High Glucose-Induced Upregulation of Osteopontin Is Mediated via Rho/Rho Kinase Pathway in Cultured Rat Aortic Smooth Muscle Cells

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Objective—Osteopontin is upregulated in the diabetic vascular wall and in vascular smooth muscle cells cultured under high glucose concentration. In the present study, we analyzed the mechanism of high glucose-induced upregulation of osteopontin in cultured rat aortic smooth muscle cells.

Methods and Results—We found that an inhibitor of Rho-associated protein kinase, Y-27632, suppressed osteopontin mRNA expression under high glucose concentration. Transfection of cells with a constitutive active Rho mutant, pSRAmyc-RhoDA, enhanced osteopontin mRNA expression. Furthermore, incubation of cells under high glucose concentration activated Rho, indicating that Rho/Rho kinase pathway mediates high-glucose–stimulated osteopontin expression. Treatment of cells with an inhibitor of protein kinase C, GF109203X, and azaserine, an inhibitor of the hexosamine pathway, suppressed high glucose-induced Rho activation. Glucosamine treatment was shown to activate Rho. Treatment of cells with an inhibitor of MEK1, PD98059, suppressed osteopontin mRNA expression under high glucose concentration. Incubation of cells under high glucose concentration activated ERK. Finally, transfection of cells with pSRAmyc-RhoDA also activated ERK.

Conclusions—In conclusion, our present findings support a notion that Rho/Rho kinase pathway functions downstream of protein kinase C and the hexosamine pathways and upstream of ERK in mediating high-glucose–induced upregulation of osteopontin expression. (Arterioscler Thromb Vasc Biol. 2004;24:276-281.)

Key Words: osteopontin ■ Rho ■ glucose ■ atherosclerosis ■ smooth muscle cells

Osteopontin (OPN) is a multifunctional phosphoprotein secreted by many cell types such as osteoclasts, lymphocytes, macrophages, epithelial cells, and vascular smooth muscle cells (SMC). Overexpression of OPN has been found in several physiological and pathological conditions, including immunologic disorders, neoplastic transformation, progression of metastasis, formation of urinary stones, and wound healing.

It was reported that OPN protein and mRNA were expressed in the neointima and in calcified atheromatous plaque. A neutralizing antibody against OPN was found to inhibit rat carotid neointimal formation after endothelial denudation. These results have suggested that OPN promotes the development of atherosclerosis. Recently, we found upregulation of OPN expression in diabetic human and rat vascular walls. It was also noted that high glucose concentrations stimulated OPN expression via a protein kinase C (PKC)-dependent pathway and the hexosamine pathway in cultured rat aortic SMC. Furthermore, OPN was found to stimulate migration and enhance platelet-derived growth factor-mediated DNA synthesis of cultured rat aortic SMC. Based on these data, we suggest that OPN plays a role in accelerated atherogenesis in diabetes mellitus.

In the present study, we further analyzed the mechanism of high glucose-induced upregulation of OPN in cultured rat aortic SMC. We show that Rho/Rho kinase pathway functions downstream of PKC and the hexosamine pathways and upstream of ERK in mediating high glucose-stimulated OPN expression.

Methods

Reagents

GGTI-298, an inhibitor of geranylgeranyltransferase I, FTI-277, an inhibitor of farnesyltransferase, Y-27632, an inhibitor of Rho-associated protein kinase, GF109203X, an inhibitor of PKC, PD98059, an inhibitor of MEK1, SB203580, an inhibitor of p38 mitogen-activated protein (MAP) kinase, and SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), were purchased from Calbiochem (La Jolla, CA). Azaserine, an inhibitor of glutamine:fructose-6-phosphate amidotransferase (GFAT) was from Sigma (St. Louis, MO). The p44/42 MAP kinase assay kit, p38 MAP kinase assay kit, and SAPK/JNK assay kit were from Cell Signaling Technology (Beverly, MA). Rho activation assay kit was from UBI (Lake Placid, NY).
NY). pSRo-myc-RhoDA, an expression vector containing a constitutive active Rho mutant, was kindly provided by Dr Yoshimi Takai (Osaka University, Osaka, Japan). Rat OPN cDNA was from Dr Mark Thiede (Pfizer, Groton, CT). Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was from Dr Masashi Yamazaki (Chiba University, Chiba, Japan). Pitavastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, was from Dr Masaki Kitahara (Nissan Chemical, Saitama, Japan).

**Cell Culture**

Primary cultures of rat aortic SMC were isolated as described by the explant method from adult male Wistar rats weighing ~200 grams. Cells were maintained in Dulbecco modified Eagle medium containing 5.5 mmol/L glucose, 10% fetal bovine serum, and 40 µg/mL gentamicin (Schering-Plough, Kenilworth, NJ) in a humidified atmosphere at 37°C in 5% CO2. Cells at passages 7 to 9 were used for the present experiments.

**Transient Transfection**

At 50% confluency in 100-mm dishes, cells were transfected with pSRo-myc-RhoDA by using Fugene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). pSRo-myc-RhoDA was mixed with Fugene 6 transfection reagent at the ratio of 1:3 and incubated at room temperature for 15 to 40 minutes. Then, cells were transfected by incubation with the mixture for 24 hours. After additional 48 hours of incubation under normal glucose concentration (5.5 mmol/L glucose), cells were processed for Northern blotting and MAP kinase activity assays.

**Northern Blotting**

Subconfluent cells growing in 100-mm dishes were treated with the indicated concentrations of specific inhibitors under normal or high (30 mmol/L) glucose concentrations. After 48 hours of incubation, total RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan). Northern hybridization was performed essentially as described using 32P-labeled rat OPN cDNA probe. The blots were stripped and subsequently re-hybridized with 32P-labeled rat GAPDH cDNA probe to assess the amount of RNA loaded in each lane, or with 32P-labeled Rho cDNA probe to estimate the efficiency of transfection with pSRo-myc-RhoDA. Densitometric analysis of fluorograms and autoradiograms were performed using the imaging scanner (EPSON ES 8000) with the NIH Image 1.44 software.

**Assay of ERK1/2, p38 MAP Kinase and SAPK/JNK Activities**

Subconfluent cells growing in 100-mm dishes were serum-starved for 24 hours and then incubated under different glucose concentrations for the indicated times. After conditioning, activities of ERK1/2 and p38 MAP kinase in cell lysates were measured by immune complex kinase assay using the p44/42 MAP kinase assay kit with an immobilized phospho p44/42 MAP kinase antibody and Elk-1 protein as substrate, or using the p38 MAP kinase assay kit with an immobilized phospho p38 MAP kinase antibody and ATF-2 protein as substrate, respectively, according to the manufacturer’s instructions. After phosphorylation reactions, samples were processed for Western blotting with phospho Elk-1 antibody or phospho ATF-2 antibody. After transfection with pSRo-myc-RhoDA, JNK activity was also evaluated by immune complex kinase assay using the SAPK/JNK assay kit with an c-Jun fusion protein beads followed by Western blotting with phospho c-Jun antibody, according to manufacturer’s instructions.

**Rho Activation Assay**

Subconfluent cells growing in 150-mm dishes were treated with the indicated concentrations of GF109203X or azaerine under high glucose concentration, or with the indicated concentrations of glucosamine under normal glucose concentration for 24 hours. Thereafter, Rho activity was measured using the Rho activation assay kit according to the manufacturer’s instructions. GTP-Rho in cell lysates was adsorbed to GST-Rhoetkin Rho binding domain, which binds selectively to GTP-Rho, not GDP-Rho. After precipitation, samples were processed for Western blotting with a specific anti-Rho antibody.

**Western Blotting**

Samples were dissolved in SDS sample buffer and boiled for 5 minutes, and the proteins were separated by SDS-PAGE on 15% (wt/vol) polyacrylamide resolving gels and electrophoretically transferred to nitrocellulose membranes (Hybond-ECL; Amersham Biosciences, Piscataway, NJ). For blocking nonspecific binding, membranes were incubated in Block Ace (Dainippon Chemicals, Tokyo, Japan) at room temperature for 1 hour. Then, the membranes were probed with the phospho Elk-1 antibody (dilution 1:1000), the phospho ATF-2 antibody (dilution 1:1000), or the anti-Rho antibody (3 µg/mL) in a dilution buffer consisting of phosphate-buffered saline containing 10% Block Ace at 4°C overnight. After being washed with phosphate-buffered saline containing 0.1% Tween-20, the membranes were incubated with an anti-rabbit IgG horseradish peroxidase-linked whole antibody (dilution 1:1000, Amersham Biosciences) in the dilution buffer at room temperature for 1 to 2 hours. After washing, the antibody binding bands were detected using an enhanced chemiluminescence system (ECL Western blotting detection reagents and analysis system; Amersham Biosciences) and visualized by exposure to Hyperfilm-ECL (Amersham Biosciences). Each experiment presented in this study was repeated at least twice under the identical conditions to confirm the reproducibility of the observations.

**Results**

**Pitavastatin Suppresses OPN Expression Under High Glucose Concentration**

Recently, we found upregulation of OPN expression in diabetic human and rat vascular walls. Furthermore, oral administration of Pitavastatin, an HMG-CoA reductase inhibitor, effectively suppressed abnormally upregulated expression of OPN mRNA in the aorta and kidney of streptozotocin-induced diabetic rats. These findings prompted us to examine in vitro effect of Pitavastatin on high glucose-induced upregulation of OPN expression in cultured rat aortic SMC. Cells were incubated with different concentrations of Pitavastatin at 37°C for 48 hours under high glucose concentration (30 mmol/L glucose). After incubation, the cells were processed for Northern blotting. As shown in Figure 1A, Pitavastatin dose-dependently decreased OPN mRNA level. Pitavastatin did not show cytotoxic effect at the examined doses as evaluated by trypan blue dye exclusion assay (data not shown).

**Isoprenylation Is Required for OPN Expression**

Inhibition of HMG-CoA reductase prevents the biosynthesis of isoprenoids, such as geranylgeranylpyrophosphate and farnesylpyrophosphate, and thereby inhibits subsequent isoprenylation. It is thus conceivable that the observed effect of Pitavastatin may result from inhibition of isoprenylation. To prove this assumption, we examined effects of inhibitors for geranylgeranyltransferase I and farnesyltransferase, GGTL-298 and FTI-277, respectively, on high glucose-induced upregulation of OPN expression in cultured rat aortic SMC. As shown in Figure 1B and C, GGTL-298 and FTI277 dose-dependently decreased OPN mRNA level under high glucose concentration, as expected.
Rho/Rho Kinase Pathway Mediates High Glucose-Induced Upregulation of OPN Expression

It is well known that geranylgeranylation is prerequisite for Rho, a small GTP-binding protein, to exert its cellular function. Therefore, Rho seemed to be a possible candidate involved in mediating a positive signal for OPN expression. To evaluate a role of Rho, we first examined effect of an inhibitor of Rho-associated protein kinase, Y-27632, on high glucose-induced upregulation of OPN expression in cultured rat aortic SMC. As shown in Figure 2A, Y-27632 dose-dependently decreased OPN mRNA level under high glucose concentration, suggesting a critical role of Rho kinase activity in OPN expression.

Next, we examined effect of transient transfection of a constitutive active Rho mutant, pSRα-myc-RhoDA, on OPN expression in cultured rat aortic SMC. As shown in Figure 2B, transfection of pSRα-myc-RhoDA enhanced OPN mRNA expression in proportion to the efficiency of its transfection, confirming that Rho mediates a positive signal for OPN expression.

Finally, we examined effect of high glucose on Rho activation in cultured rat aortic SMC. As shown in Figure 2C, the amount of GTP-Rho in cells cultured in 30 mmol/L glucose was found to be much higher than that in 5.5 mmol/L glucose. No difference was found in total Rho protein levels between 5.5 mmol/L glucose and 30 mmol/L glucose.

Figure 1. Effects of inhibitors for HMG-CoA reductase, geranylgeranyltransferase, and farnesyltransferase on OPN expression in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of Pitavastatin (A), GGTL-298 (B), or FTI-277 (C) in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 48 hours. After incubation, cells were processed for Northern blotting with 32P-labeled rat OPN and GAPDH cDNA probes. The level of OPN mRNA expression was estimated by the ratio of OPN signal to GAPDH signal. Data are expressed as fold increase relative to the value obtained in 30 mmol/L glucose without inhibitors. Data shown in this figure are representative of at least 2 independent experiments providing essentially similar results.

Figure 2. A, Effect of a Rho kinase inhibitor on OPN expression in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of Y-27632 in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 48 hours. After incubation, cells were processed for Northern blotting as described in the legend to Figure 1. B, effect of transient transfection of a constitutive active Rho mutant (CA Rho) on OPN expression in cultured rat aortic SMC. At 50% confluency, cells were transfected with 1 to 3 μg of pSRα-myc-RhoDA and incubated for 48 hours, as described in Methods. After incubation, cells were processed for Northern blotting. The blots were re-proved with 32P-labeled Rho cDNA probe to estimate the efficiency of transfection. Data are expressed as fold increase relative to the value obtained in the absence of CA Rho. C, High glucose-induced Rho activation in cultured rat aortic SMC. After serum starvation for 24 hours, cells were incubated in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 24 hours. After incubation, GTP-Rho in cell lysates was adsorbed to GST-Rhotekin Rho-binding domain and subjected to Western blotting with an anti-Rho antibody. Data are expressed as fold increase relative to the value obtained in 5.5 mmol/L glucose. Data shown in this figure are representative of at least 2 independent experiments providing essentially similar results.
glucose (data not shown). In contrast, treatment of cells with osmotic controls (5.5 mmol/L D-glucose plus 24.5 mmol/L L-glucose or 5.5 mmol/L D-glucose plus 24.5 mmol/L D-mannitol) providing an equivalent osmolality as 30 mmol/L glucose, did not change Rho activity (data not shown), indicating that the observed enhanced effect on Rho activity is specific to glucose. Taken together, these data strongly support a notion that Rho/Rho kinase pathway mediates high glucose-induced upregulation of OPN expression.

Rho/Rho Kinase Pathway Is a Common Downstream of PKC and Hexosamine Pathways
It was previously noted that high glucose concentrations stimulated OPN expression via a PKC-dependent pathway and the hexosamine pathway in cultured rat aortic SMC. Therefore, our next question was whether Rho/Rho kinase pathway functions downstream of these pathways. As shown in Figure 3A, treatment of cells with GF109203X, an inhibitor of PKC, dose-dependently inhibited high glucose-stimulated increase in Rho activity, suggesting the involvement of PKC activation in the process. Likewise, treatment with azaserine, an inhibitor of GFAT, the key enzyme of the hexosamine pathway, dose-dependently inhibited high glucose-stimulated increase in Rho activity. Total Rho protein levels were unchanged by addition of high glucose, 1 μM GF109203X or 5 μmol/L azaserine (data not shown). Furthermore, as shown in Figure 3B, glucosamine dose-dependently enhanced Rho activity. These data also suggest the involvement of the hexosamine pathway in the process.

ERK Functions Downstream of Rho in Mediating High Glucose-Induced Upregulation of OPN Expression
Small GTP-binding proteins have been demonstrated to induce a variety of responses, including activation of MAP kinase cascades in various cells. Therefore, to trace a signaling pathway that mediates OPN expression downstream of Rho, we first examined effects of inhibitors for MEK1 (PD98059), p38 MAP kinase (SB203580), and JNK (SP600125) on high glucose-induced upregulation of OPN expression in cultured rat aortic SMC. As shown in Figure 4A, PD98059 and SB203580 dose-dependently decreased OPN mRNA level under high glucose concentration, whereas SP600125 had no effect.

Next, we examined whether high glucose induces activation of ERK and p38 MAP kinase in cultured rat aortic SMC. After incubation of cells under normal (5.5 mmol/L) or high (30 mmol/L) glucose concentrations for 24 to 48 hours, activities of ERK1/2 and p38 MAP kinase were determined by immune complex kinase assay. As shown in Figure 4B, exposure to high glucose for 48 hours led to the increase in ERK activity, as assessed by phosphorylation of Elk-1, whereas activity of p38 MAP kinase, as assessed by phosphorylation of ATF-2, did not change under high glucose condition. Treatment with osmotic control (24.5 mmol/L L-glucose + 5.5 mmol/L D-glucose) had no effect on ERK activity (data not shown), indicating that the observed enhanced effect on ERK activity is specific to glucose.

Figure 3. A, Effects of GF109203X (GFX) and azaserine on high glucose-induced Rho activation in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of GFX or azaserine in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 24 hours. After incubation, cells were processed for Rho activation as described in the legend to Figure 2. B, Glucosamine-induced Rho activation in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of glucosamine in serum-free medium containing 5.5 mmol/L glucose for 24 hours. After incubation, cells were processed for Rho activation assay. Data are expressed as fold increase relative to the value obtained in the absence of glucosamine. Data shown in this figure are representative of at least 2 independent experiments providing essentially similar results.

Finally, to confirm that ERK functions downstream of Rho, we examined ERK activity after transient transfection of cultured rat aortic SMC with a constitutive active Rho mutant. As shown in Figure 4C, transfection of pSRα-myc-RhoDA dramatically enhanced ERK activity, whereas transfection of pSRα-myc-RhODA did not increase either p38 MAP kinase or JNK activities. Based on these data, we concluded that ERK functions downstream of Rho in mediating high glucose-induced upregulation of OPN expression.

Discussion
In the present study, we demonstrate that Rho/Rho kinase pathway functions downstream of PKC and the hexosamine pathways and upstream of ERK in mediating high glucose-induced upregulation of OPN expression. Involvement of Rho in mediating a positive signal for OPN expression has also been reported by Chaul et al. They showed that extracellular UTP increased OPN expression in cultured rat aortic SMC and thereby induced migration of the cells. Blockade of ERK1/2 or Rho pathways led to the inhibition of...
UTP-induced OPN increase and migration, demonstrating the central role of OPN in this process. The finding, together with our present observation, underscores the importance of Rho in OPN expression.

Our present finding that high glucose induces Rho activation sheds new light on the mechanism of the accelerated atherogenesis in diabetes mellitus, because involvement of Rho/Rho kinase pathway has been implicated in a wide variety of atherosclerotic processes, including neointimal formation, vasospastic response, proliferation, and anti-apoptosis of vascular SMC, and vascular gene expression of monocyte chemoattractant protein-1 and inducible nitric oxide synthase. Besides our present study using rat aortic SMC, high glucose-induced Rho activation was also observed in cultured rat mesangial cells and in basilar artery derived from streptozotocin-induced diabetic rats. It is thus conceivable that high glucose promotes diabetic vascular complications not only by upregulation of OPN but also by more diverse effects resulting from Rho activation.

It was reported that transfection of vascular SMC with the c-Ha-rasEJ oncogene induced overexpression of OPN. It is well known that farnesylation is prerequisite for Ras to exert its cellular effect; therefore, our present finding that the inhibitor of farnesyltransferase, FTI-277, suppressed OPN expression might be ascribed to the inhibition of Ras function by the drug. In our previous study, however, the inhibitory effect of Pitavastatin on OPN expression in cultured rat aortic SMC was almost completely reversed by the addition of mevalonate or geranylgeranylpiphosphatase but not by farnesylpyrophosphatase. Studies using other types of cells, fibroblasts, or keratinocytes showed that transfection of dominant-negative Rho or dominant-negative Rac suppressed Ras-induced activation of Raf-MEK-ERK pathway, indicating that Ras requires either Rho or Rac function in activation of Raf-MEK-ERK pathway. Based on these findings, it is speculated that the inability of farnesylpyrophosphate to rescue the cells from the inhibition of OPN expression by Pitavastatin might be caused by suppression of Rho family function in Pitavastatin-treated cells. Further study is necessary to prove this possibility.

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