p38 Mitogen-Activated Protein Kinase Mediates Hyperosmolarity-Induced Vasoconstriction through Myosin Light Chain Phosphorylation and Actin Polymerization in Rat Aorta

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Hyperosmotic stress induces the contractile response of vascular smooth muscle cells (VSMCs). Previous studies have demonstrated that cytoskeleton reorganization and Rho/Rho-kinase-mediated inactivation of myosin light chain phosphatase (MLCP) play an important role in hyperosmotic vasoconstriction, but the precise mechanism is unknown. This study aimed to investigate the contractile response of endothelium-denuded rings of rat aortas to hyperosmolar sucrose (160 mOsms) in the presence or absence of inhibitors for various protein kinases. We found that the hyperosmotic constriction of aortic rings was attenuated not only by ML-7 or hydroxyfasudil, specific inhibitor for myosin light chain kinase (MLCK) or Rho-kinase, respectively, but also by SB203580, a specific inhibitor for p38 mitogen-activated kinase (p38 MAPK). Hyperosmolar sucrose evoked a transient increase in cytosolic free Ca²⁺ in rat VSMCs, and this response was not affected by SB203580. Western blot analysis of proteins extracted from rings showed that the hyperosmolar sucrose stimulated phosphorylation of the Rho-kinase-mediated myosin phosphatase target subunit 1, myosin light chain (MLC), and p38 MAPK. The experiments performed using a combination of the kinase inhibitors showed that hyperosmolarity-induced MLC phosphorylation is partially mediated via the SB203580-sensitive pathway and is independent of both MLCK and Rho-kinase-mediated inactivation of MLCP. Furthermore, the hyperosmolarity-induced increase in the F-actin/G-actin ratio in rings was attenuated not only by hydroxyfasudil but also by SB203580. These results suggest that p38 MAPK is involved in hyperosmotic vasoconstriction via stimulation of MLC phosphorylation and cytoskeleton reorganization through pathways independent of activation of MLCK and/or Rho-kinase-mediated mechanisms.

Key words hyperosmolarity; p38 mitogen-activated protein kinase; Rho-kinase; actin polymerization; vasoconstriction

In mammalian cells, exposure to hyperosmotic conditions leads to excretion of water according to the osmotic gradient, resulting in cell shrinkage as a result of decreased cell volume. The shrinkage of cells activates intracellular signaling pathways, which in turn modify a multitude of cell functions, including the regulation of cell volume.¹–³ Many studies have demonstrated that hyperosmotic shrinkage triggers various intracellular cascades of protein kinases, such as mitogen-activated protein kinase (MAPK),⁴–⁸ protein kinase C,⁹,¹⁰ calmodulin kinase,¹¹ and myosin light chain kinase (MLCK).¹²,¹³ MAPKs are a part of the 3-component kinase module consisting of a MAPK, an extracellular signal-regulated kinase (ERK), and a MEK kinase that couples the signals from receptors to trigger the downstream pathways. Three major subfamilies of MAPKs, that is, the extracellular signal-regulated kinase 1/2, c-Jun N-terminal kinase (JNK), and p38 MAPKs, are activated by various stimuli, including hyperosmotic stress in myocytes,⁴,⁸ corneal epithelial cells,⁶,⁷ and smooth muscle cells.⁵

Previous studies have demonstrated that hyperosmotic stress induces vasoconstriction by Rho/Rho-kinase-mediated inactivation of myosin light chain phosphatase (MLCP) and cytoskeletal reorganization in vascular smooth muscle cells (VSMCs).⁸,¹⁵ In addition, hyperosmotic stress induces an increase in the cytosolic free Ca²⁺ [Ca²⁺]i in cardiomyocytes⁶,¹⁷ or neutrophils⁸ and also induces phosphorylation of the myosin light chain (MLC) in aortic endothelial cells.¹⁹ These findings suggest that hyperosmotic stress evokes a variety of signaling pathways that may induce tension as a consequence of MLC phosphorylation and cytoskeletal reorganization in VSMCs. Despite these findings, the role of MAPKs in hyperosmolarity-induced vasoconstriction remains unknown. To clarify this, we investigated the contractile response of endothelium-denuded rat aortas to hyperosmolar sucrose in the presence or absence of inhibitors for various protein kinases. Our results demonstrate that vasoconstriction induced by hyperosmotic stress is regulated at least in part by p38 MAPK-mediated mechanisms.

MATERIALS AND METHODS

Chemicals The chemicals were purchased from the commercial sources indicated: sucrose from Sigma-Aldrich (St. Louis, MO, U.S.A.); SB203580, AS601245, PD98059 ML-7, and hydroxyfasudil from Calbiochem (U.S.A.); antibodies for myosin phosphatase target subunit 1 (MYPT1), MYPT1 phosphorylated at Thr853 (p-MYPT), p38 MAPK, and phosphorylated p38 MAPK from Santa Cruz Biotechnology (U.S.A.); and antibodies for myosin light chain and MLC phosphorylated at Thr18 and Ser19 from Cell Signaling (U.S.A.); globular (G)-actin/filamentous (F)-actin in vivo assay biochem kit from Cytoskeleton Inc. (U.S.A.); and cytochalasin B from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and Organ Chamber Experiments Male Wistar rats were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed according to the...
guidelines of the Kobe Gakuin University Experimental Animal Care and Use Committee.

Organ chamber experiments were performed as previously described.20 Briefly, aortic rings were prepared from the thoracic aortas of 7-week-old male Wistar rat (4 rings/animal), and the endothelium was removed by carefully rotating a manipulator inside the ring lumen. The rings (3–4 mm length) were vertically fixed under a resting tension of 1.0 g in a 5 mL organ bath filled with Krebs–Henseleit solution (37°C, pH 7.4) continuously aerated with a gas mixture of 95% O₂ and 5% CO₂. Sucrose was dissolved in Krebs–Henseleit and added to the organ bath up to a concentration of 320 mM. The isometric tension changes were measured as described previously.29 Inhibitors were dissolved in dimethyl sulfoxide and added to the bath either 15 min (SB203580, AS601245, PD98059 and hydroxyfasudil) or 30 min (ML-7) before exposure to hyperosmolar sucrose. The contractile responses observed have been expressed as a percentage of the maximum constriction evoked by 40 mM KCl.

Measurement of [Ca²⁺]ι in Rat VSMCs VSMCs were isolated from rat thoracic aortas and cultured in SmGM-2 medium (Lonza Japan) as previously described.21 Changes in the [Ca²⁺]ι of VSMCs loaded with a fluorescent Ca²⁺ indicator dye, Fluo-4 NW, were measured using a confocal laser scanning microscope equipped with a diode laser (FLUOVIEW FV1000; Olympus), as described previously.20 The change in fluorescence intensity in response to hyperosmolar sucrose was monitored every 10 s for a period of 3 min.

Measurement of Phosphorylated MYPT1, p38 MAPK, and MLC in Aortic Rings Aortic rings equilibrated in the organ bath were exposed to sucrose as described above and then frozen in liquid nitrogen. Total proteins were extracted; subsequently, they were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes as described previously.29 The blots were incubated with primary antibodies for MYPT1, MYPT1 phosphorylated at Thr853, p38 MAPK, and p38 MAPK phosphorylated at Tyr182, MLC, and MLC phosphorylated at Thr18 and Ser19. Subsequently, bound antibodies were detected with peroxidase-conjugated anti-rabbit immunoglobulin G antibodies by using enhanced chemiluminescence (Chemilum One Super; Nacalai Tesque, Kyoto, Japan).

Measurement of the F- to G-Actin Ratio in the Rat Aorta The F- to G-actin ratio in aortic rings was measured using the G-actin/F-actin in vivo kit according to the manufacturer’s instructions (Cytoskeleton Inc.). Briefly, aortic rings were treated with sucrose with or without inhibitors for 10 min and then frozen in liquid nitrogen. The ring (2–3 mg) was homogenized in 100 µL of F-actin stabilizing buffer containing ATP and protease inhibitors (Cytoskeleton Inc.) followed by centrifugation at 10000 × g for 5 min. The supernatant was fractionated into G-actin (supernatant fraction) and F-actin (sedimented fraction) by differential ultracentrifugation (100000 × g for 60 min). The F-actin fraction was dissolved in 500 µL of F-actin depolymerization buffer. Ten microliters of each fraction was subjected to SDS-PAGE followed by immunoblot analysis of G- and F-actin with specific antibodies. The F-actin/G-actin ratio in each sample was determined by densitometry of actin bands.22

Statistical Analysis All data are expressed as mean ± S.E. Differences between mean values were tested for significance using one-way ANOVA, and the Tukey–Kramer test was used for post hoc comparison. Differences were considered significant at p<0.05.

RESULTS

Aortic Constriction by Hyperosmolar Sucrose Since the hyperosmolality produced by different osmolytes, such as sucrose, glucose and NaCl, evokes a similar contractile force in rat aortic rings, present study has been carried out using sucrose as an osmolyte. Figure 1A is a representative tracing of the rat aortic ring after exposure to 160 mM sucrose. Aortic ring was treated with 160 mM sucrose for 15 min, and then washed by isosmotic Krebs–Henseleit solution (W). After equilibration for 1 h, the ring was again exposed to 160 mM sucrose for 15 min. (B) Concentration–response curve of the aortic rings was constructed by cumulative addition of sucrose to organ bath every 10 min. The contractile response is expressed as a percentage of the maximal force to 40 mM KC. Four rings were prepared from each animal, and data of 5 animals are expressed as mean ± S.E.

Effects of Various Inhibitors on Hyperosmolar Sucrose-Induced Aortic Constriction To evaluate the role of MAPKs in the hyperosmotic vasoconstriction, the aortic rings were treated with the p38 MAPK inhibitor SB203580 (2 and 10 µM), the JNK inhibitor AS601245 (10 µM), or the MEK inhibitor PD98059 (50 µM) 15 min before exposure to 160 mM sucrose. Addition of 2 µM SB203580 blocked sucrose-induced constriction by 35% (p<0.01), whereas AS601245 and PD98059 had no effect (Fig. 2A). When SB203580 was used at
the concentration of 10 µM, it attenuated the sucrose-induced constriction to the same extent (37%) as observed with 2 µM SB203580. Subsequent experiments were performed using only 10 µM SB203580.

Smooth muscle contraction is principally regulated by the phosphorylation and dephosphorylation of the regulatory light chains of myosin, catalyzed by MLCK and MLCP, respectively. Therefore, to test whether these signaling pathways are involved in hyperosmotic constriction, we examined 2 specific inhibitors: ML-7 for MLCK and hydroxyfasudil for Rho-kinase-dependent MLCP inactivation. Sucrose-induced constriction was partially blocked by 21% with ML-7 at 2 or 10 µM; the combination of ML-7 (10 µM) with SB203580 further attenuated the constriction by another 25% (Fig. 2B). Hydroxyfasudil also significantly blocked sucrose-induced constriction by 47% when used at 2 or 10 µM concentration (Fig. 2C). and combined treatment with SB203580 further attenuated the constriction (Fig. 2C). In addition, the reduced constriction under combined treatment with ML-7 and hydroxyfasudil to 61% was further attenuated by another 30% by combined treatment with SB203580 (Fig. 2D). These results suggest that the hyperosmolar sucrose-induced constriction attenuated by SB203580 is mediated by a pathway distinct from those blocked by ML-7 and hydroxyfasudil.

Hyperosmolar Sucrose-Induced Increase in [Ca2+]i in Rat VSMCs A previous study by Anfinogenova et al. showed that hyperosmotic vasoconstriction is partially attenuated in aortic rings after 1-hour equilibration in Ca2+-free medium, but not affected by L-type Ca2+ channel blockers, implicating that the mobilization of cytosolic free Ca2+ from intracellular sources, but not from extracellular space, plays a role at least in part in the hyperosmotic constriction. However, there is no evidence so far whether hyperosmotic stress alters the cytosolic free Ca2+ levels in VSMCs. As shown in Fig. 3A, we monitored [Ca2+]i in rat VSMCs that had been loaded with Fluo-4. Phenylephrine, an alpha-receptor agonist (positive control), evoked a rapid increase in [Ca2+]i within 20 s (Fig. 3A). Hyperosmotic sucrose was found to induce a transient increase in [Ca2+]i within 20 s, although the effect was modest compared to that produced by phenylephrine (Fig. 3A). Treatment with SB203580 did not alter the sucrose-induced increase in [Ca2+]i (Fig. 3B).
Changes in \([\text{Ca}^{2+}]_i\) concentration in the VSMCs were studied by loading the cells with Fluo-4 and then measuring the fluorescence (excitation at 473 nm/emission at 510 nm) every 10 s for 3 min using confocal laser scanning microscopy. Panel A shows changes in Fluo-4 fluorescence in vehicle-treated (○), in phenylephrine (Phe; 1 μM)-treated (■), and in sucrose (Suc; 160 mM)-treated cells (□). Panel B shows changes in sucrose (Suc; 160 mM)-treated (□) and sucrose (160 mM) plus SB203580 (SB; 10 μM)-treated cells (●). Data (n=4) are expressed as mean±S.E. Significant differences were observed in the \([\text{Ca}^{2+}]_i\) for vehicle-treated and sucrose-treated cells within 30 s (p<0.01 in panel A). Treatment with SB203580 did not affect any change in \([\text{Ca}^{2+}]_i\) due to sucrose alone (p>0.05 in panel B).

**DISCUSSION**

Hyperosmotic stress induces several adaptive responses in cells to regulate their volume precisely by mechanisms that have been elucidated by numerous studies.\(^{20-31}\) Of the multiple signaling pathways involved in these adaptive responses, the p38 MAPK pathway is considered to be an essential component in the osmotic responses of mammalian cells, such as apoptosis, cytokine production, transcription regulation and cytoskeleton reorganization.\(^{20}\) Apart from these studies, our present study is the first to provide evidence that p38 MAPK is also involved in hyperosmolarity-induced tension development in VSMCs.

It is known that activation of the p38 MAPK signaling pathway by the contractile agonists angiotensin II,\(^{32,33}\) vasopressin,\(^{34}\) and endothelin-1\(^{35}\) results in actin remodeling-dependent tension development through downstream phosphorylation of HSP27 in VSMCs.\(^{36}\) In the present study, we found that a selective p38 MAPK inhibitor SB203580 not only attenuated the contractile response to hyperosmotic stress but also inhibited the hyperosmolarity-induced increase in the F-actin/G-actin ratio, suggesting that hyperosmotic stress induces vasoconstriction via p38 MAPK-mediated actin polymerization, probably through the same downstream pathway as described above. A similar observation was reported in the rat kidney medullary thick ascending limb in that the hyperosmotic stimuli induced p38 MAPK-mediated actin remodeling.\(^{37}\) The present study also showed that the selective pathway. As expected, hyperosmolar sucrose induced the phosphorylation of p38 MAPK in aortic rings, and SB203580 completely blocked p38 MAPK phosphorylation (Fig. 4B). Neither ML-7 nor hydroxyfasudil affected the phosphorylation of p38 MAPK.

**Hyperosmolar Sucrose-Induced Increase in the F-Actin to G-Actin Ratio in Aortas**

Recent studies have documented a critical role for actin polymerization and cytoskeletal dynamics in the regulation of active tension development in smooth muscle.\(^{22,28}\) Actin exists in both a soluble monomers (G-actin) and filamentous polymeric (F-actin) state. Increase in the cellular F-actin to G-actin ratio that undergoes polymerization has been demonstrated in many types of smooth muscle during contraction, including VSMCs in response to hyperosmotic stress.\(^{14,20}\) To determine whether the hyperosmotic activation of Rho/Rho-kinase, MLCK, and p38 MAPK is linked to actin polymerization in aortic smooth muscle cells, we analyzed the proportions of G-actin and F-actin in extracts from aortic rings. The F-actin/G-actin ratio significantly increased after exposure to hyperosmolar sucrose (4.41±0.14 in vehicle-treated rings versus 5.14±0.11 in sucrose-treated rings; p<0.01; Fig. 5). Cytochalasin B, an inhibitor of actin filament elongation, not only blocked the sucrose-induced increase in the F-actin/G-actin ratio (4.41±0.13 in sucrose plus cytochalasin B-treated rings versus sucrose-treated rings; p<0.01; Fig. 5) but also inhibited the sucrose-induced constriction by ~80% (data not shown). The increase in the F-actin/G-actin ratio by hyperosmolar sucrose was not affected by ML-7 but was significantly blocked by hydroxyfasudil and SB203580 (4.69±0.07 in sucrose plus hydroxyfasudil-treated and 4.71±0.07 in sucrose plus SB203580-treated rings versus sucrose-treated rings; p<0.05; Fig. 5).
Rho-kinase inhibitor hydroxyfasudil inhibited not only hyperosmolarity-induced vasoconstriction but also increase in the F-actin/G-actin ratio in the rat aortas. This result is consistent with previous studies demonstrating a role of the Rho-family small guanosine 5'-triphosphatases in actin reorganization. Since hydroxyfasudil failed to inhibit the hyperosmolarity-induced phosphorylation of p38 MAPK and SB203580 did not affect the hyperosmolarity-induced phosphorylation of MYPT1, it seems likely that the hyperosmolarity-induced cytoskeleton reorganization is mediated by 2 independent pathways comprising p38 MAPK and Rho/Rho-kinase.

In addition to the downstream signaling in actin remodeling, the activation of p38 MAPK appears to be involved in MLC phosphorylation because SB203580 significantly attenuated the hyperosmolarity-induced MLC phosphorylation in the presence of sufficient concentrations of hydroxyfasudil and ML-7, thereby blocking the MLC phosphorylation via Rho-mediated inactivation of MLCP and Ca²⁺-dependent activation of MLCK, respectively. Ca²⁺/calmodulin-dependent activation of MLCK is the primary pathway by which changes in the phosphorylation level of MLC are regulated. Although the mobilization of cytosolic free Ca²⁺ from the intracellular sources has been suggested in VSMCs after exposure to hyperosmotic stress, no evidence has so far been provided. As shown in the present study, we confirmed that hyperosmolar sucrose stimulated a transient rise of cytosolic free Ca²⁺ in VSMCs, suggesting a role of Ca²⁺/calmodulin-mediated pathway in hyperosmotic vasoconstriction. This is supported by our findings that a MLCK inhibitor ML-7 partially inhibited not only the contractile response but also phosphorylation of MLC in aortic rings after exposure to hyperosmolar sucrose. In addition, we observed that a calmodulin inhibitor W-7 (10 µM) attenuated the hyperosmolar sucrose-induced aortic constriction by ca. 17% (data not shown), supporting further a role of pathway involving Ca²⁺/calmodulin-mediated pathway in the hyperosmotic vasoconstriction. A recent study by Gatidis et al. showed that hyperosmotic shock induces eryptosis in human erythrocytes via a p38 MAPK-mediated increase in

Fig. 4. Effect of Protein Kinase Inhibitors on Hyperosmolar Sucrose-Induced Phosphorylation of Myosin Phosphatase Target Subunit 1 (MYPT1) (A), Myosin Light Chain (MLC) (B and C), and p38 Mitogen-Activated Protein Kinase (MAPK) in Rat Aortas
Thus, neither a pathway mediated by the Ca\(^{2+}\) is independent of the Rho-mediated inactivation of MLCP, indicating that the p38 MAPK-mediated MLC phosphorylation is not involved in the mobilization of cytosolic free Ca\(^{2+}\) in response to hyperosmolar sucrose.

Another potential pathway regulated MLC phosphorylation by p38 MAPK is the inactivation of MLCP through Rho-kinase activation. However, SB203580 did not affect the hyperosmolar sucrose-induced increase in cytosolic Ca\(^{2+}\) of VSMCs was not affected by SB203580, indicating that the p38 MAPM is not involved in the mobilization of cytosolic free Ca\(^{2+}\) in response to hyperosmolar sucrose.

The studies published to date have suggested that p38 MAPK, such as zipper-interacting protein kinase 42) and integrin-linked kinase, 43) we examined the effect of 2 inhibitors with respect to hyperosmolarity-induced aortic constriction: (4Z)-4-(3-Pyridylmethylene)-2-styryl-oxazol-5-one 44,45) for zipper-interaction protein kinase and \(N\)-methyl-3-(1-(4-((piperazin-1-yl)phenyl)-5-(4′-(trifluoromethyl)biphenyl-4-yl)1H-pyrazol-3-yl)propanamide 46) for integrin-linked kinase. However, these inhibitors did not affect the hyperosmolar sucrose-induced aortic constriction at the concentration of 10\(\mu\)M (data not shown). Therefore, further studies will be required to determine the mechanisms by which p38 MAPK is involved in MLC phosphorylation.

The studies published to date have suggested that p38 MAPK plays an important role in the pathogenesis of diabetic cardiovascular damages through endothelial dysfunction, pro-inflammatory cytokine production, apoptosis, immune cell activation, and VSMC growth. 47) Although oxidative stress has been considered to cause the activation of vascular p38 MAPK in hyperglycemia, 48) hyperosmotic stress may also be linked to diabetic vascular complications through p38 MAPK 49) because the serum osmolarity in diabetic hyperglycemia is more than 320mosmol/L and reaches up to 450mosmol/L in some severe cases 50,51) Since the osmolarity of 160mosmol/L (ca. 450mosmol/L) corresponds to severe hyperglycemia, the potential role of osmolarity in the pathogenesis of p38 MAPK-mediated changes in vascular functions cannot be excluded.

Taken together, our results demonstrate 3 independent pathways for hyperosmolarity-induced MLC phosphorylation in VSMCs (Fig. 6), that is, the pathways involved in activation of MLCK, Rho/Rho-kinase-dependent inactivation of MLCP, and p38-MAPK-mediated MLC phosphorylation. Further, we have also demonstrated 2 independent pathways for hyperosmolarity-induced actin polymerization in VSMCs: one mediated through the Rho/Rho-kinase and the other through p38 MAPK (Fig. 6). Although the importance of these kinases in the regulation of MLC phosphorylation and cytoskeleton reorganization has been well documented, our present study is the first to demonstrate the role of the p38 MAPK-mediated pathway in hyperosmolarity-induced vasoconstriction.

**REFERENCES**


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