Objective—Rho/Rho-kinase pathway plays pivotal roles in cardiovascular diseases including arteriosclerosis and hypertension. Recently it has become evident that C-reactive protein (CRP), a powerful marker for cardiovascular events, has direct proatherothrombotic effects on vascular cells. However, its molecular mechanism has not been fully investigated. We examined the involvement of Rho/Rho-kinase signaling in CRP-induced plasminogen activator inhibitor-1 (PAI-1) expression in bovine aortic endothelial cells (BAECs).

Methods and Results—PAI-1 expression was determined by Western blotting. RhoA activation was determined by an affinity pull-down assay using Rho-binding fragment of rhotekin. NF-κB activity was determined using the luciferase reporter gene.

Incubation of BAECs with human recombinant CRP (≥25 μg/mL) induced a significant increase in PAI-1 expression. Stimulation of BAECs with CRP significantly increased RhoA activation. Pretreatment with TAT-C3 (a membrane-permeable RhoA inhibitor) and Y-27632 (Rho-kinase inhibitor) significantly inhibited CRP-induced PAI-1 expression. NF-κB activity was markedly enhanced by CRP and pretreatment with Y-27632 inhibited its activation. Parthenolide, SN50, and BAY 11-7082 (NF-κB inhibitors) significantly blocked CRP-mediated PAI-1 expression.

Conclusions—These data suggested that CRP activates Rho/Rho-kinase signaling, which in turn activates NF-κB activity, resulting in PAI-1 expression in BAEC. These observations provide evidence for the possible involvement of Rho/Rho-kinase signaling in CRP-induced atherothrombogenesis. (Arterioscler Thromb Vasc Biol. 2005;25:2088-2093.)

Key Words: C-reactive protein ■ endothelial cells ■ NF-κB ■ PAI-1 ■ Rho/Rho-kinase
Rho/Rho-kinase signaling, which in turn activates NF-κB activity, resulting in PAI-1 expression. These findings provide evidence for the possible involvement of Rho/Rho-kinase signaling in CRP-induced atherothrombogenesis.

Methods

Materials

Recombinant human CRP was purchased from Calbiochem. Endotoxin was removed from CRP with a detoxigel column (Pierce Biochemicals) and quantified as <0.008 endotoxin unit/mL by Limulus assay (WAKO). Y-27632 was generously provided by Mitsubishi Pharma Corp (Osaka, Japan). Y-27632 is a specific inhibitor for Rho kinases and its inhibitory potency is at least 20 to 30 times higher than those for 2 other Rho effector kinases.17 Three kinds of NF-κB inhibitors were used: Parthenolide (Calbiochem), a specific inhibitor for NF-κB activity;18 SN50 (BIOMOL), a cell-permeable specific inhibitory peptide that inhibits nuclear translocation of NF-κB complex;19 BAY 11-7982 (BIOMOL), a selective and irreversible inhibitor of IκB-α phosphorylation with additional effects that include activation of stress-activated protein kinase (p38 and JNK-1), and protein tyrosine phosphorylation.20

Cell Culture

BAECs were harvested from thoracic aortas and maintained as described previously.21 Cells were grown to confluence in DMEM (Sigma) with 10% fetal bovine serum (Biowest, Ringmer, UK), 100 U/mL penicillin, and 100 µg/mL streptomycin in 95% O2/5% CO2 at 37°C and were used between passages 3 and 9.

Preparation of Cell Extracts

Confluent cells were washed twice with phosphate-buffered saline and scraped in modified buffer (50 mmol/L Tris- HCl at pH 7.4, 1% NP-40, 150 mmol/L NaCl, 0.25% wt/vol Na-deoxycholate, 1 mmol/L EDTA) with protease inhibitors (1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µmol/L p-amidinophenylmethanesulfonyl fluoride hydrochloride). Cells were lysed using a 23-gauge needle. After centrifugation at 10 000g for 10 minutes at 4°C, the supernatants were applied to SDS-PAGE and subsequent Western blotting.

Determination of RhoA Activation

RhoA activation was determined by affinity precipitation of the active GTP-bound RhoA using a glutathione S-transferase (GST)-fusion protein of the Rho-binding domain of the Rho effector rhokinase (GST-RBD) as previously described.22 The plasmid for GST-RBD was a generous gift from Dr Martin Alexander Schwartz (The Scripps Research Institute, La Jolla, Calif). In brief, BAECs stimulated with CRP were lysed with RhoA-RBD buffer, and then GST-RBD bound to glutathione Sepharose 4B was added to the lysate to selectively bind activated RhoA for the pull-down assay. Detection of RhoA was performed by Western blot using anti-RhoA antibody (Santa Cruz Biotechnology Inc).

Western Blotting Analysis

Protein-matched samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore). The primary antibodies used in this study were mouse monoclonal anti-PAI-1, anti-α-tubulin (Oncogene), and rabbit polyclonal anti-RhoA antibodies. Immunodetection was accomplished using appropriate horseradish peroxidase-linked secondary antibodies (Amersham) and enhanced chemiluminescence kit (Amersham). The blots were exposed to films (Fuji RX) for various times to obtain a linear response with the enhanced chemiluminescence method and band density was estimated by densitometry, as described previously.23

Plasmid Constructions and TAT-C3

Protein Purification

cDNA encoding for Clostridium botulinum C3 exoenzyme (kindly provided by Dr Alan Hall, Institute of Cancer Research, London) was cloned in frame, from the C-terminal of the HIV TAT protein transduction domain in vector pTAT- HA (kindly provided by Dr Steven F. Dowdy, Washington University, St. Louis, Mo). Recombinant TAT-C3 protein was produced in Escherichia coli and purified as previously described.24

NF-κB Luciferase Reporter Gene Assay

The pNF-κB-Luc reporter plasmid (BD Biosciences Clontech), which contains 5 NF-κB binding sites as an enhancer and the firefly luciferase reporter gene was purchased from Clontech. BAECs cultured in a 6-well dish were transfected 1 µg of the pNF-κB-Luc using FuGENE 6 (Roche). At 24 hours after transfection, the cells were pretreated with Y-27632 for 30 minutes, then stimulated with CRP. The cells were lysed and the luciferase activity was measured in a Lumat LB9501 luminometer (Berthold Technologies) using a Luciferase Assay System (Promega). The results were normalized to the β-galactosidase activity of the cotransfected 1 µg of the pSV-β-galactosidase control vector (Promega) using the β-galactosidase Enzyme Assay System (Promega) in all experiments.

Statistics

All values are expressed as mean±SEM. Data were analyzed by 2-way ANOVA or Student t test when appropriate. A level of P<0.05 was considered significant.

Results

PAI-1 Expression Induced by CRP in BAECs

Incubation of BAECs with human recombinant CRP resulted in a significant increase in PAI-1 expression. The upregulation of PAI-1 by CRP was observed both in the absence (1.30±0.19-fold versus control; P<0.05) and the presence of 10% fetal bovine serum (2.05±0.45-fold versus control; P<0.05), although higher levels of PAI-1 expression were achieved in the presence of serum (Figure 1A). A significant
An increase in PAI-1 expression was detected after 6 hours, peaked at 12 hours, and its plateau expression was observed until 24 hours of incubation (data not shown). The levels of PAI-1 expression were dependent on CRP concentration (25 to 100 µg/mL) (data not shown). Boiling of CRP (100°C for 5 minutes) abrogated its effect on PAI-1 expression (data not shown). As shown in Figure 1B, sodium azide, which was included in commercial preparation, had no effect on PAI-1 expression in BAECs at the concentration of 50 µg/mL CRP equivalent, although a higher concentration (100 µg/mL CRP equivalent) of sodium azide could significantly inhibit PAI-1 expression (67 ± 2% of control; *P < 0.01, n = 3). These data confirmed the stimulatory effect of CRP on PAI-1 expression in human aortic endothelial cells.

Activation of RhoA in BAECs Stimulated by CRP

The level of the active GTP-bound form of RhoA was examined by a pull-down assay with the Rho-binding fragment of rhotekin. As shown in Figure 2, stimulation of the cells with CRP significantly activated RhoA (3.4 ± 0.5-fold versus control), suggesting that CRP stimulation may activate RhoA in BAEC.

Effects of TAT-C3 on PAI-1 Expression by CRP

The effect of TAT-C3 (membrane-permeable RhoA inhibitor) on CRP-induced PAI-1 expression was investigated. As shown in Figure 3, pretreatment with TAT-C3 significantly inhibited the CRP-induced PAI-1 expression by 60 ± 15%. These data support the involvement of RhoA activation in CRP-induced PAI-1 expression in BAECs.

Effect of Y-27632 on the Expression Level of PAI-1 Induced by CRP

We examined the involvement of Rho-kinase, one of the major downstream targets of RhoA, on CRP-induced PAI-1 expression in BAEC. As shown in Figure 4, the Rho-kinase inhibitor, Y-27632, dose-dependently inhibited the PAI-1 expression induced by CRP. Pretreatment with 1 and 10 µmol/L Y-27632 blocked PAI-1 expression by 43 ± 26% and 78 ± 11%, respectively. Y-27632 could also inhibit basal (without CRP stimulation) PAI-1 expression.

Secreted PAI-1 antigen levels in the culture medium of human aortic endothelial cells were also increased by CRP (control, 464 ± 34 ng/mL; 50 µg/mL CRP stimulation for 12 hours, +P < 0.01).

Figure 2. Activation of RhoA by CRP in BAECs. Representative Western blots show the extents of the active form of RhoA (GTP-RhoA; top panel) and total RhoA (middle panel). Bottom panel, Summarized data of the densitometrical analyses of RhoA activation. RhoA activity was represented as the relative ratio of the density of GTP-RhoA against that of total RhoA. Relative ratio in the control was expressed as 1 arbitrary unit. ctl, BAECs without CRP stimulation; CRP, BAECs stimulated with CRP (50 µg/mL, for 12 hours). n = 4, *P < 0.05.

Figure 3. Effect of TAT-C3 on CRP-induced PAI-1 expression. Top and middle panels, Representative Western blots using anti-PAI-1 and anti-α-tubulin antibodies, respectively. Bottom panel, Summarized densitometrical data. Mean ratio of the densities of PAI-1 vs α-tubulin in the control was expressed as 1 arbitrary unit. ctl, BAECs without CRP stimulation; CRP, BAECs stimulated with CRP (50 µg/mL, for 12 hours); CRP + TAT-C3, BAECs pretreated with TAT-C3 (5 µg/mL, for 12 hours), followed by stimulation with CRP. n = 4, *P < 0.01.

Figure 4. Effect of Y-27632 on CRP-induced PAI-1 expression. Top and middle panels, Representative Western blots using anti-PAI-1 and anti-α-tubulin antibodies, respectively. Bottom panel, summarized densitometrical data. Mean ratio of the densities of PAI-1 vs α-tubulin in control (without Y-27632 pretreatment and CRP stimulation) was expressed as 1 arbitrary unit. BAECs were pretreated with or without Y-27632 (for 30 minutes), then stimulated by either CRP (50 µg/mL) or its solvent phosphate-buffered saline. After 12 hours, incubation cells were harvested, followed by Western blot. n = 4, *P < 0.05, **P < 0.01.
We examined whether CRP-induced PAI-1 expression in BAECs was dependent on NF-κB. Pretreatment with Y-27632 (10 μmol/L) inhibited its activation by 53±9.0% (Figure 5). These results suggest the involvement of Rho-kinase in CRP-induced PAI-1 expression in BAECs as well as in human aortic endothelial cells.

**Effect of Rho-Kinase Inhibitor on CRP-Induced NF-κB-Dependent Transcriptional Activity**

We examined the ability of CRP to induce NF-κB-mediated reporter gene expression and the effect of Rho-kinase inhibitor on CRP-mediated NF-κB activity. As shown in Figure 5, NF-κB activity was markedly enhanced (1.5±0.3 folds) by the stimulation with CRP. Pretreatment with Y-27632 (10 μmol/L) inhibited its activation by 53±9.0% (Figure 5). These results suggest the involvement of Rho-kinase in NF-κB activation by CRP.

**Effect of NF-κB Inhibitors on the Expression Level of PAI-1 Induced by CRP**

We examined whether CRP-induced PAI-1 expression in BAECs was dependent on NF-κB activation using 3 NF-κB inhibitors. As shown in Figure 6A, pretreatment with 1 and 10 μmol/L parthenolide significantly blocked the level of CRP-induced PAI-1 expression by 38±15% and 70±20%, respectively. Similarly, pretreatment with either SN50 (10 μmol/L) or BAY 11-7082 (10 μmol/L) also significantly blocked its expressions by 50±18% and 72±3.9%, respectively (Figure 6B and 6C). These data support the involvement of NF-κB activation in CRP-induced PAI-1 expression in BAECs.

**Discussion**

Inflammation is involved in all stages of atherogenesis, in which various mediators, including cytokines, chemokines, growth factors, and adhesion molecules, play important roles.\(^1,2\) In addition to being an independent cardiovascular disease risk factor,\(^3\) recent accumulating evidence implicates CRP as a mediator of atherosclerosis.\(^4,5\)

The concept of CRP as a mediator of atherogenesis is based on in vitro data mostly from endothelial cells and, in some reports, VSMCs and monocyte–macrophages.\(^6\) In endothelial cells, CRP elicited proinflammatory and prothrombotic effects through increased cell adhesion molecules,\(^25\) monocyte chemoattractant protein (MCP-1),\(^26\) endothelin-1 (ET-1),\(^27\) interleukin (IL)-6,\(^27\) IL-8,\(^8\) and PAI-1,\(^6\) and decreased nitric oxide\(^28,29\) and prostacyclin.\(^30\) Despite the many reports regarding the cellular and biological phenomena induced by CRP, their intracellular signal transduction roles are quite limited and remain to be determined. There were only several reports that CRP elicited NF-κB and MAP kinase signaling, in endothelial cells, VSMC, and U937 histiocytes.\(^8–10,31\)

In this study we investigated whether Rho/Rho-kinase signaling is involved in CRP-induced PAI-1 expression. Similar to a report that used human aortic endothelial cells,\(^15\) human recombinant CRP increased PAI-1 expression in a time- and dose-dependent manner in BAEC. The main findings of this study are: (1) that CRP activated RhoA activity; (2) that the RhoA inhibitor TAT-C3 blocked PAI-1 expression induced by CRP; (3) that the Y-27632 inhibited the PAI-1 upregulation and NF-κB activity induced by CRP; and (4) that the NF-κB inhibitors inhibited PAI-1 expression in BAEC induced by CRP. All these results indicated that...
Rho/Rho-kinase signaling is activated by CRP and plays a pivotal role in CRP-induced PAI-1 expression at least through NF-κB activation.

The cellular effects of CRP remain controversial and the issue is whether the observed cellular responses are either direct effects of CRP itself or caused by contaminants and/or additives in the CRP solution. Recently, it has been reported that most of the responses were induced by the contaminants lipopolysaccharide (LPS) or azide. However, in vivo infusion of pure CRP, free from LPS and sodium azide, into humans was reported to elicit the activation of inflammation and coagulation. In this experiment, contaminating LPS in CRP solution was almost completely removed using detoxigel column, by which the contaminating LPS in CRP solution was dramatically reduced to <0.8 pg/mL. Using this purified CRP, the final concentration of LPS in the medium was <0.04 pg/mL, of which the concentration of LPS was far less than that which could induce PAI-1 expression in our culture condition (data not shown). In addition, boiling of CRP (100°C for 5 minutes) abolished its effect on PAI-1 expression. Sodium azide itself could not induce PAI-1 expression but rather inhibited its expression in BAEC. Furthermore, CRP during extensive dialysis has been reported to induce significant PAI-1 expression (data not shown). All these results indicated that CRP itself has biological activity and can induce PAI-1 expression in endothelial cells.

Some studies have indicated the requirement of serum for cellular responses to CRP. Although the extent of PAI-1 upregulation was higher in the presence of serum, CRP-induced PAI-1 expression could be detected in the absence of serum in this study. The reasons for this discrepancy are not clear, though there is little doubt that CRP could activate endothelial cells in the presence of serum.

In this study, the activation of RhoA by CRP was uncovered by the pull-down assay and the inhibitory effect of a membrane permeable Rho inhibition, TAT-C3. Rho is activated through Gαq/11, a member of the heterotrimeric G protein family coupled with receptors such as those of lysophosphatidic acid, ET-1 and thrombin, and their downstream targets, guanine nucleotide exchange factors. Rho is also activated by Gαq and Gαq-coupled receptors and by intracellular Ca2+ taken in through a voltage-gated Ca2+ channel. The effects of CRP on these Rho regulatory cascades remain to be investigated.

Rho activity is negatively regulated by cAMP and cyclic GMP, possibly via the phosphorylation of Rho by their downstream kinases. To the contrary, Rho negatively regulates endothelial nitric oxide synthase expression, mainly by decreasing the stability of endothelial nitric oxide synthase mRNA. Indeed, CRP, which was shown to activate RhoA in this study, could cause a direct reduction in endothelial nitric oxide synthase expression, resulting in decreased nitric oxide synthesis and a concomitant lowering of cyclic GMP levels. Therefore, CRP-induced Rho activation may be caused and/or effect of impaired nitric oxide/cGMP signaling in BAECs.

We showed that Y-27632 significantly blocked the levels of intracellular PAI-1 expression (in BAECs) and secreted PAI-1 antigen (in human aortic endothelial cells) induced by CRP, indicating the involvement of Rho-kinase signaling in CRP-induced PAI-1 expression. The concentration of Y-27632 (10 μmol/L) used in this study is a reasonable concentration to inhibit Rho-kinase in cultured cells. The inhibitory effects of Y-27632 on PAI-1 expression were also observed in BAECs without CRP stimulation. Because serum was added to the all experiments in this study, the inhibitory effects seemed to be caused by the inhibition of Rho/Rho-kinase signaling activated by serum stimulation.

In cardiovascular tissues, Rho/Rho-kinase signaling has been revealed to be involved in many pathological conditions, including vascular smooth muscle hypercontractility, endothelial permeability, and barrier dysfunction and cardiac hypertrophy. Regarding vascular remodeling and atherosclerosis, treatment with a Rho-kinase inhibitor prevented neointimal formation after balloon injury, and reduced early atherosclerotic lesion formation in mice. These results lead one to the conclusion that Rho-kinase is involved in atherogenesis.

The transcription factor NF-κB has an important function in the regulation of many genes involved in the inflammatory and proliferative responses of cells, and recent studies indicate that NF-κB is involved in the pathogenesis of atherosclerosis. In this study, NF-κB-mediated reporter gene expression was enhanced by the stimulation with CRP. Pretreatment with Y-27632 blocked CRP-induced NF-κB reporter gene expression. All these results suggest that CRP activates Rho/Rho-kinase signaling, which in turn activates NF-κB transcriptional activity. The mechanism for Rho/Rho-kinase–mediated NF-κB activation is not well-understood, although some reports showed the RhoA-induced phosphorylation of inhibitory subunit Iκ-Bα and the activation of Iκ-B kinase α by Rho-kinase, with the association of these 2 molecules. The involvement of Rho-kinase in PAI-1 expression was also reported in angiotensin II-induced VSMC and LPS-stimulated monocytes.

The link between CRP stimulation and NF-κB activation in BAECs was demonstrated by the result that three different kinds of NF-κB inhibitors, namely parthenolide, SN50, and BAY 11-7082, significantly inhibited PAI-1 expression induced by CRP. The concentrations of these NF-κB inhibitors required for the inhibition of CRP-induced PAI-1 expression are almost identical to the values reported for the inhibition of NF-κB activity. Similar results have been observed in rat VSMCs and human aortic and saphenous vein endothelial cells. In VSMCs, CRP-induced NF-κB activation may lead to the induction of MCP-1, IL-6, and inducible nitric oxide synthase gene expression.

In summary, Rho/Rho-kinase is one of the critical signals involved in CRP-induced PAI-1 expression, at least through NF-κB activation in BAEC. Therefore, the Rho/Rho-kinase pathway may come into play in CRP-induced atherothrombotic processes, and the inhibition of Rho/Rho-kinase signals seems to be a novel and fruitful strategy for the prevention and treatment of atherosclerosis.

Acknowledgments

This work was supported in part by grants-in-aid for Scientific Research from the Japan Society for the Promotion of Science, the Mie University COE Project Fund, and Takeda Science Foundation.
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2093

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Rho/Rho-Kinase Pathway Contributes to C-Reactive Protein–Induced Plasminogen Activator Inhibitor-1 Expression in Endothelial Cells
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Arterioscler Thromb Vasc Biol. 2005;25:2088-2093; originally published online August 25, 2005;
doi: 10.1161/01.ATV.0000183607.50230.9f
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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