

sity, and type 2 diabetes (2,4). Although WS patients are usually not obese by the definition of BMI, the visceral fat specifically accumulated by an unknown mechanism (3) might cause high TNF- α and low adiponectin levels, characteristics similar to morbid obesity.

We recently reported the successful improvement of glycemic control and insulin sensitivity by pioglitazone in diabetic WS patients (5). Therefore, we next assessed adipocytokines before and after 16 weeks on pioglitazone (15 mg/day) in three diabetic WS patients. The treatment significantly elevated adiponectin levels from 2.57 ± 1.36 to 7.07 ± 2.48 $\mu\text{g/ml}$ ($P = 0.03$ by paired t test). TNF- α and HbA_{1c} levels showed a tendency to decline from 16.1 ± 4.75 to 3.53 ± 0.58 pg/ml ($P = 0.052$) and from 7.7 ± 0.6 to $6.4 \pm 0.5\%$ ($P = 0.17$), respectively.

To our knowledge, this is the first study to examine serum adipocytokine levels in WS patients. Reduced insulin sensitivity with increased visceral adiposity is the hallmark of both WS and normal aging. Because pioglitazone achieved improvement of glycemic control as well as correction of adiponectin and TNF- α levels, these cytokines are likely to be at least in part responsible for insulin resistance in WS. Adipocyte function may be a key element linking WRN mutation and the metabolic abnormalities observed in WS. It is also of our interest to know whether pioglitazone and other thiazolidinediones can prevent or delay the onset of diabetes in WS by modulating adipocytokines. Our present findings raise a possibility that pioglitazone could extend the lifespan of WS patients by improving metabolism and preventing early cardiovascular death.

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Effect of α -Linolenic Acid-Containing Linseed Oil on Coagulation in Type 2 Diabetes

Blood coagulation in diabetes is known to be increased (1,2). Because levels of n-3 and n-6 polyunsaturated fatty acids (PUFAs) influence

the parameters of blood coagulation, the aim of this study was to determine the effects of n-3 PUFA supplementation on coagulation and fibrinolytic factors in type 2 diabetic subjects. While it is not clear what the appropriate intake ratio of n-6 to n-3 PUFAs should be for diabetic subjects, it is known that the dietary intake ratio of n-6 to n-3 PUFAs is roughly 4:1 in Japanese subjects (3).

Ten subjects (six women and four men, average age 59.6 years) with type 2 diabetes participated in this study as inpatients. Their average BMI and HbA_{1c} values were 20.9 ± 3.8 kg/m² and $10.8 \pm 1.1\%$, respectively. Their daily energy intake during the course of the study was $1,490 \pm 166$ kcal. After 2 weeks on the control diet, our subjects were placed on a diet in which 5 g linseed oil was added (in salads, miso soup, etc., without heating) in exchange for 5 g cooking oil. The ratio of PUFAs to saturated fatty acids in the subjects' prestudy and study diets were 1.2 and 1.6, respectively, while the ratios of n-6 to n-3 PUFAs in their prestudy and study diets were 3.6 and 1.5, respectively. Blood samples were collected before and 14 days after initiation of the study. Plasmin α 2-plasmin inhibitor complex (PPI) level and plasminogen activator inhibitor-1 (PAI-1) activity in plasma was measured using a latex photometric immunoassay, while thrombin anti-thrombin III complex (TAT) level was measured using an enzyme-linked immunoassay. Differences in these parameters obtained at the start and end of the study were analyzed using a paired t test; values were considered to be significant if the P value was <0.05 . Values are expressed as the mean \pm SD.

After 2 weeks on a linseed oil-supplemented diet, PPI level, PAI-1 activity, and TAT level fell significantly (0.72 ± 0.19 vs. 0.47 ± 0.14 $\mu\text{g/ml}$, $P = 0.0009$; 73.3 ± 37.5 vs. 51.6 ± 25.0 ng/ml, $P = 0.02$; and 9.6 ± 9.1 vs. 2.5 ± 1.1 ng/ml, $P = 0.04$; respectively).

Boberg et al. (4) reported that PAI-1 activity was increased in type 2 diabetic subjects after supplementation of their diet with 10 g eicosapentaenoic acid. Kelly et al. (5) reported that a diet containing flaxseed oil (60% α -linolenic acid) did not alter indexes of blood coagulation, i.e., bleeding time, prothrombin time, and partial prothrombin time. Chan et al. (6) showed that altering the dietary n-6-to-n-3 PUFA ratio had no effect on

clinical trials or meta-analysis. The epidemiological literature did not need a clinical trial to conclude that smoking causes lung cancer. In the case of propoxyphene, clinical trials have already indicated its lack of efficacy advantages over other alternatives, and epidemiological studies have shown its potential to increase risk of fractures in addition to other known side effects. We appreciate Dr. Morton's clinical experience of treating many patients with propoxyphene and achieving acceptable analgesic efficacy with only rare adverse experiences over the last 25 years. But, again, this by itself is not rigorous scientific evidence of the efficacy and safety of propoxyphene in elderly patients; in fact, in epidemiological terms, Dr. Morton's own clinical experience can be considered an example of only a case series study design.

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METABOLIC IMPROVEMENT AND ABDOMINAL FAT REDISTRIBUTION IN WERNER SYNDROME BY PIOGLITAZONE

To the Editor: Werner syndrome is a rare autosomal recessive disorder known for its premature aging phenotype in-

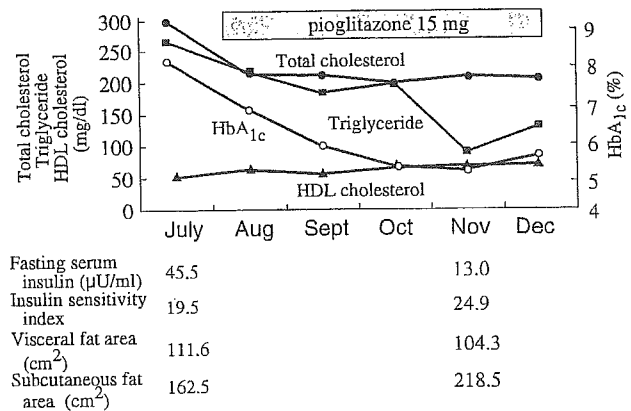


Fig. 1 Metabolic parameters and abdominal fat areas before and during pioglitazone treatment. HDL = high-density lipoprotein; Hb = hemoglobin.

cluding loss of hair, cataracts, atrophy of peripheral soft tissue, diabetes mellitus, and atherosclerosis. Mutations in the deoxyribonucleic acid (DNA) helicase gene have been identified as the cause of this disease.¹ One common feature of Werner syndrome is insulin resistance, but the mechanism by which insulin resistance occurs in this syndrome is unknown. We have previously described that visceral fat accumulation is strongly associated with insulin resistance in Werner syndrome.² We report a case of Werner syndrome in which administration of pioglitazone, a thiazolidinedione derivative, improved insulin sensitivity, glucose tolerance, lipid metabolism, and abdominal fat distribution.

A 46-year old woman with Werner syndrome came to our hospital for glycemic control. After obtaining written informed consent, we analyzed genomic DNA from peripheral leukocytes, which revealed that the patient was homozygote for type 4 mutation in the Werner helicase gene.³ She was thin (body mass index = 16.5 kg/m²) but had accumulated visceral fat in excess, as determined using a computed tomography scan at the umbilical level (visceral fat area = 111.6 cm², normal range for Japanese women <90).⁴ She also had type IIb hyperlipidemia according to World Health Organization classification. She had significant insulin resistance, as determined using an insulin sensitivity index calculated from the value of steady state plasma glucose (19.4, normal range 55–162).⁵ After 1 week of treatment with diet, pioglitazone 15 mg daily was initiated. After 16 weeks of pioglitazone treatment, the patient's fasting plasma glucose had decreased from 198 mg/dL to 115 mg/dL, glycated hemoglobin A1c from 8.4% to 5.9% (normal = 5.9% or less), serum total cholesterol from 270 mg/dL to 209 mg/dL (normal = 130–220 mg/dL), serum triglyceride from 301 mg/dL to 90 mg/dL (normal = 80–150 mg/dL), and serum high-density lipoprotein-cholesterol increased from 52 mg/dL to 64 mg/dL (normal ≥40 mg/dL). Fasting serum insulin decreased from 45.5 μU/mL to 13.0 μU/mL (normal = 6–26 μU/mL), and insulin sensitivity index had improved to 24.9 (Figure 1, July to November). Although the patient gained weight, from 35.9 kg to 39.0 kg, during the period, her visceral fat area (V) decreased to 104.3 cm². In contrast, abdominal subcutaneous fat area (S) increased from 162.5 cm² to 218.5 cm². As a result, her V/S ratio decreased from 0.69 to 0.48 (normal range for Japanese

<0.4).⁴ Liver function monitored using serum transaminase level did not show abnormality throughout the period.

These results suggest that pioglitazone was effective in ameliorating impaired insulin sensitivity, glycemic control, and hyperlipidemia in the patient. Human and animal studies have shown that a possible mechanism for thiazolidinedione to improve insulin sensitivity is through the specific promotion of subcutaneous adipocyte differentiation through the activation of peroxisome proliferator-activated receptor- γ .⁶ It has also been reported that troglitazone-treatment of type 2 diabetic patients resulted in subcutaneous fat increase in accordance with improvement of glucose tolerance.⁷ It was also proven experimentally that, in lipoatrophic diabetes mellitus, lack of fat is directly associated with insulin resistance and hyperglycemia.⁸ Marked atrophy of soft tissues in the extremities, a characteristic feature of Werner syndrome, may at least in part account for the insulin resistance. Leptin administration was recently reported to ameliorate severe insulin resistance in leptin-deficient lipodystrophic patients,⁹ but in our patient, serum leptin levels were in the normal range before and during the pioglitazone treatment (data not shown). Therefore, in this case, induction of subcutaneous fat using pioglitazone would have accompanied production of another mediator than leptin to improve insulin sensitivity.

Recently, accumulating evidence suggests that thiazolidinedione has direct antiatherosclerotic effects on vascular cells.¹⁰ Because atherosclerotic vascular disease is a leading cause of middle-age mortality in Werner syndrome, pioglitazone may disorder an ideal choice for the treatment of metabolic disorders to improve prognosis of this syndrome.

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TREATMENT OF FRACTURES OF THE ATROPHIC MANDIBLE IN THE ELDERLY

To the Editor: The surgical treatment of facial injuries in elderly patients becomes increasingly important in geriatric medicine.¹ The number of injuries increases in the geriatric population due to a longer life expectation of the people in our society, in combination with a higher frequency of leisure activities and mobility of older people. The therapeutic concept of injuries in elderly patients has some special aspects; often, the general condition of the patients and some local factors complicate surgical management and increase comorbidity. The preferably fast reconstruction of function with as little invasiveness as possible is the most important aim of surgical care of the aged.²

The surgical management of maxillofacial fractures in elderly patients has the same problems. Whereas fractures of the midface can be treated surgically using conventional miniplates, mandibular fracture treatment, being biomechanically more complex and challenging, imposes some unresolved problems. The mandible in the elderly is often edentulous and atrophic. The reduced cross section and small contact area of the fractured ends produce minor primary stability, and the correct anatomical reposition is often difficult.³ Moreover, increasing atrophy frequently represents a risk during bone healing because of the sclerotic bone and the lack of blood circulation.⁴ These unfavorable conditions contribute to the high morbidity of atrophic mandibular fractures.⁵

In the literature, various therapeutic concepts are described. Although some authors favor noninvasive, conservative management, plate osteosynthesis of the fractured atrophic mandible is the most commonly preferred treatment option in trauma centers.⁶ Surgeons who use bicortical plate osteosynthesis systems report difficulties in inserting the thick screws into the thin bone fragments. Additionally, the exact reposition with direct contact between the thin bone fragments is often impossible with the strong metal plates. A major disadvantage of thick plates is the inability of patients to wear full dentures during the time of plate incorporation (Figure 1, top right), but the application of conventional miniplates does not generally result in enough stability of the bone fragments⁷ (Figure 1, top left).

Therefore, none of the therapeutic concepts discussed in the literature has gained general acceptance. Whereas some controversy existed in the literature on the stability of osteosynthetic fracture treatment, recent research indicates a range of optimized micromovements (500–2,000 μ strain) in the fracture gap.⁸ A unique plate system fulfilling the individual biological, biomechanical, and clinical requirements

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

Harukiyo Kawamura, Koutaro Yokote, Sunao Asami, Kazuki Kobayashi, Masaki Fujimoto, Yoshiro Maezawa, Yasushi Saito, Seijiro Mori

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, pSR α -myc-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 pSR α -myc-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)¹ is a multifunctional phosphoprotein secreted by many cell types such as osteoclasts, lymphocytes, macrophages, epithelial cells, and vascular smooth muscle cells (SMC).^{1,2} Overexpression of OPN has been found in several physiological and pathological conditions, including immunologic disorders,³ neoplastic transformation,⁴ progression of metastasis,⁵ formation of urinary stones,⁶ and wound healing.⁷

It was reported that OPN protein and mRNA were expressed in the neointima and in calcified atheromatous plaque.⁸ A neutralizing antibody against OPN was found to inhibit rat carotid neointimal formation after endothelial denudation.⁹ 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.¹⁰ 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.¹¹ Furthermore, OPN was found to stimulate migration and enhance platelet-derived growth factor-mediated DNA synthesis of cultured rat aortic SMC.¹⁰

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,

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NY). pSR α -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 glyceraldehydes-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 described¹² by the explant method from adult male Wistar rats weighing \approx 200 grams. Cells were maintained in Dulbecco modified Eagle medium containing 5.5 mmol/L glucose, 10% fetal bovine serum, and 40 μ g/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 pSR α -myc-RhoDA by using Fugene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). pSR α -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 described¹¹ 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 pSR α -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 pSR α -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 azaserine 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-Rhotekin 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 μ g/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.¹⁰ 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.¹³ 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, GGTI-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, GGTI-298 and FTI277 dose-dependently decreased OPN mRNA level under high glucose concentration, as expected.

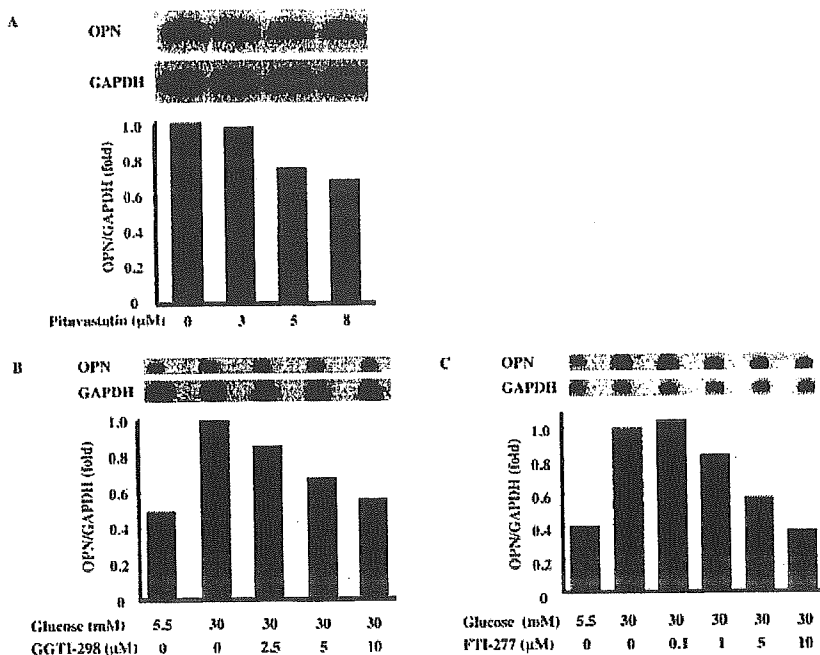


Figure 1. Effects of inhibitors for HMG-CoA reductase, geranylgeranyltransferase, and farnesyltransferase on OPN expression in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of Pitavastatin (A), GGTI-298 (B), or FTI-277 (C) in serum-free 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.

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, pSR α -myc-RhoDA, on OPN expression in cultured rat aortic SMC. As shown in Figure 2B, transfection of pSR α -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

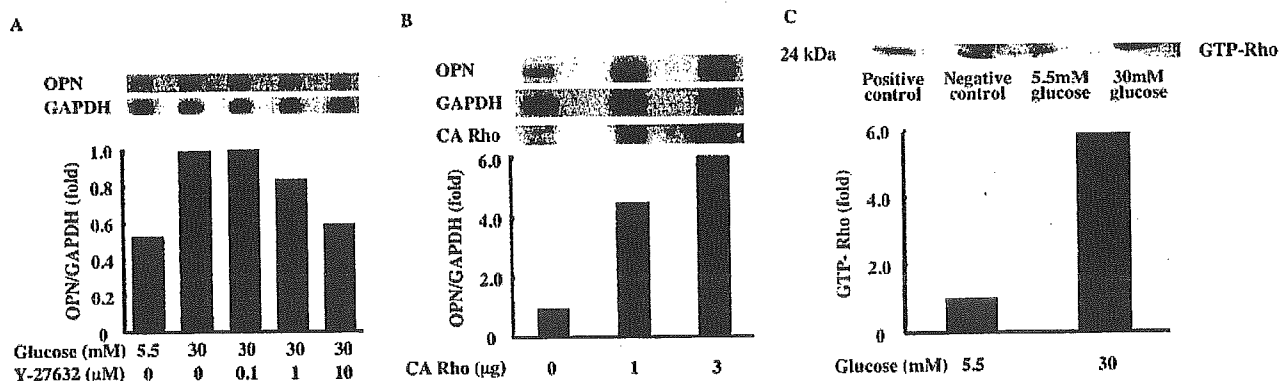


Figure 2. A, Effect of a Rho kinase inhibitor on OPN expression in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of Y-27632 in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 48 hours. After incubation, cells 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 μ g of pSR α -myc-RhoDA and incubated for 48 hours, as described in Methods. After incubation, cells were processed for Northern blotting. The blots were re-probed 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 osmolarity 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.¹¹ 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 μ M GF109203X or 5 μ mol/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.

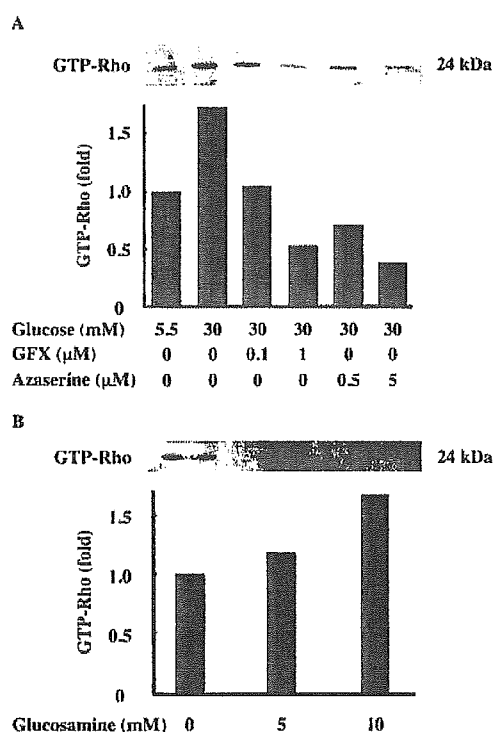


Figure 3. A, Effects of GF109203X (GFX) and azaserine on high glucose-induced Rho activation in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of GFX or azaserine in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 24 hours. After incubation, cells were processed for Rho activation assay 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 pSR α -myc-RhoDA dramatically enhanced ERK activity, whereas transfection of pSR α -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 Chalet et al.¹⁴ 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

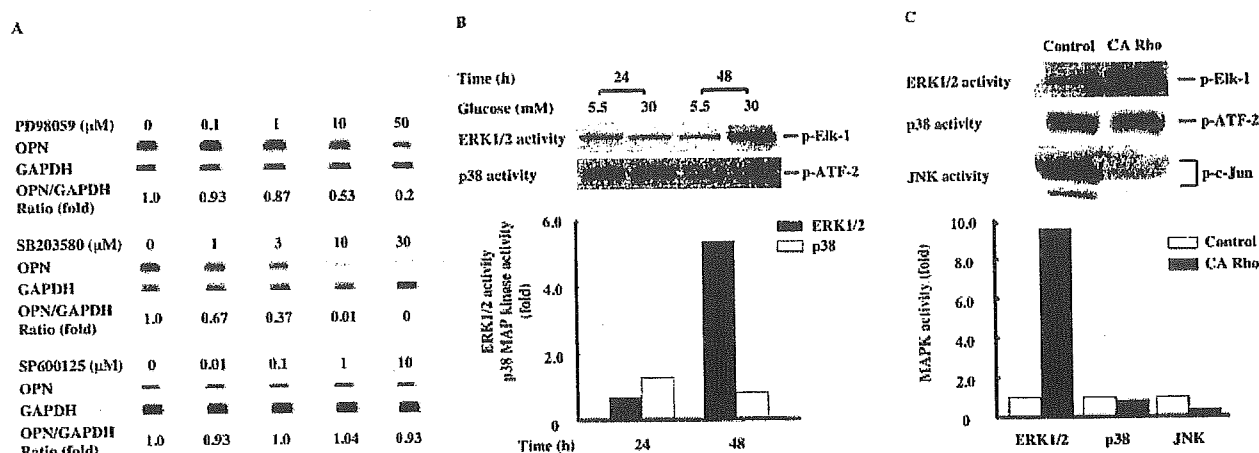


Figure 4. A, Effects of MAP kinase inhibitors on OPN expression in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of PD98059, SB203580, or SP600125 in serum-free medium containing 30 mmol/L glucose for 48 hours. After incubation, cells were processed for Northern blotting as described in the legend to Figure 1. B, High glucose-induced ERK 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 to 48 hours. After incubation, activities of ERK1/2 and p38 MAP kinase in cell lysates were measured by immune complex kinase assay with an immobilized phospho p44/42 MAP kinase antibody and Elk-1 protein as substrate, or with an immobilized phospho p38 MAP kinase antibody and ATF-2 protein as substrate, respectively. After phosphorylation reactions, samples were processed for Western blotting with phospho Elk-1 antibody or phospho ATF-2 antibody. Data are expressed as fold increase relative to the value obtained in 5.5 mmol/L glucose at the indicated times. C, Effect of transient transfection of a constitutive active Rho mutant (CA Rho) on activation of MAP kinases in cultured rat aortic SMC. Cells were transfected with 3 μg of pSRα-myc-RhoDA and incubated for 48 hours as described in the legend to Figure 2. After incubation, MAP kinase activities in cell lysates were determined. Data are expressed as fold increase relative to the value obtained in the absence of CA Rho. Double bands in the JNK activity assay correspond to 37- and 35-kilodalton forms of phosphorylated c-Jun fusion proteins. Data shown in this figure are representative of at least 2 independent experiments providing essentially similar results.

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 atherosclerosis in diabetes mellitus, because involvement of Rho/Rho kinase pathway has been implicated in a wide variety of atherosclerotic processes, including neointimal formation,¹⁵ vasospastic response,^{16,17} proliferation,^{18,19} migration,^{19,20} and anti-apoptosis^{20,21} of vascular SMC, and vascular gene expression of monocyte chemoattractant protein-1,²² transforming growth factor-β1,²² and inducible nitric oxide synthase.²³ Besides our present study using rat aortic SMC, high glucose-induced Rho activation was also observed in cultured rat mesangial cells²⁴ and in basilar artery derived from streptozotocin-induced diabetic rats.²⁵ 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.²⁶ 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 geranylgeranylpyrophosphate but not by farnesylpyrophosphate.¹³ Studies using other types of cells,

fibroblasts,²⁷ or keratinocytes²⁸ 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.

Acknowledgments

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Metabolic improvement and abdominal fat redistribution
in Werner syndrome by pioglitazone.

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To the Editor: Werner syndrome is a rare autosomal recessive disorder known for its premature aging phenotype including loss of hair, cataracts, atrophy of peripheral soft tissue, diabetes mellitus and atherosclerosis. Mutations in the deoxyribonucleic acid helicase gene have been identified as the cause of this disease¹. One common feature of Werner syndrome is insulin resistance. However, the mechanism by which insulin resistance occurs in this syndrome is unknown. We have previously described that visceral fat accumulation is strongly associated with insulin resistance in Werner syndrome². We report a case of Werner syndrome in which administration of pioglitazone, a thiazolidinedione derivative, improved insulin sensitivity, glucose tolerance, lipid metabolism as well as abdominal fat distribution.

A 46-year old woman with Werner syndrome came to our hospital for glycaemic control. Analysis of genomic DNA from peripheral leukocytes, after obtaining a written informed consent, revealed that the patient was homozygote for type 4 mutation in the Werner helicase gene³. She was thin (body-mass index 16.5 kg/m²), but accumulated visceral fat in excess as determined by computed tomography scan at the umbilical level (visceral fat area 111.6 mm², normal range for Japanese women: <90)⁴. She also had type IIb hyperlipidemia according to World classification. She had a significant insulin resistance as judged by Insulin Sensitivity Index (ISI) calculated from the value of

steady state plasma glucose (19.4, normal range:55-162)⁵. After 1 week of treatment on diet, pioglitazone 15 mg daily was initiated. Following 16 weeks of pioglitazone treatment, the patient's fasting plasma glucose had decreased from 198 mg/dl to 115 mg/dl, glycated hemoglobin (Hb) A_{1c} from 8.4% to 5.9%, serum total cholesterol from 270 mg/dl to 209 mg/dl, serum triglyceride from 301 mg/dl to 90 mg/dl and serum high density lipoprotein (HDL)-cholesterol increased from 52 mg/dl to 64 mg/dl. Fasting serum insulin decreased from 45.5 μ U/ml to 13.0 μ U/ml, and ISI had improved to 24.9 (Figure, July to November). Although the patient gained weight from 35.9 kg to 39.0 kg during the period, her visceral fat area (V) decreased to 104.3 mm². In contrast, abdominal subcutaneous fat area (S) increased from 162.5 mm² to 218.5 mm². As the result, V/S ratio decreased from 0.69 to 0.48 (normal range for Japanese: < 0.4)⁴. Liver function monitored by serum transaminase level did not show abnormality throughout the period.

These results suggest that pioglitazone was effective in ameliorating impaired insulin sensitivity, glycaemic control as well as hyperlipidemia in the patient. Both human and animal studies have shown that a possible mechanism for thiazolidinedione to improve insulin sensitivity is through the specific promotion of subcutaneous adipocyte differentiation through the activation of peroxisome proliferator-activated receptor- γ ⁶. It has also been reported that

troglitazone-treatment of type2 diabetic patients resulted in subcutaneous fat increase in accordance with improvement of glucose tolerance⁷. It was also proven experimentally that, in lipoatrophic diabetes, a lack of fat is directly associated with insulin resistance and hyperglycemia⁸. Marked atrophy of soft tissues in the extremities, a characteristic feature of Werner syndrome, may at least in part account for the insulin resistance. Leptin administration was recently reported to ameliorate severe insulin resistance in leptin-deficient lipodystrophic patients⁹. However, in our patient, serum leptin levels were in a normal range both before and during the pioglitazone treatment (data not shown). Therefore, in this case, induction of subcutaneous fat by pioglitazone would have accompanied production of a yet other mediator than leptin to improve insulin sensitivity. Recently, accumulating evidence suggests that thiazolidinedione has direct anti-atherosclerotic effects on vascular cells¹⁰. Since atherosclerotic vascular disease is a leading cause of middle age mortality in Werner syndrome, pioglitazone may provide an ideal choice for the treatment of metabolic disorders to improve prognosis of this syndrome.

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Footnotes

The abbreviations used are: ISI, insulin sensitivity index; Hb, hemoglobin; HDL, high density lipoprotein; V, visceral fat area; S, subcutaneous fat area.

The normal ranges are: total cholesterol, 130 to 220 mg/dl; triglyceride, 80 to 150 mg/dl; HDL-cholesterol, 40 mg/dl or more; HbA1c, 5.9% or less; fasting serum insulin, 6 to 26 μ U/ml; insulin sensitivity index, 55 to 162, visceral fat area, less than 100 cm²; V/S ratio, less than 0.4.

Figure Legend

Metabolic parameters and abdominal fat areas before and during pioglitazone treatment.

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

Harukiyo Kawamura, Koutaro Yokote, Sunao Asami, Kazuki Kobayashi, Masaki Fujimoto, Yoshiro Maezawa, Yasushi Saito, Seijiro Mori

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, pSR α -myc-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 pSR α -myc-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)¹ is a multifunctional phosphoprotein secreted by many cell types such as osteoclasts, lymphocytes, macrophages, epithelial cells, and vascular smooth muscle cells (SMC).^{1,2} Overexpression of OPN has been found in several physiological and pathological conditions, including immunologic disorders,³ neoplastic transformation,⁴ progression of metastasis,⁵ formation of urinary stones,⁶ and wound healing.⁷

It was reported that OPN protein and mRNA were expressed in the neointima and in calcified atheromatous plaque.⁸ A neutralizing antibody against OPN was found to inhibit rat carotid neointimal formation after endothelial denudation.⁹ 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.¹⁰ 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.¹¹ Furthermore, OPN was found to stimulate migration and enhance platelet-derived growth factor-mediated DNA synthesis of cultured rat aortic SMC.¹⁰

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,

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NY). pSR α -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 glyceraldehydes-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 described¹² by the explant method from adult male Wistar rats weighing \approx 200 grams. Cells were maintained in Dulbecco modified Eagle medium containing 5.5 mmol/L glucose, 10% fetal bovine serum, and 40 μ g/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 pSR α -myc-RhoDA by using Fugene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). pSR α -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 described¹¹ 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 pSR α -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 pSR α -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 azaserine 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-Rhotekin 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 μ g/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.¹⁰ 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.¹³ 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, GGTI-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, GGTI-298 and FTI277 dose-dependently decreased OPN mRNA level under high glucose concentration, as expected.

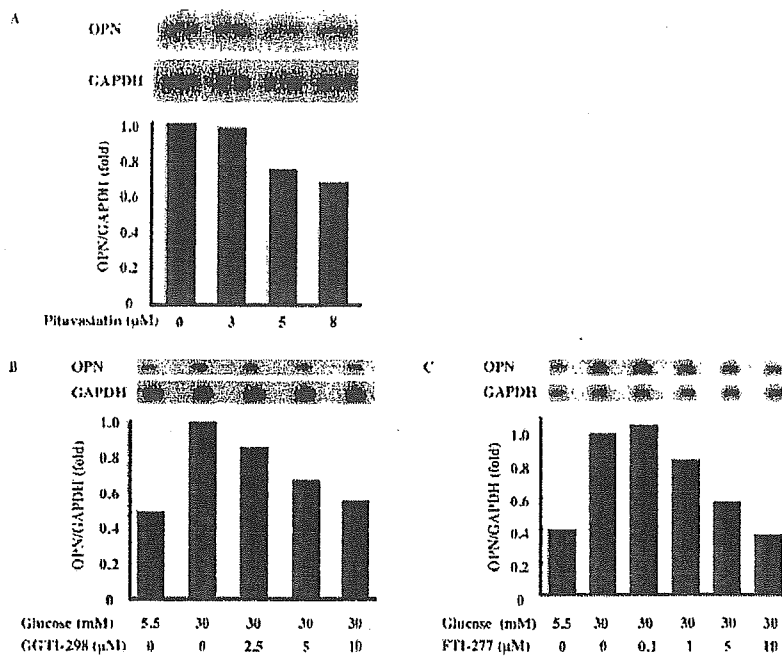


Figure 1. Effects of inhibitors for HMG-CoA reductase, geranylgeranyltransferase, and farnesyltransferase on OPN expression in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of Pitavastatin (A), GGTI-298 (B), or FTI-277 (C) in serum-free 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.

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, pSR α -myc-RhoDA, on OPN expression in cultured rat aortic SMC. As shown in Figure 2B, transfection of pSR α -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

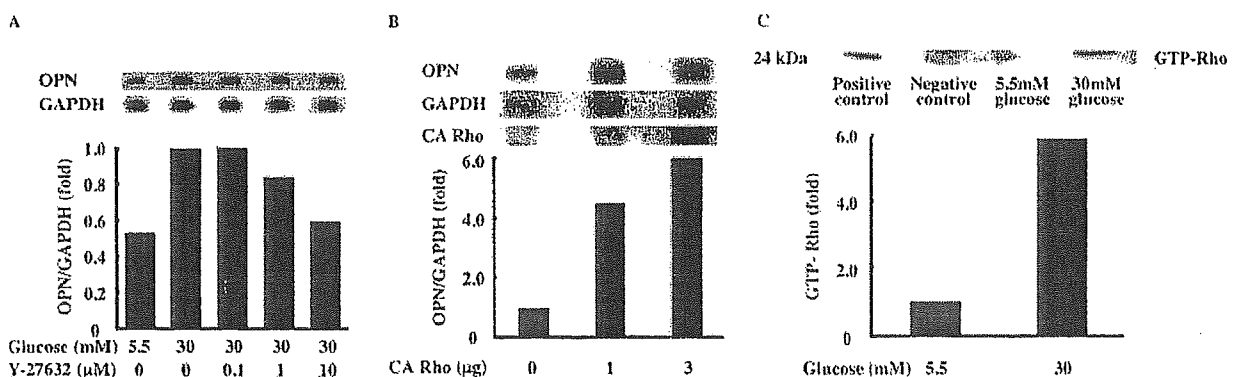


Figure 2. A, Effect of a Rho kinase inhibitor on OPN expression in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of Y-27632 in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 48 hours. After incubation, cells 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 μ g of pSR α -myc-RhoDA and incubated for 48 hours, as described in Methods. After incubation, cells were processed for Northern blotting. The blots were re-probed 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 osmolarity 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.¹¹ 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 μ M GF109203X or 5 μ mol/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.

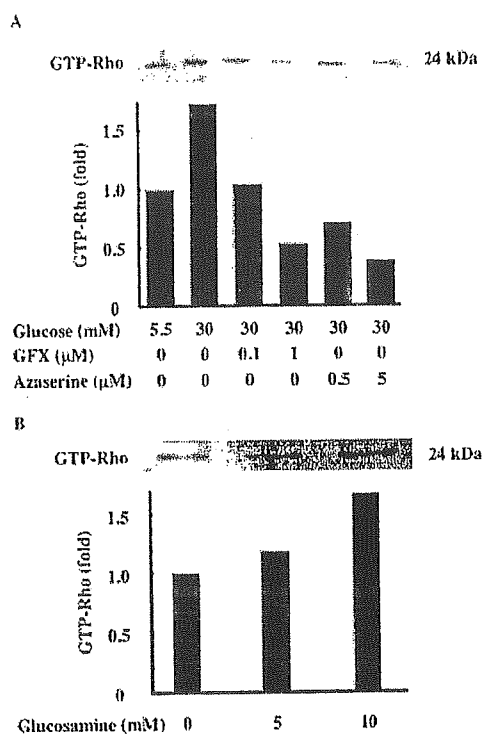


Figure 3. A, Effects of GF109203X (GFX) and azaserine on high glucose-induced Rho activation in cultured rat aortic SMC. After serum-starvation for 24 hours, cells were incubated with the indicated concentrations of GFX or azaserine in serum-free medium containing either 5.5 mmol/L or 30 mmol/L glucose for 24 hours. After incubation, cells were processed for Rho activation assay 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 pSR α -myc-RhoDA dramatically enhanced ERK activity, whereas transfection of pSR α -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 Chaulet et al.¹⁴ 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