vehicle. #P<0.05 compared with EPO 120 IU/ml without pravastatin.

Fig. 5 Effect of pravastatin (PRV) and mevalonic acid on EPO-induced VSMC proliferation.

(A) DNA synthesis was evaluated by measuring [³H]thymidine uptake. Values are the mean \pm S.E.M. (n=3). *P<0.05, **P<0.01 compared with vehicle. #P<0.05 compared with EPO 120 IU/ml. §P<0.05 compared with EPO 120 IU/ml + PRV 100 nM.

(B) Cell proliferation was measured by WST-1 proliferation assay. Values are the mean \pm S.E.M. (n=6). *P<0.05 compared with vehicle. #P<0.05 compared with EPO 120 IU/ml. §P<0.05 compared with EPO 120 IU/ml + PRV 100 nM.

(C) Representative immunoblots and densitometric analysis of p44/42 MAPK and phosphorylated p44/42 MAPK. Values are the mean \pm S.E.M. (n=3). *P<0.05 compared with vehicle. #P<0.05 compared with EPO 120 IU/ml. §P<0.05 compared with EPO 120 IU/ml + PRV 100 nM.

Fig. 6 Effect of simvastatin (SMV) and mevalonic acid on EPO-induced VSMC proliferation.

(A) DNA synthesis were evaluated by measuring [³H]thymidine uptake. Values are the mean \pm S.E.M.(n=3). **P<0.01 compared with vehicle. ###P<0.01

compared with EPO 120 IU/ml. $^{\$}$ P<0.01 compared with EPO 120 IU/ml + SMV100 nM.

(B) Cell proliferation was measured by WST-1 proliferation assay. Values are the mean \pm S.E.M. (n=6). *P<0.05, **P<0.01 compared with vehicle. #P<0.05 compared with EPO 120 IU/ml. $^{\$}$ P<0.05 compared with EPO 120 IU/ml + SMV100 nM.

(C) Representative immunoblots and densitometric analysis of p44/42MAPK and phosphorylated p44/42 MAPK. Values are the mean \pm S.E.M.(n = 3). **P<0.01 compared with vehicle. ##P<0.01 compared with EPO 120 IU/ml. $^{\$}$ P<0.01 compared with EPO 120 IU/ml + SMV100 nM.

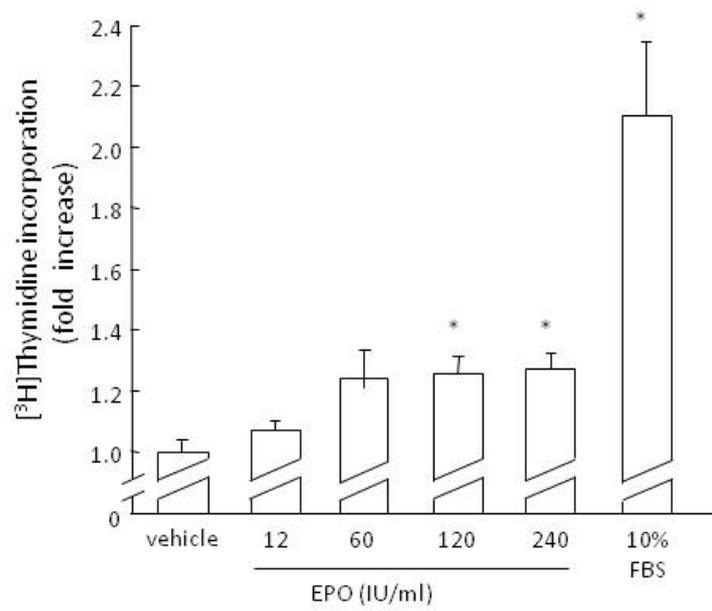


Fig.1

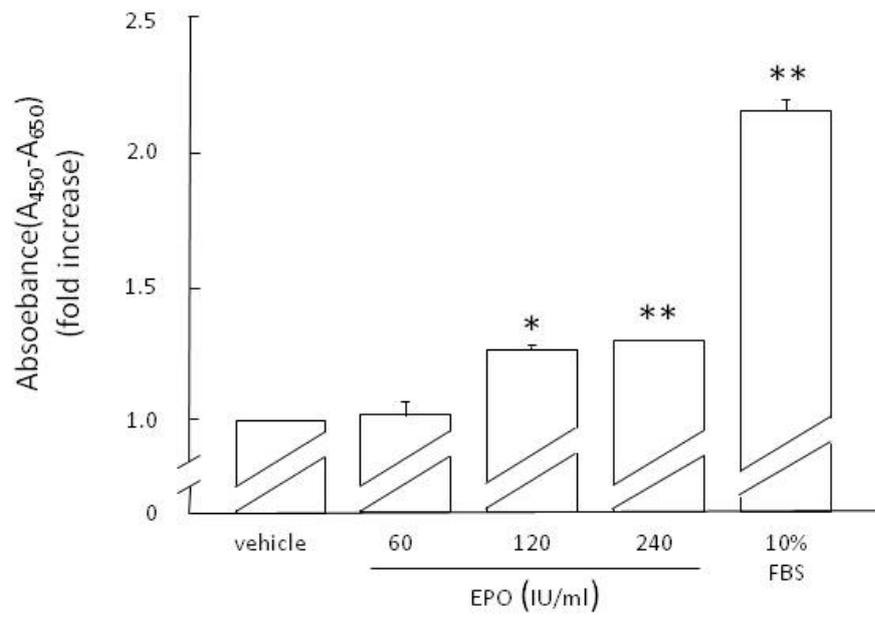


Fig.2

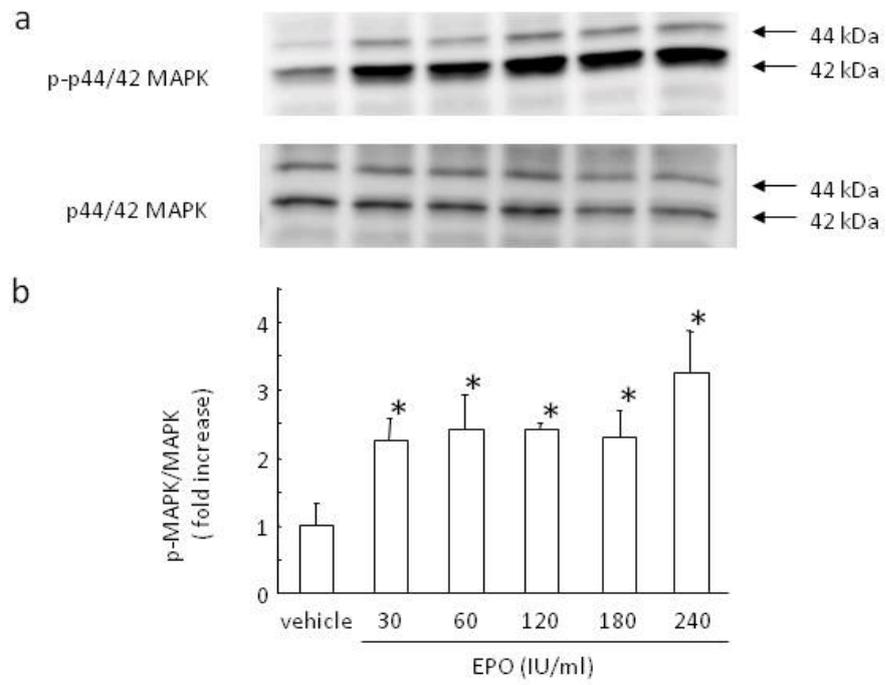


Fig.3

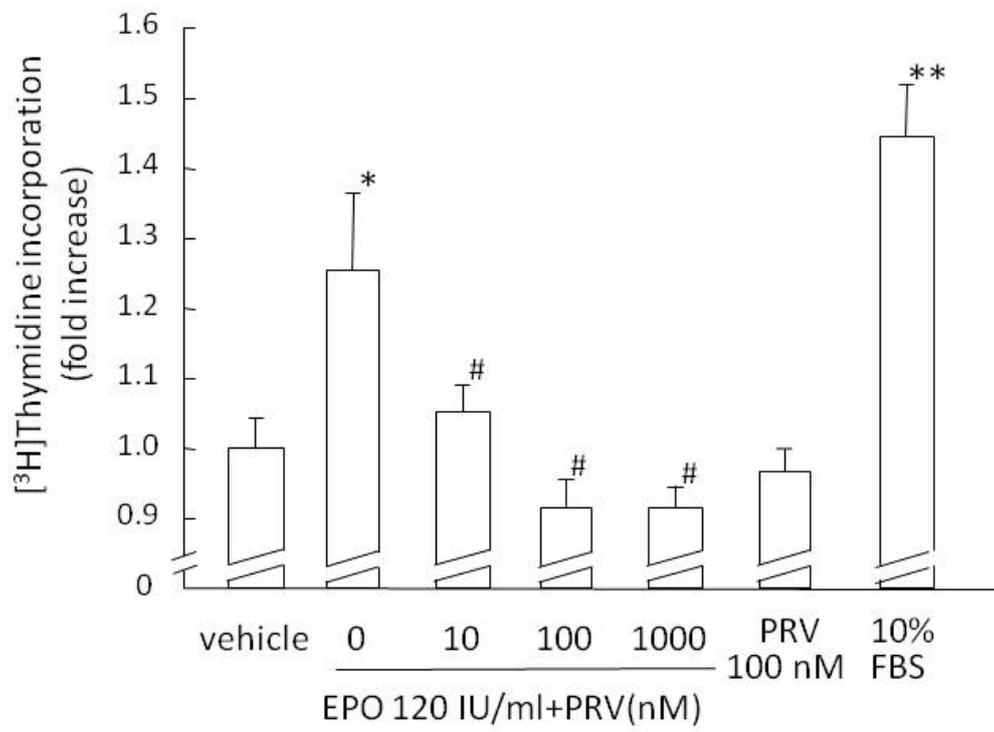


Fig.4

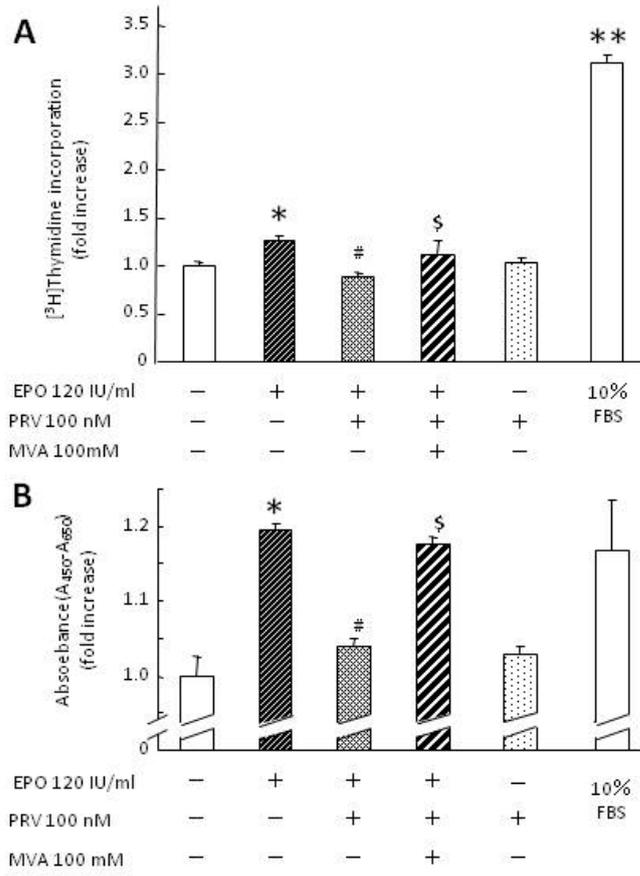


Fig.5AB

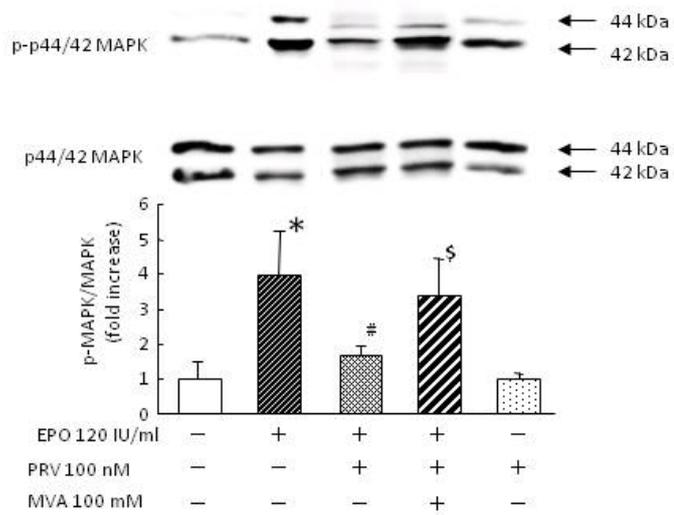


Fig.5C

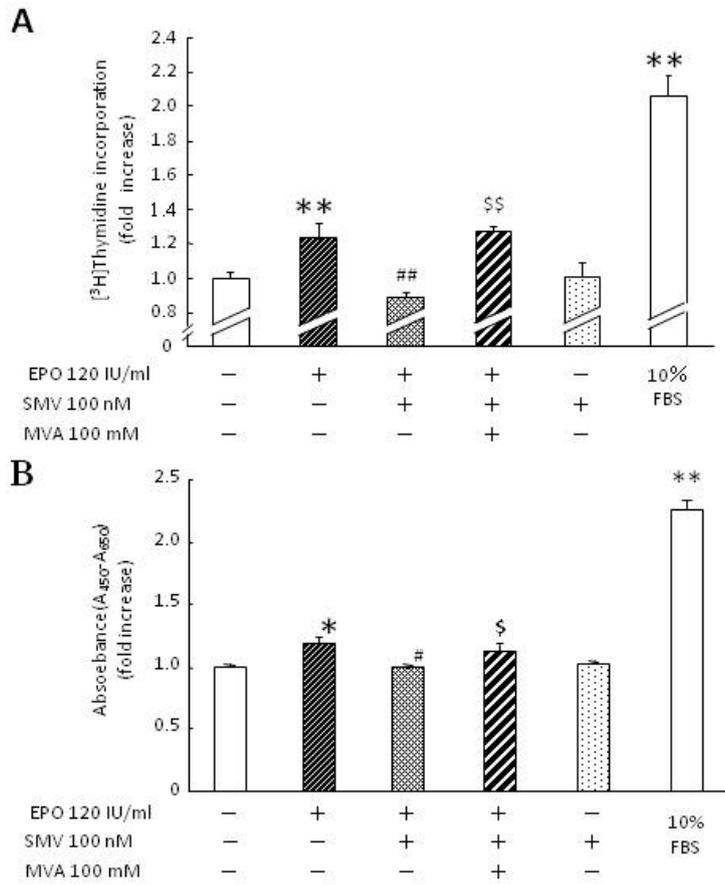


Fig.6AB

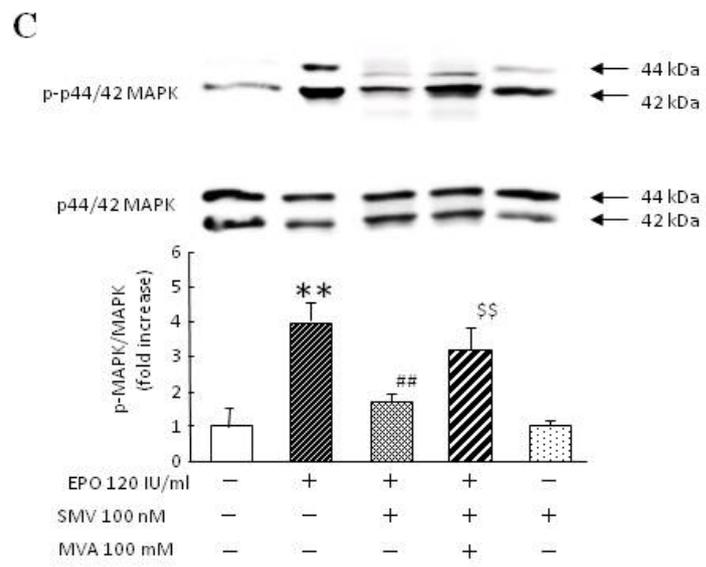


Fig.6C