Indoxyl sulfate stimulates proliferation of rat vascular smooth muscle cells

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Vascular smooth muscle cell (VSMC) proliferation is a key event in the progression of arteriosclerosis. Clinical studies show that uremic toxins deteriorate the arteriosclerosis in renal failure patients. Indoxyl sulfate (IS) is a strong protein-bound uremic toxin, but the effect of IS on VSMC proliferation has not been studied. We examined the effect of IS on rat VSMC proliferation, assessed by a cell counting kit (4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2*H*-5-tetrazolio-1, 3-benzene disulfonate] assay) and by [^{3}H]thymidine incorporation *in vitro*. We further evaluated a contribution of mitogen-activated protein kinase (MAPK; p44/42 MAPK) to VSMC proliferation by IS. Immunohistochemical staining was performed for VSMCs using antirat organic anion transporter (OAT)3 antibody. The mRNA expressions of platelet-derived growth factor (PDGF)-A and -C chains, and PDGF-β receptor were evaluated by real-time PCR. IS stimulated the proliferation of VSMCs in a concentration-dependent manner and activated p44/42 MAPK. Concentration of IS needed to stimulate the proliferation of rat VSMC was about 250 μM, which is compatible with that in the serum of end-stage renal failure patients. PD98059 (10 μM), a selective inhibitor of MAPK/extracellular signal-regulated kinase, inhibited the IS-induced (250 μM) VSMC proliferation and phosphorylation of MAPK. Probenecid (0.5 mM), an inhibitor and substrate of OAT, inhibited the IS-induced (250 μM) VSMC proliferation. Rat OAT3 was detected in VSMCs. The mRNA expressions of PDGF-C chain and PDGF-β receptor were significantly increased by IS. We conclude that IS directly stimulates rat VSMC proliferation and activates MAPK *in vitro*. This might be one of the mechanisms underlying the progression of atherosclerotic lesions in end-stage renal disease patients.

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Improvements in dialysis treatment have resulted in prolongation of the survival period of hemodialysis patients, but their prognosis is still poor compared to that in the general population.[1] The major cause of death in patients undergoing dialysis is cardiovascular disease.[2] The pathophysiology of cardiovascular disease in end-stage renal disease patients is not completely understood; however, accumulation of uremic toxins that are difficult to be removed from the body by current dialysis procedures is partly involved in the condition.[3,4] Indoxyl sulfate (IS) is one of the organic anions metabolized in the liver from indole, which is produced by the intestinal bacteria as a metabolite of tryptophan.[5] Although the molecular weight of IS is small, the rate of binding to albumin is high in the blood,[6] which, in turn, leads to its large secretion from the proximal tubule cells in urine.[7] Various organic anion transporters (OATs) exist in proximal tubules, and it has recently been revealed that IS is secreted in urine mainly by OAT3, one of the members of the OAT family.[8] Therefore, IS accumulates in the body of patients with reduced renal function.[5]

Removal of IS by hemodialysis is difficult because the size of the IS–albumin complex molecule in blood is larger than the pore size of a dialysis membrane.[5] Thus, IS is considered to be one of the main uremic toxins that are difficult to be removed from the body by current dialysis procedures.[5] It has been reported that IS accelerates the progression of renal failure,[9] but only one datum is available about the effects of IS on the cardiovascular system.[10]

In the progression of atherosclerotic lesions, the proliferation of vascular smooth muscle cells (VSMCs) is of particular importance. The mitogen-activated protein kinase (MAPK) cascades are a well-documented family of serine/threonine kinases that include p44/42 MAPK (also called extracellular signal-regulated kinases (ERK1/2)), p38 MAPK, and c-Jun N-terminal kinase.[11] The p44/42 MAPK cascade is the most well characterized and is shown to mediate proliferative responses in various cells, including mesangial cells[12] and VSMCs.[13] Growth factors, such as platelet-derived growth factor (PDGF),[14] angiotensin II,[15] erythropoietin,[16] and uremic toxins such as homocysteine,[17] and uric acid[18] can activate intracellular signaling cascades, leading to the proliferation of VSMCs. However, the effects of IS on VSMC proliferation and activation of MAPK have not been studied.

In this study, we therefore directly investigated the effects of IS *in vitro* on VSMC proliferation and MAPK activation using cultured rat VSMCs.

RESULTS

IS increases rat VSMC proliferation and DNA synthesis

First, we examined the effect of IS on the number of VSMCs. IS significantly increased the number of VSMCs in a concentration-dependent manner (Figure 1). PDGF (10 ng/ml) also increased the number of VSMCs.

We next evaluated whether the increase in cell proliferation was accompanied with DNA synthesis. Figure 2a shows that [^{3}H]thymidine incorporation was also significantly increased by IS (100–500 μM). Because IS is mainly bound to albumin in the serum, we also evaluated the effect of IS in the presence of 4 g/dl albumin in the medium. Although basal value was significantly lower with albumin, IS at 250 and 500 μM significantly increased the uptake (Figure 2b). This result indicates that IS stimulated VSMC proliferation even in the presence of 4 g/dl albumin. IS in the body is made from tryptophan in the intestine[5] and the medium used in this study contained tryptophan (78 μM), which may affect the results. Thus, we further examined the effect of the removal of tryptophan from the medium. As shown in Figure 3, 250 μM IS increased the [^{3}H]thymidine incorporation by VSMCs in both the presence and absence of tryptophan (78 μM). Therefore, IS, but not its precursor, directly stimulated the proliferation of VSMC *in vitro*.

As IS is reported to be the substrate of OAT3 in the proximal tubule cell membrane,[8] we next examined whether the proliferation of VSMCs is mediated by cellular transport of IS via the OAT. As shown in Figure 4, the increase of [^{3}H]thymidine incorporation in VSMCs by 250 μM IS was partly prevented by co-administration of 0.5 mM probenecid, an inhibitor and substrate of OAT,[8] whereas probenecid alone did not affect it. Next, to determine whether OAT3 is present in rat VSMCs, we performed immunostaining of rat VSMCs by rat OAT3 antibody. As shown in Figure 5, the rat VSMCs had strong signals of rat OAT3, mainly in the cell membrane.

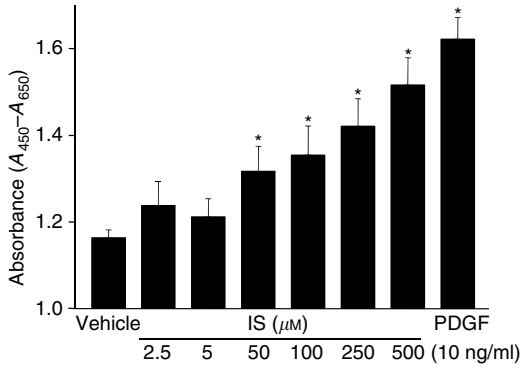

Figure 1 | Effect of IS on the proliferation of rat VSMCs by 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] **proliferation assay.** Growth-arrested VSMCs were stimulated by IS and PDGF for 24 h. 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] was added for the last 4 h. PDGF (10 ng/ml) was used as a positive control. Absorbance was measured by an enzyme-linked immunosorbent assay reader. Values are mean ± s.e.m. ($n = 8$). *$P < 0.05$ compared with the vehicle.

IS activates the p44/42MAPK pathway in rat VSMCs

To evaluate further intracellular signaling event in the effects of IS on the rat VSMC proliferation, we investigated the

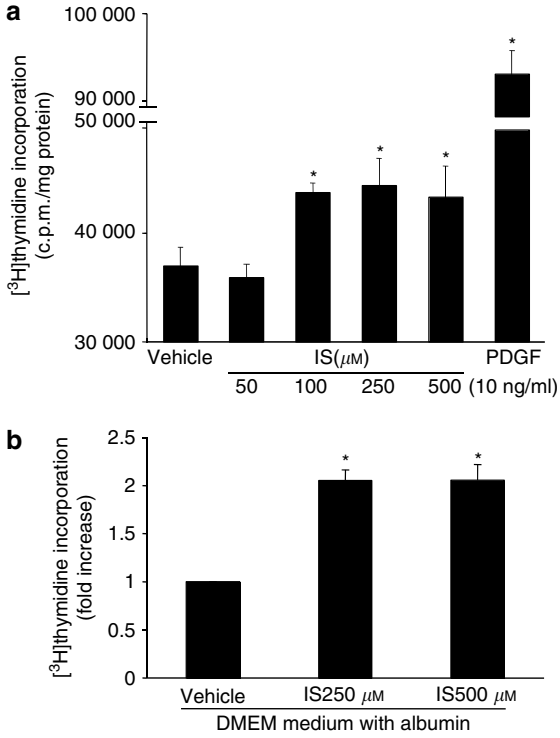

Figure 2 | Effect of IS on the proliferation of rat VSMCs assessed by DNA synthesis. (**a**) Growth-arrested VSMCs were stimulated by IS and PDGF for 24 h. [^{3}H]Thymidine was added for the last 6 h. PDGF (10 ng/ml) was used as a positive control. (**b**) Effect of IS on the proliferation of rat VSMCs in the presence of 4 g/dl albumin in the medium assessed by DNA synthesis. Values are mean ± s.e.m. ($n = 8$). *$P < 0.05$ compared with the vehicle.

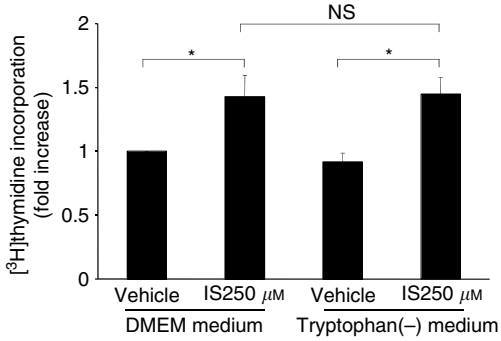

Figure 3 | Effect of the removal of tryptophan on IS-induced increase of DNA synthesis in rat VSMCs. Growth-arrested VSMCs were incubated for 24 h in DMEM or tryptophan (–) medium with or without IS (250 μM). [^{3}H]Thymidine was added for the last 6 h. Values are mean ± s.e.m. ($n = 8$). *$P < 0.05$ compared with vehicle.

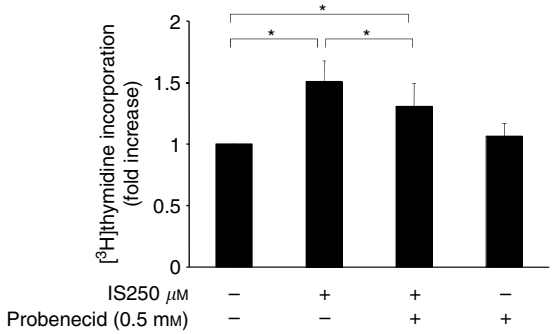

Figure 4 | Effect of probenecid on IS-induced increase of DNA synthesis in rat VSMC. Growth-arrested VSMCs were pretreated for 1 h with probenecid (0.5 mM) and then stimulated by IS (250 μM) for 24 h. Values are mean ± s.e.m. ($n = 8$). *$P < 0.05$.

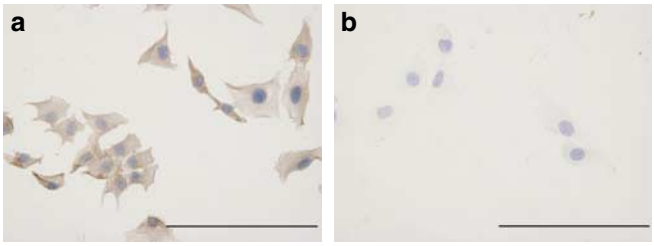

Figure 5 | Immunohistochemistry of rat OAT3 in rat VSMCs. (**a**) VSMCs were stained with polyclonal antibodies against rat OAT3 (**b**) There was no staining in the negative control with rabbit immunoglobulin fraction. Bar = 200 μm.

activation of p44/42 MAPK in VSMCs. As plasma concentration of IS in hemodialysis patients is reported to be about 250 μM,[5] we selected this dose for the following experiments. IS at 250 μM induced p44/42 MAPK phosphorylation in the cells, with a maximal intensity at 5 min (Figure 6). To confirm that the p44/42 MAPK pathway was actually involved in the IS-induced mitogenesis, we pretreated VSMCs with 10 μM PD98059, an MAPK/ERK kinase inhibitor,[14] to inhibit the pathway. The IS-induced phosphorylation was significantly inhibited by PD98059 (Figure 7). We further evaluated the effect of pretreatment with PD98059 at the same dose on the increase in IS-induced [^{3}H]thymidine incorporation. PD98059 inhibited the increase in DNA synthesis by IS, whereas PD98059 alone had no effect on DNA synthesis (Figure 8).

IS increases mRNA expressions of PDGF-C chain and PDGF-β receptor

To discuss the mechanisms of IS-induced proliferation of VSMC, we evaluated the mRNA expressions of PDGF-A and -C chains, and PDGF-β receptor by real-time PCR. We found that mRNA expressions of PDGF-C chain and PDGF-β receptor were significantly increased by the addition of IS (Figure 9). Therefore, the increased expressions by IS are partly involved in the mechanism of IS-induced VSMC proliferation.

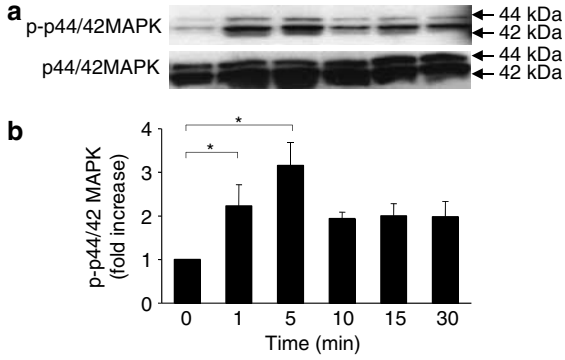

Figure 6 | Effect of IS on p44/42 MAPK phosphorylation. Growth-arrested VSMCs were stimulated by IS (250 μM) for the indicated times. (**a**) Representative immunoblots are shown with antibodies that recognize phosphorylated p44/42 MAPK and total p44/42 MAPK. (**b**) Densitometric analysis of phosphorylated p44/42 MAPK. Values are mean ± s.e.m. ($n = 5$). *$P < 0.05$.

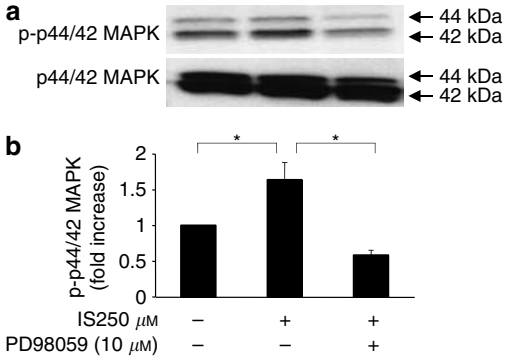

Figure 7 | Effect of PD98059 on IS-induced phosphorylation of p44/42 MAPK. Growth-arrested VSMCs were pretreated with PD98059 (10 μM) for 1 h and then stimulated by IS (250 μM) for 5 min. (**a**) Representative immunoblots are shown with antibodies that recognize phosphorylated p44/42 MAPK and total p44/42 MAPK. (**b**) Densitometric analysis of phosphorylated p44/42 MAPK. Values are mean ± s.e.m. ($n = 5$). *$P < 0.05$.

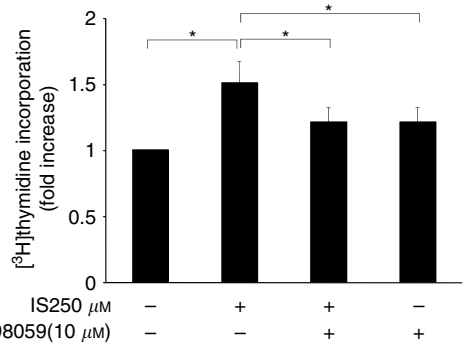

Figure 8 | Effect of PD98059 on IS-induced proliferation of rat VSMCs assessed by DNA synthesis. Growth-arrested VSMCs were pretreated for 1 h with PD98059 (10 μM) and then stimulated with IS (250 μM) for 24 h. Values are mean ± s.e.m. ($n = 8$). *$P < 0.05$.

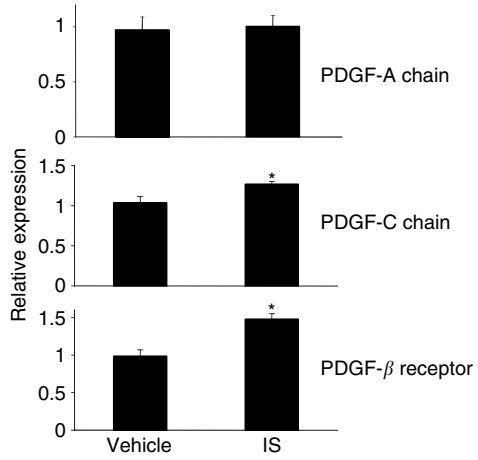

Figure 9 | Effect of IS on mRNA expressions of PDGF-A and -C chains and PDGF-β receptor. Growth-arrested VSMCs were stimulated with IS (250 μM) for 6 h and then total RNA was isolated. Real-time quantitative PCR was performed as described in Materials and Methods. Values are mean ± s.e.m. of two identical samples performed in at least triplicate ($n = 4$ in each). *$P < 0.05$ compared with the vehicle.

DISCUSSION

It is known that IS is a protein-bound uremic toxin that accelerates the progression of renal failure,[5] but data are limited about its actions on other organs. A recent *in vitro* study has shown that IS has a suppressive effect on the repair of damage in human umbilical vein endothelial cells, suggesting that the agent is involved in endothelium dysfunction in renal failure.[10] The present study shows for the first time that IS directly promotes VSMC proliferation. This is the most important finding in the present study. Serum concentration of IS in patients with end-stage renal failure is about 250 μM,[5] which is compatible with that needed to stimulate the proliferation of rat VSMCs in this study. As the enhanced proliferation of VSMCs in vascular walls is believed to promote hypertrophy of arteries, it is possible that VSMC proliferation induced by IS is one of the causes of cardiovascular complications that occur in dialysis patients with a high serum IS.

As IS is known to be transported by OATs on tubular cell membranes, the effect of probenecid, an inhibitor and substrate of OATs, on VSMC proliferation was investigated. In this experiment, we found that probenecid had an inhibitory effect on the proliferation of VSMCs. We also confirmed the presence of OAT3 in VSMCs by immunostaining. These findings suggest that IS is at least in part taken into VSMCs via OAT. Proliferation of VSMCs plays some role in the development of hypertension and arteriosclerosis,[19] and the p44/42 MAPK pathway is thought to be the intracellular signaling pathway involved in the proliferation of VSMCs.[13] When we evaluated the involvement of MAPK in the effect of IS on VSMCs, we found that pretreatment of VSMCs with PD98059, an inhibitor of MAPK, caused inhibition of VSMC proliferation. We also found that IS induced p44/42 MAPK phosphorylation, which was inhibited by PD98059. These findings indicate that the p44/42 MAPK pathway is involved in the induction of VSMC proliferation by IS.

In the present study, serum was not used in experiments examining the effects of IS stimulation, although no significant difference was found between the degrees of VSMC proliferation in serum with and without IS (data not shown). However, serum contains various growth factors, some of which might have a strong proliferative effect in addition to IS on VSMCs. Indeed, we found that the mRNA expressions of PDGF-C chain and PDGF-β receptor were increased by IS. Therefore, the increased expressions by IS are partly involved in the mechanism of IS-induced VSMC proliferation. The culture medium used in the present study contained tryptophan, a precursor of IS, but the possibility that the presence of tryptophan in the medium affected the results was ruled out by comparison of results of experiments using a medium containing tryptophan and a medium from which tryptophan had been removed.

The mechanisms by which IS induces proliferation of VSMC and activation of MAPK are not known. Uric acid has also been reported to be a uremic toxin that induces proliferation of VSMCs. Rao *et al.*[18] reported that uric acid induces proliferation of VSMCs by promoting the production of PDGF-A chain, and Johnson *et al.*[20,21] reported that uric acid is taken up by VSMCs via an OAT. As a potential mechanism for VSMC proliferation, uric acid, after being taken into VSMCs, activates MAPK and induces the production of cyclooxygenase-2 and the expression of PDGF-A chain, -C chain, and PDGF-α receptor mRNA.[20] We found that the induction of VSMC proliferation by IS was inhibited by probenecid and that MAPK was involved in the induction of VSMC proliferation by IS. Therefore, the mechanism of IS-induced VSMC proliferation might be similar to that of uric acid. To our knowledge, other protein-bound uremic toxins that have been shown to induce VSMC proliferation *in vitro* are homocysteine[22] and leptin.[23] Further study is needed to determine whether MAPK activation by IS is mediated by various growth factor receptors on the cell membrane surface or by an as yet unidentified receptor. To understand the pathophysiology of cardiovascular disease in end-stage renal disease patients, the effect of IS on other cell types of blood vessels and their crosstalk must be evaluated in the future.

In summary, the present *in vitro* study shows for the first time that IS directly induces cell proliferation of rat VSMC. Concentration of IS required to induce VSMC proliferation is similar to its blood concentration in patients on maintenance dialysis. Part of the action of IS on VSMC is mediated by OAT3, and the induction of VSMC proliferation by MAPK activation caused by IS. Increases of mRNA expressions of PDGF-C chain and PDGF-β receptor are also involved in the phenomenon. This might be one of the mechanisms underlying the progression of atherosclerotic lesions in end-stage renal disease patients.

MATERIALS AND METHODS

Materials

A p44/42 MAPK assay kit, including anti-p44/42 MAP kinase and anti-phospho-p44/42 MAP kinase (Thr202/Try204) antibodies, was purchased from Cell Signaling Technology (Beverly, MA, USA). A Cell Counting Kit was purchased from DOJINDO Laboratories (Kumamoto, Japan). [^{3}H]Thymidine was purchased from Perkin Elmer Life Science (Boston, MA, USA). Anti-rat OAT3 polyclonal antibody was purchased from TransGenic Inc. (Kumamoto, Japan). All other materials including IS were purchased from Sigma (St Louis, MO, USA). IS was dissolved with distilled water as vehicle in all experiments.

Cell culture

VSMCs were isolated from the aortas of male Sprague–Dawley rats (150–200 g) as previously described.[13] In brief, 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% CO_2 incubator at 37°C. VSMCs at 70–80% confluence were growth-arrested by incubation in DMEM with 0.5% FBS for 48 h. The cells were used between passages 3 and 8. 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). We measured endotoxin concentration in the medium by limulus amebocyte 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.

Cell proliferation assay

VSMC proliferation was assessed using the Cell Counting Kit.[24] 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 IS or PDGF 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.

[^{3}H]Thymidine incorporation

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, and [^{3}H]thymidine (1 μCi/ml; specific activity, 79 Ci/mmol) was added to 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, Japan).

Western blot analysis

Growth-arrested cells cultured in 100-mm dishes were stimulated by 250 μM IS for the indicated times. For the inhibitor studies, cells were pretreated for 1 h with PD98059 (10 μM), an MAPK/ERK kinase inhibitor.[14] The cells were washed with ice-cold phosphate-buffered saline and lysed in 400 μl 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 β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/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 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrophoretically transferred to polyvinylidine 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) (Cell Signaling Technology, Beverly, MA, USA) 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 KODAK 1D Image Analysis Software (KODAK, Rochester, NY, USA).

Immunohistochemical staining

Immunostaining of OAT3 in rat VSMCs was performed by the labeled streptavidin biotin (LSAB) method using a DAKO LSAB Kit (Dako, Carpinteria, CA, USA). Briefly, VSMCs were cultured on eight-well Lab-Tek chamber slides (Nalge Nunc International) and fixed with paraformaldehyde lysine periodate solution (containing 0.01 M $NaIO_4$, 0.075 M lysine, 0.0375 M phosphate buffer with 2% paraformaldehyde; pH 6.2) for 1 h at 4°C. After fixation, the cells were permeabilized in 0.1% Triton X-100 in phosphate-buffered saline for 5 min and incubated with 3% hydrogen peroxide for 30 min to suppress endogenous peroxidase activity and then with blocking solution for 10 min. The cells were incubated with a polyclonal antibody against rat OAT3[8] (1:20) at 4°C overnight. The negative controls were treated with the rabbit immunoglobulin fraction (Dako, Carpinteria, CA, USA). After rinsing with TBS-T, the cells were incubated with a biotinylated link antibody against rabbit immunoglobulin for 30 min. The cells were washed with TBS-T and incubated with horseradish peroxidase-conjugated streptavidine solution for 30 min. Horseradish peroxidase labeling was detected using a peroxidase substrate diaminobenzidine, and then counterstaining was performed with hematoxylin.

RNA extraction and real-time quantitative PCR

The isolation of total RNA was achieved using the RNeasy Mini Kit (Quiagen, Valencia, CA, USA). Reverse transcription was performed with 1.2 μg of total RNA, random hexamer primers, and RevertAid M-MuLV reverse transcriptase (Fermentas, Hanover, MD, USA). The resulting cDNA equivalent to 60 ng of RNA was used for real-time quantitative PCR in the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) as previously described.[25] All of the specific sets of primers and TaqMan probes in the present study were obtained from Applied Biosystems (Assays-on-Demand Gene Expression Products and TaqMan Rodent GAPDH Control Reagants). All primer sets but that of TaqMan Rodent GAPDH Control Reagents were designed to be located in two exons to avoid amplification of potentially contaminating

genomic DNA. To control the variation in the amount of DNA available for PCR in the different samples, gene expressions of the target sequence were normalized in relation to the expression of an endogenous control, glyceraldehyde-3-phosphate dehydrogenase. As the efficiency of the target amplification was approximately equal to that of the glyceraldehyde-3-phosphate dehydrogenase amplification, data were analyzed using the comparative threshold cycle method.[26] Because the intra- and interassay coefficients of variation of the relative expression values were $<20\%$, we considered the mean relative values of less than 0.8 or more than 1.2 to be significant in this study.

Statistics

The results are expressed as the mean $\pm$ s.e.m. 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 personal computer with StatView version 5.0 (SAS Institute, Cary, NC, USA). Differences with $P<0.05$ were considered to be significant.

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