Up-regulation of CPI-17 phosphorylation in diabetic vasculature and high glucose cultured vascular smooth muscle cells

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Abstract

Objective: Contractile responses are significantly increased in vascular smooth muscle tissues isolated from type 2 diabetic db/db mice (hyperreactivity). However, the molecular mechanisms underlying this hyperreactivity are largely unknown. The current study investigates the roles of RhoA, ROCK (rho kinase), PKC (protein kinase C), and CPI-17 (protein kinase C-potentiated phosphatase inhibitor of 17 kDa), molecules shown to play pivotal physiological roles regulating smooth muscle contraction, in diabetes-associated vascular smooth muscle hyperreactivity.

Methods: Experiments utilized db/db mouse mesenteric arteries and aortas and primary rat aortic smooth muscle cells (VSMCs) cultured in high or normal glucose. RhoA, ROCK, and CPI-17 protein expression and activity were determined by immunoblotting for total or phosphorylated proteins. RhoA activity was determined by subcellular fractionation and pull-down assays. Isometric contractions were determined using isolated mesenteric artery strips.

Results: Active phosphorylated CPI-17 and total and active membrane-bound RhoA were significantly increased in db/db mouse mesenteric arteries and aortas. High glucose time-dependently activated RhoA, ROCK, and CPI-17 in VSMCs. Moreover, inhibiting either RhoA with C3 exoenzyme or ROCK with Y-27632 or H-1152 for 30 min diminished high glucose-induced CPI-17 phosphorylation. Inhibiting protein kinase C (PKC) with GF109203X for 30 min did not inhibit high glucose-induced CPI-17 phosphorylation. Interestingly, when added at the same time as high glucose for a total of 48 h, GF109203X diminished high glucose-induced RhoA and ROCK activation as well as CPI-17 phosphorylation, suggesting PKC is required for high glucose-induced RhoA/ROCK activation and consequently CPI-17 phosphorylation. Importantly, in isolated db/db mouse mesenteric arteries, inhibiting ROCK with Y-27632 or H-1152 significantly alleviated the contractile hyperreactivity in response to phenylephrine or high potassium.

Conclusions: Diabetes and high glucose activate RhoA, ROCK, and CPI-17, which in turn contribute to diabetic vascular smooth muscle hyperreactivity.

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Keywords: Diabetes; Vascular smooth muscle contraction; Protein phosphorylation; Signal transduction; G-protein

1. Introduction

Vascular complications are the major causes of increased mortality and morbidity in diabetic patients. Abnormalities in blood vessel constriction or dilatation (vasomotion), detected in early diabetes, can result in blood flow dysregulation and increased peripheral resistance, thereby contributing to diabetic retinopathy, nephropathy, neuropathy and a higher rate of
hypothesis. In addition to endothelial dysfunction, vascular smooth muscle tissues isolated from db/db mice, an extensively studied monogenic type 2 diabetic mouse model, exhibit a significantly increased contractile response to stimuli [1–6]. Moreover, we and others recently found that cyclooxygenase-2 (COX-2) is up-regulated in db/db mouse vascular smooth muscle tissues and inhibition of COX-2 partially but significantly alleviates the vascular smooth muscle contractile hyperreactivity [6,7]. While these data suggest that COX-2 contributes to the contractile hyperreactivity, they also indicate that additional mechanisms are involved. The current study investigates the roles of RhoA, ROCK, PKC and CPI-17, molecules shown to play physiological pivotal roles in regulating smooth muscle contraction, in diabetes associated vascular smooth muscle hyperreactivity.

RhoA is a small molecular weight G-protein and rho kinase (ROCK) is a serine/threonine kinase that is activated by RhoA. The RhoA/ROCK pathway is activated by various G-protein-coupled receptor agonists and plays a pivotal role in multiple cellular functions including vascular smooth muscle contraction, migration and differentiation [8,9]. Recent studies have demonstrated the participation of the RhoA/ROCK signaling pathway in several cardiovascular pathologies, including hypertension, coronary artery spasm, effort angina, atherosclerosis and restenosis (reviewed in [8,10]). However, it remains to be established whether the RhoA/ROCK pathway is activated and contributes to the vascular smooth muscle contractile hyperreactivity in type 2 diabetes.

PKCs are a family of serine/threonine kinases. It is well-recognized that certain isoforms of PKC(s) are activated in vascular tissue by hyperglycemia/diabetes and that activated PKC(s) plays an important role in multiple diabetic cellular dysfunction [11,12]. However, the signaling mechanisms linking PKC activation to a variety of smooth muscle dysfunctions remain to be identified.

CPI-17 (PKC-potentiated myosin phosphatase inhibitor of 17 kDa) is a phosphorylation-dependent myosin phosphatase inhibitory protein that is primarily expressed in smooth muscle tissue, especially in arteries [13,14]. A growing body of evidence suggests that CPI-17 plays an important role in smooth muscle contraction, endothelial cell and fibroblast cytoskeletal reorganization, and cerebellar long-term synaptic depression [8,15]. Phosphorylation of Thr-38 increases CPI-17’s phosphatase inhibitory potency more than 1000-fold [13,16]. Under normal conditions, CPI-17 is phosphorylated by PKC and/or ROCK in response to agonist stimulation. However, it is unknown if CPI-17 is phosphorylated under diabetic conditions and if so, which kinase is responsible.

Using mesenteric artery and aorta isolated from type 2 diabetic db/db mice and primary rat aortic smooth muscle cells cultured in normal- and high-glucose medium, the current study tests the hypothesis that RhoA, ROCK and CPI-17 are activated and contribute to diabetes-associated vascular smooth muscle contractile hyperreactivity. Our results suggest an important role of RhoA, ROCK and CPI-17 activation in diabetic vascular smooth muscle dysfunction.

2. Methods

2.1. Animals and materials

Male C57BL/KsJ db/db mice (db/db −/−), age/gender-matched non-diabetic (db/db +/+) C57BL/KsJ control mice and Sprague–Dawley rats were purchased from Jackson Laboratory (Bar Harbor, ME) and Harlan (Indianapolis, IN), respectively. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. The Rhotekin rho-binding domain pGEX-2T cDNA construct was kindly provided by Dr. Martin Schwartz (The Scripps Research Institute). The antibodies for CPI-17 and for Thr-38 phosphorylated CPI-17 were produced and affinity purified as described previously [17,18]. Anti-RhoA and anti-p-MYPT1 (Thr-853) were from Santa-Cruz Biotech (Santa Cruz, CA) and anti-myosin phosphatase (total) was from Babco (Richmond, CA). Y-27632 and H-1152 were from Calbiochem (La Jolla, CA). GF109203X was from Sigma (St. Louis, MO).

2.2. Isometric tension measurement

Mesenteric arteries were isolated from 12- to 14-week-old male db/db and gender/age-matched control mice and a third-order branch was cut into small spiral strips. After denuding endothelium, isometric contractions were measured using a “bubble chamber” as described previously [6].

2.3. Primary cell culture

Rat aortic smooth muscle cells were isolated by enzymatic dissociation, as described [17,19]. These cultured smooth muscle cells were used within the first five passages as we have shown previously that the expression of smooth muscle α-actin, smooth muscle myosin heavy chain, caldesmon and calponin are expressed at levels comparable to that of mature smooth muscle tissue in the initial five passages [17].

2.4. Immunoblot analysis

db/db or control mouse aortas and mesenteric arteries or primary cultured rat aortic smooth muscle cells were treated by 10% trichloroacetic acid and proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Specific proteins were detected using immunoblot, as described previously [20]. The signals were captured by using a Kodak Image Station 4000 MM and quantified by using a Kodak Molecular Imaging Software. Special care was taken to ensure that the immunoblot signals were within linear range. CPI-17 or MYPT1 phosphorylation levels were quantified by the ratios of phosphorylated versus total.
CPI-17 or MYPT1 signals from two sets of paralleled immunoblots.

2.5. Construction and injection of recombinant adenovirus containing C3

The recombinant adenovirus containing C3 was constructed as previously described [17].

2.6. RhoA activation assays

RhoA activation was analyzed by quantifying the membrane-bound RhoA and the GTP-bound RhoA as previously described [21,22].

2.7. Statistical analysis

Each experiment was repeated a minimum of three times. Data were expressed as mean ± S.E.M. Statistical analysis was performed by using an unpaired t test or ANOVA (GraphPad, Prim 4 software).

3. Results

3.1. RhoA and CPI-17 are activated in db/db mouse mesenteric arteries and aorta

To investigate the molecular mechanisms underlying the vascular smooth muscle contractile hyperreactivity, we isolated mesenteric arteries from db/db and control mice and immunoblotted proteins that have recognized roles in smooth muscle contraction. As shown in Fig. 1A and B, we found active phosphorylated CPI-17 (CPI-17-P) was significantly increased in db/db mouse mesenteric arteries. In contrast, no significant differences were detected in the protein levels of total CPI-17 (CPI-17-T), RhoA, ROCK, protein kinase Cα (PKCα) and the myosin binding subunit of myosin phosphatase (MBS) between db/db and control mouse mesenteric arteries. Because CPI-17 can be phosphorylated by the RhoA/ROCK pathway, we quantified the active membrane-bound RhoA and found it was significantly increased in the diabetic db/db mouse mesenteric arteries (Fig. 1C), indicating activation of the RhoA in db/db mice.

To test if similar changes were present in the aorta, a conduit vessel that also exhibited contractile hyperreactivity [6], we isolated aorta and blotted the same panel of proteins. As shown in Fig. 2A and B, phosphorylated CPI-17, total CPI-17 protein and RhoA protein were increased in db/db mouse aortas when compared to that of control mice. Together, these data demonstrate that the RhoA and CPI-17 were activated in both mesenteric arteries and aortas of diabetic db/db mice.

3.2. CPI-17 phosphorylation is up-regulated by high glucose in primary cultured vascular smooth muscle cells

To elucidate the mechanisms underlying the observed RhoA and CPI-17 activation in db/db mouse vasculature, we used cultured VSMCs in 25 mM high glucose. High glucose is the hallmark of diabetes and has been well recognized to play an important role in diabetic vascular complications (For example, see review [23]). 25 mM glucose was used because it is within the range that can be
reached in vivo in diabetes and it has been extensively used in the literature. For example, blood glucose of the 12- to 14-weeks-old db/db mice used in this study is 31.4 mM. To determine if elevated glucose was able to increase CPI-17 phosphorylation, we cultured vascular smooth muscle cells in normal (5.5 mM) or high glucose (25 mM) medium. We found that high glucose alone caused time-dependent CPI-17 phosphorylation (Fig. 3A and B) with increased CPI-17 phosphorylation detected after a 3-h incubation in high glucose containing medium and peaked (10.3 ± 0.54 (n = 3) fold of the basal level) at 48 h. In contrast, the same concentration of mannitol did not cause a detectable increase in CPI-17 phosphorylation (Fig. 3C and D), suggesting that high glucose induces CPI-17 phosphorylation via mechanisms other than hyperosmolarity.

3.3. The RhoA/ROCK pathway is activated by high glucose in vascular smooth muscle cells

Under normal conditions, the RhoA/ROCK pathway has been shown to contribute to agonist-induced CPI-17 phosphorylation [15,17,24]. Therefore, we tested if high

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Fig. 2. RhoA and CPI-17 are increased in db/db mice aorta. Aortas were isolated from six pairs of 12- to 14-weeks-old male diabetic db/db and control mice and two aortas were pooled into one sample. The tissues were processed for immunoblot as described in Fig. 1. (A) Original immunoblots. (B) Quantitative summary of the immunoblots shown in A. *p < 0.05; **p < 0.001.

Fig. 3. High glucose, but not high mannitol, induces time-dependent CPI-17 phosphorylation in vascular smooth muscle cells. 70% to 80% confluent VSMCs were incubated in serum-free medium for 48 h and then in DMEM medium with varying glucose levels: normal glucose (5.5 mM, NG), high glucose (25 mM, HG) or high mannitol (5.5 mM glucose plus 19.5 mM mannitol) for various times as indicated. Cells were collected and proteins were separated using SDS-PAGE. Phosphorylated CPI-17 and total CPI-17 were detected using phosphorylation-specific and total antibodies, respectively. (A) and (C) representative immunoblots of four independent experiments. (B) and (D) summary of the experiments shown in A and C. ***p < 0.001 when compared to controls (time point 0).
glucose was sufficient to activate the RhoA/ROCK pathway in cultured smooth muscle cells. We determined the activities of RhoA and ROCK after high glucose incubation. RhoA activity was assessed by determining the active membrane-bound RhoA and GTP-bound RhoA. As shown in Fig. 4A and B, the active membrane-bound RhoA was significantly increased to 2.0 ± 0.12 (n = 4, p < 0.01) fold of control level by 48 h of high-glucose incubation. Consistently, there was a 3.4 ± 0.54 (n = 5, p < 0.01) fold increase in GTP-RhoA in cells that have been incubated with high glucose (Fig. 4C and D) detected using GST-rhotekin Rho-binding domain pull-down assay.

To determine if the RhoA activation promotes the activation of its downstream effector ROCK, we determined the time course of MYPT1 Thr-853 phosphorylation. MYPT1 Thr-853 has been shown to be a selective ROCK phosphorylation site [25]. Our results show that high glucose induced ROCK phosphorylation at Thr-853 (Fig. 5A and B) in a time course that parallels high glucose-induced CPI-17 phosphorylation (Fig. 3A and B) without affecting the total ROCK protein level. Together, these data indicate that elevated glucose levels are sufficient to induce activation of RhoA and ROCK.

3.4. Inhibition of the RhoA/ROCK pathway diminishes high glucose-induced CPI-17 phosphorylation in vascular smooth muscle cells

To examine if the RhoA/ROCK pathway mediates the high glucose-induced CPI-17 phosphorylation, we tested the effects of inhibiting RhoA or ROCK on high glucose-induced CPI-17 phosphorylation. We inhibited ROCK with Y-27632 or H-1152, two selective and potent ROCK inhibitors with distinct structures. As shown in Fig. 6, both Y-27632 and H-1152 potently and dose-dependently inhibited high glucose-induced CPI-17 phosphorylation, indicating that ROCK phosphorylates CPI-17.

Fig. 4. High glucose activates RhoA in rat aortic smooth muscle cells. Cells were incubated in serum-free medium for 48 h and then in DMEM medium with normal glucose (5.5 mM, NG) or high glucose (25 mM, HG) for 48 h. For the determination of membrane-bound RhoA, the cytosol and membrane fractions were separated by centrifugation at 100,000 x g for 30 min at 4 °C. For GTP-RhoA pull-down assay, the cells were lysed and incubated with GST-rhotekin Rho-binding domain agarose beads for 1 h at 4 °C. After washing, the beads were collected and the GTP-RhoA bound to the beads were detected by immunoblot. (A) and (C) Representative immunoblots of membrane-bound RhoA, GTP-RhoA and total RhoA of four to seven independent experiments. (B) and (D) summary of densitometric analysis of results shown in (A) and (C). **p < 0.01; ***p < 0.001. when compared to NG.

Fig. 5. High glucose induces time-dependent MYPT1 Thr-853 phosphorylation. VSMCs were incubated in serum-free medium for 48 h and then in DMEM medium with normal glucose (5.5 mM) or high glucose (25 mM) for various times as indicated. The cells were collected and proteins were separated by SDS-PAGE. Total- and phosphorylated-MYPT1 were detected by immunoblotting. (A) Representative immunoblots of six independent experiments. (B) Summary of densitometric analysis of results shown in (A). **p < 0.01; ***p < 0.001.
To inhibit RhoA function, smooth muscle cells were infected with recombinant adenovirus-encoding C3 exoenzyme and then incubated with high glucose for 48 h. As shown in Fig. 7A and B, infection of cells with C3-containing adenovirus but not with a control empty adenovirus nearly abolished high glucose-induced CPI-17 phosphorylation. Thus RhoA is required for high glucose-induced CPI-17 phosphorylation.

3.5. Protein kinase C activity is required for high glucose-induced RhoA/ROCK activation and CPI-17 phosphorylation

It is well-recognized that some PKC isoforms are selectively activated by high glucose [11,12] and PKC have been shown to phosphorylate CPI-17 under normal glucose condition by agonist stimulation [11,26]. We therefore tested if PKC also plays a role in high glucose-induced CPI-17 phosphorylation. We inhibited PKC with GF109203X, a potent and selective inhibitor of conventional and novel PKCs [27], and determined the effects on high glucose-induced CPI-17 phosphorylation. Smooth muscle cells were either cultured in the presence of 25 mM glucose for a total of 48 h with GF109203X added during the last 30 min or were cultured for 48 h in the presence of GF109203X and 25 mM glucose, which were added at the same time. In contrast to the dramatic inhibition of CPI-17 phosphorylation by a 30-min treatment with H-1152 or Y-27632 (Fig. 6), the 30-min treatment with up to 3 μM GF109203X did not inhibit high glucose-induced CPI-17 phosphorylation (Fig. 8E and F, lane 3 vs. lane 2),
suggesting that PKC is not the kinase that directly phosphorylates CPI-17 in the presence of high glucose. As a control, 30-min GF109203X treatment completely reversed PMA-induced CPI-17 phosphorylation (data not shown), suggesting that GF109203X, at the concentration used, was able to effectively inhibit PKC in our system. In contrast, when present for the entire 48 h during high-glucose incubation, GF109203X dose-dependently inhibited high glucose induced CPI-17 phosphorylation to 86%, 73% and 27% of the control level at the concentrations of 0.3, 1 and 3 μM. Interestingly, 48 h GF109203X treatment also inhibited high glucose-induced RhoA activation (Fig. 8A and B, lane 4 vs. lane 2), ROCK activation (Fig. 8C and D, lane 4 vs. lane 2) as well as CPI-17 phosphorylation (Fig. 8E and F, lane 4 vs. lane 2). This suggests that PKC is required for high glucose-induced RhoA/ROCK activation and consequently CPI-17 phosphorylation.

3.6. ROCK contributes to the contractile hyperreactivity in db/db mouse mesenteric artery

To further explore a potential causal role of the RhoA/ROCK pathway in vascular smooth muscle contractile hyperreactivity, we determined the effects of inhibiting ROCK on db/db mouse mesenteric artery contractile hyperreactivity. Consistent with our previous observations, the contractile responses to phenylephrine (Fig. 9) and to high potassium (Fig. 10) were significantly enhanced in the mesenteric arteries isolated from db/db mice compared to control mice [6]. Y-27632 or H-1152, at concentrations

Fig. 8. Protein kinase C(s) is required for high glucose-induced RhoA/ROCK activation and CPI-17 phosphorylation. VSMCs were incubated in serum-free medium for 48 h and then in DMEM medium with normal glucose (lane 1), high glucose alone (lane 2) or high glucose plus GF109203X (lanes 3 and 4). GF109203X (3 μM) was added either for the last 30 min of the 48 h incubation in high glucose (lane 3) or at the same time when the high glucose incubation was initiated (lane 4). For detection of MYPT1 or CPI-17 phosphorylation, the cells were collected after 10% TCA treatment. For detection of GTP-RhoA, the cells were collected and analyzed by pull-down assay. (A), (C) and (E) Representative immunoblots of GTP-RhoA, phosphorylated MYPT-1 and CPI-17 of five to seven independent experiments. (B), (D) and (F) Summary of densitometric analysis of results shown in (A), (C) and (E). **p < 0.01; ***p < 0.001 when compared to high glucose (HG) in the absence of GF109203X.

Fig. 9. Inhibiting ROCK induces greater suppression of phenylephrine-induced contractions in db/db mouse mesenteric artery than that in control. Mesenteric arteries isolated from 12- to 14-weeks-old male diabetic db/db and control mice were cut into spiral strips and denuded of endothelium. After obtaining stable contractions in response to high K+ and phenylephrine (10 μM), the strips were incubated with 10 μM Y-27632 or 1 μM H-1152 for 30 min and then stimulated with phenylephrine again. Phenylephrine-induced contractions in the presence and absence of Y-27632 or H-1152 were plotted. N=5–11; **p < 0.01; ***p < 0.001.
shown to maximally inhibit ROCK in smooth muscle tissue [28], substantially inhibited the phenylephrine-induced contractions in the tissues isolated from db/db or control mice. This indicates an important role for ROCK in both physiologic and diabetes-enhanced contractions to agonists.

High potassium-induced contractions are biphasic in mouse mesenteric artery, consisting of an initial transient phasic phase, followed by a slow, tonic phase that reaches a plateau. Both phases of contractions were significantly increased in db/db mouse mesenteric arteries compared to controls. Y-27632 or H-1152 significantly inhibited the tonic phase of high potassium-induced contractions (Fig. 10A) without significantly affecting the initial phasic phase of contractions (Fig. 10B) in the tissues isolated from db/db or control mice. This indicates an important role for ROCK in both physiologic and diabetes-enhanced contractions to high potassium.

4. Discussion

The major novel findings of the current study are that 1) RhoA and CPI-17 are activated in db/db mouse mesenteric arteries and aortas; 2) ROCK is the kinase that directly phosphorylates CPI-17 in the presence of high glucose; 3) PKC is required for high glucose-induced RhoA/ROCK activation and consequently CPI-17 phosphorylation, and 4) enhanced ROCK activity contributes to the contractile hyperreactivity of db/db mouse mesenteric artery.

4.1. Diabetes and high glucose increase CPI-17 phosphorylation

Under physiological conditions, agonist stimulates the phosphorylation of CPI-17, which in turn contributes to the inhibition of myosin phosphatase and smooth muscle contraction [8]. However, whether CPI-17 is activated under diabetic conditions has not been studied. Our results first show that CPI-17 phosphorylation is significantly increased in type 2 diabetic db/db mouse mesenteric arteries and aortas (Figs. 1 and 2) and in vascular smooth muscle cells cultured in the presence of high glucose (Fig. 3). These data raise the possibility that, in addition to physiologically regulating vascular smooth muscle function, CPI-17 is involved in diabetic vascular pathology.

Two mechanisms may be responsible for the observed increase in active phosphorylated CPI-17 in db/db mice vasculature: 1) activation of the kinase pathway(s) that phosphorylates CPI-17 and 2) increase in total CPI-17 protein. These two mechanisms are not mutually exclusive and both seem to be operating in db/db mice. Total CPI-17 protein is increased in db/db mouse aorta (Fig. 2). However, no increase of total CPI-17 protein is detected in db/db mouse mesenteric artery (Fig. 1) or in aortic smooth muscle cells cultured in high glucose (Fig. 3). This discrepancy may reflect that, in addition to high glucose, other components in the db/db serum are required to increase CPI-17 expression. Alternatively, this discrepancy may reflect that there is a variation in the regulation of CPI-17 protein expression in different smooth muscle cells: aorta versus mesenteric artery. Mesenteric arteries are resistant peripheral vessels and have different functional properties than the large conduit vessel such as aorta.

Different from the rapid and transit CPI-17 phosphorylation induced by agonist, high glucose induced CPI-17 phosphorylation is sustained with a long 3 h delay. There are at least two potential mechanisms may account for the differences. First, high glucose can increase diacylglycerol by a de novo metabolic pathway whereas agonist increases diacylglycerol by hydrolysis of phosphatidylinositolides or...
phosphatidylcholine by phospholipase C or phospholipase D. It has been shown that high glucose induced diacylglycerol increase does not occur immediately; it reaches maximal level by 2 days and remains at high level by 6 days (the longest time tested) [29]. Such delayed and sustained increase in diacylglycerol and thus activation of PKC and ROCK is potentially responsible for the delayed and sustained CPI-17 phosphorylation induced by high glucose (Fig. 3). Second, high glucose may inhibit the phosphatase that dephosphorylates CPI-17.

4.2. The signaling pathway mediating high glucose-induced CPI-17 phosphorylation

Multiple kinases have been shown to phosphorylate CPI-17 in test tube using purified kinases and CPI-17 [8]. Under physiological conditions, both PKC and ROCK have been shown to mediate agonist-induced CPI-17 phosphorylation [15,17,24]. However, several kinases, including PKCα/δ [30], ROCK [31,32 and current study], and ILK [33], are activated by diabetes or high glucose. It is unknown which of these kinases is responsible for high glucose-induced CPI-17 phosphorylation. While our data do not exclude a role for ILK, our results provide evidence that activation of the PKC and subsequently RhoA/ROCK pathway mediates high glucose-induced CPI-17 phosphorylation.

While only active RhoA was increased in db/db mouse mesenteric arteries and VSMCs cultured in high glucose, both active RhoA and total RhoA protein expression level were increased in db/db mouse aorta. An increase in GTP-bound active RhoA can result from 1) increased total RhoA and/or 2) increased loading of GTP to the same amount of total RhoA protein. Although the modulation of RhoA expression level has been less extensively described, accumulating studies reported that the RhoA mRNA or protein level is increased in arteries from hypertensive [34], aged [35], and diabetic [36] rats. Increase of transcription and increase of RhoA protein stability have been reported to regulate RhoA protein levels [37,38].

Another novel finding of the current study is that PKC activation is also required, though indirectly, for high glucose-induced CPI-17 phosphorylation in vascular smooth muscle cells. Although PKC can directly phosphorylate CPI-17 upon some agonist stimulation in vascular smooth muscle tissue under physiological conditions [15], our data show that PKC is not the kinase that directly phosphorylates CPI-17 in the presence of high glucose. Inhibition of PKC with GF109203X (30 min) could not reverse the CPI-17 phosphorylation caused by high glucose incubation (Fig. 8). However, when added at the same time as high glucose, GF109203X abolished high glucose-induced RhoA/ROCK activation and CPI-17 phosphorylation. These results suggest that under diabetic conditions, RhoA/ROCK and CPI-17 are downstream effectors of PKC. So how does the activation of PKC lead to the activation of RhoA/ROCK? One possibility is that PKC mediates high glucose-induced RhoA activation by phosphorylating RhoA [37,38], Rho-GDI [39] or Rho-GEF [40].

4.3. ROCK partially mediates the contractile hyperreactivity

In addition to impaired endothelium-mediated relaxation, vascular smooth muscle contractile responses are dramatically increased in type 2 diabetic db/db and ob/ob mice [1–4,41]. This vascular smooth muscle contractile hyperreactivity is not associated with detectable atherosclerotic lesions or intima formation or changes in the cross sectional areas [6,41], suggesting that changes in biochemical and signaling mechanisms in smooth muscle are responsible for the observed hyperreactivity. Several findings support this notion. First, Miao et al. showed that total RhoA protein is increased in streptozotosin-induced type 1 diabetic rat basilar artery [36]. Our data show a similar increase in total RhoA in type 2 diabetic db/db mouse vasculature and further demonstrate that the active membrane-bound RhoA is increased in db/db mouse mesenteric arteries. Second, inhibition of ROCK by Y-27632 or H-1152 significantly diminished the enhanced basal tone of db/db mouse cerebral arterioles [42] and the contractile hyperreactivity in response to phenylephrine and high potassium of db/db mouse mesenteric artery (Figs. 9 and 10). However, the inhibition of Y-27632 and H-1152 on the vascular smooth muscle hyperreactivity is incomplete, suggesting that in addition to the RhoA/ROCK and CPI-17 pathway, additional mechanisms also contribute to the hyperreactivity. Indeed, increased intracellular Ca2+ [43,44], up-regulated COX-2 [6,7] and enhanced oxidative stress [3] have been reported to be involved in diabetic vascular hyperreactivity. While the lack of available reagents to specifically inhibit CPI-17 hampers the direct test of its significance in hyperreactivity, our data clearly show that CPI-17 is activated in diabetic mice and in high glucose medium. This finding, coupled with the well recognized physiological role of CPI-17 in inhibiting myosin phosphatase, strongly implicate CPI-17 in the diabetic hyperreactivity. However, ROCK can induce contractile hyperreactivity by directly phosphorylating and inhibiting myosin phosphatase as well as phosphorylating CPI-17, the relative contribution of these two mechanisms to ROCK induced hyperreactivity remains unclear.

In summary, we show that RhoA, ROCK and CPI-17 are activated in type 2 diabetic db/db mouse vasculature and by high glucose in primary cultured rat aortic smooth muscle cells. Activation of ROCK contributes to the mesenteric artery contractile hyperreactivity in db/db mice. These data implicate ROCK and CPI-17 as potential therapeutic targets for diabetic vascular dysfunction.

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References


