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Review Article

Protein Kinase C and Rho-Associated Coiled-Coil Kinase in Mechanisms of Ca^{2+} Sensitization in Diabetes-Induced Vascular Smooth Muscle Hypercontractility

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Abstract

Diabetes is a complex syndrome which leads to multiple dysfunctions including vascular disorders. Protein kinase C (PKC) and Rho-associated coiled-coil kinase (ROCK) are important regulatory enzymes mediating signal transduction in a number of vascular functions, including vascular smooth muscle contractility. Many studies have shown that vascular smooth muscle contractile responses are dramatically enhanced in diabetes where in, PKC and ROCK significantly contribute to the process by mediating Ca^{2+} sensitization of contractile proteins. Both PKC- and ROCK-mediated pathways converge to phosphorylate the inhibitory protein CPI-17 which binds to myosin light chain phosphatase (MLCP) and inhibits its activity. Besides, ROCK phosphorylates myosin light chain phosphatase targeting subunit 1 (MYPT1) which also inhibits the phosphatase activity. Inhibition of MLCP results in a higher level of myosin light chain phosphorylation for any given intracellular level of Ca^{2+} and activity of the myosin light chain kinase (MLCK). Ca^{2+} sensitization of smooth muscle, thus, could potentially maintain vascular contraction in diabetes. A link between the ROCK and PKC pathways in diabetic vascular smooth muscle cells has been shown, assuming that the ROCK and RhoA protein are downstream effectors of PKC. Furthermore, collected data suggest that PKC and ROCK are potential therapeutic targets for treating diabetes-related complications in vascular smooth muscle cells.

Keywords: Diabetes; Protein kinase C; Rho kinase; Ca^{2+} sensitization; Vascular smooth muscle; Vascular tone

Abbreviations

AA: Arachidonic Acid; CaM: Calmodulin; CPI-17: Protein Kinase C-dependent Protein Phosphatase-1 Inhibitor of 17 kDa; DAG: Diacylglycerol; DHAP: Dihydroxyacetone Phosphate; DM: Diabetes Mellitus; EGFR: Epidermal Growth Factor Receptor; ErbB2: Epidermal Growth Factor Receptor B Family Member 2; ERK1/2: Extracellular Signal-regulated Kinases1 and 2; EP: Prostaglandin E Receptor; 5-HT: 5-Hydroxytryptamine; GAP: Glyceraldehyde-3-phosphate; $G\alpha_{q/11}$: Guanosine-5'-triphosphate-binding Proteins $G\alpha_q$ Family Subunit; $G\alpha_{12/13}$: Guanosine-5'-triphosphate-binding Proteins $G\alpha_{12/13}$ Family Subunits; GDP: Guanosine Diphosphate; GPCR: G-protein Coupled Receptor; Grb-2: Growth Factor Receptor-bound Protein 2; GT: Guanosine-5'-triphosphate; IP_3: Inositol-1,4,5-trisphosphate; IP_3R: Inositol-1,4,5-trisphosphate Receptor; $iPLA_2\beta$: Calcium-independent Phospholipase $A_2\beta$; 12/15-LOX: 12/15-lipoxygenases; LPI: Lysophosphatidylinositol; MEK1/2: Mitogen-activated Protein Kinase Kinases 1 and 2; MLCK: Myosin Light Chain Kinase; MLCP: Myosin Light Chain Phosphatase; mRNA: Messenger Ribonucleic Acid; MYPT1: Myosin-bound Myosin Light Chain Phosphatase Targeting Subunit 1; PA: Phosphatidic Acid; PIP_2: Phosphatidylinositol-4,5-bisphosphate; PKC: Protein Kinase C; PLCγ: Phospholipase Cγ; PP1: Protein Kinase C-dependent Protein Phosphatase-1; PP1cδ: Protein Kinase C-dependent Protein Phosphatase-1 Catalytic Subunit; Raf1: Rapidly Accelerated Fibrosarcoma Proto-oncogene protein kinase; Ras: Rat Sarcoma Protein; Rho: Ras Homolog Protein; RhoGAP: Rho GTPase Activating Protein; RhoGDI: Rho Guanine Nucleotide Dissociation Inhibitor; RhoGEF: Rho Guanine-nucleotide Exchange Factors; ROCK: Rho-associated Coiled-coil Kinase; ROS: Reactive Oxygen Species; SMCs: Smooth Muscle Cells; Sch: Src Homology 2 Domain Containing Transforming Protein; SOS: Son of Sevenless Homolog Protein; SR: Sarcoplasmic Reticulum; STZ: Streptozotocin

Introduction

Diabetes mellitus (DM) is a wide-spread syndrome that is rapidly rising in frequency throughout the world. Hyperglycemia and alterations of metabolism are the most severe components of DM [1,2]. Near5-10% of patients with DM have autoimmune type 1 insulin-dependent diabetes, whereas 90-95% have type 2 DM (insulin-independent), which is a consequence of lifestyle patterns contributing to obesity [1,2]. Type 2 DM typically occurs in the context of a cluster of cardiovascular risk factors [1]. DM is known to cause multiple dysfunctions including cardiovascular diseases, which are major causes of mortality, end-stage renal disease, and blindness [3]. The macrovascular manifestations of DM, such as angiopathy, atherosclerosis, medial calcification, and arterial hypertension, have been shown to mostly locate in coronary and carotid arteries [4], cerebral vessels [5], and large peripheral arteries of the lower extremities [5]. The microvascular complications of DM include

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retinopathy [6], nephropathy [7], and peripheral neuropathy [8]. Increased blood flow and elevated vascular tone have also been documented for DM [3]. A growing body of evidence indicates that endothelial and smooth muscle dysfunctions are present in various regions of the vasculature in both diabetic patients and animal models of DM [9-14].

Hyperglycemia is considered to be a key factor responsible for the development of vascular complications in DM [3,15]. Several hyperglycemia-associated mechanisms, including oxidative stress have been identified to contribute to the development of DM-associated vascular dysfunctions [16-18]. Dysfunctions in the regulation of vascular cells permeability, growth, angiogenesis, and vascular smooth muscle contractility in DM have been shown to involve protein kinase C (PKC) and Rho-associated coiled-coil kinase (ROCK)upregulation [13,18-20].

PKC

PKC is a regulatory enzyme that plays a significant role in signal transduction of several vascular functions [21]. PKC is presented with a family of serine/threonine kinases, with at least known 10 isoforms [21]. Based on homology and sensitivity to the activators, PKC isozymes are classified into three subfamilies: conventional (or classical), novel, and atypical PKC [21]. Classical isozymes include PKC-α, PKC-β1, PKC-β2 and PKC-γ [22,23]. The novel PKC subfamily consists of PKC-δ, PKC-ε, PKC-η, and PKC-θ [24,25]. The atypical isozymes are represented with PKC-ζ and PKC-ι/ [21,22]. In resting cells, PKCs primarily locate in the cytosolic fraction and the enzymes' translocation to the plasma membrane conventionally has been considered as the hallmark of PKC activation [21]. Interestingly, the former PKC-μ and PKC-υ isoforms are now classified as members of the DAG receptor protein kinase D family [26]. Expression of numerous PKC isozymes (α, β1, β2, γ, ε, η, ζ, δ, and ι/λ) in vascular tissues depends on the animal species, as well as the type and age of the vessel [18,22]. The PKC-α, PKC-β1, PKC-β2, PKC-γ, PKC-δ, PKC-ε, and PKC-ζ isoforms have been shown to be activated or overexpressed in vascular smooth muscle cells (SMCs) and the endothelium of different vascular regions in diabetes [14,27-31].

Hyperglycemia in DM results in an overproduction of reactive oxygen species (ROS) and the ensuing oxidative stress which contributes to the activation of PKC [20,32,33]. Furthermore, there is strong evidence that PKC activation is mediated, at least in part, by induction of oxidative stress and increased production of ROS [27,34,35]. Important to note, PKC is well known to be highly sensitive to oxidative stress [21]. In vascular tissues, PKC activation can also be mediated by diacylglycerol (DAG) [5,20,36] which has been shown to be elevated in DM [27,30,37,38]. Hyperglycemia can enhance the amount of DAG primarily by increasing *de novo* DAG synthesis from the glycolytic intermediate dihydroxyacetone phosphate (DHAP), as well as by inducing phosphorylation of the phospholipase C (PLCγ) [20, 38] (Figure).

ROCK

The Rho-kinases or ROCKs is a small group of serine/threonine kinases, regulatory enzymes that also play an important role in vascular function regulation [39,40]. ROCKs are represented with two isoforms encoded by two different genes, ROCK-1 (ROCK I, P160-ROCK, or ROK β) and ROCK-2 (ROCK II or ROK α) [41-43].

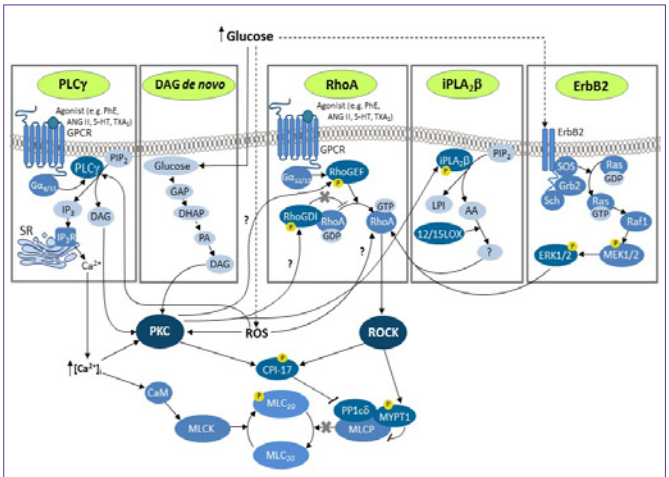

Figure: A scheme illustrating the PKC- and ROCK-mediated mechanisms of contractile proteins Ca^{2+} sensitization in vascular smooth muscle cells in DM. Hyperglycemia induces elevated ROS leading to PKC activation. Activated by hyperglycemia DAG *de novo* synthesis and ROS-mediated PLCγ-dependent DAG formation both also lead to PKC activation. Hyperglycemia increases DAG concentration by promptings *de novo* synthesis from the glycolytic intermediatesglyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), leading to the formation of phosphatidic acid (PA). High glucose, possibly mediated by ROS, and the ErbB2/ERK1/2-mediated pathway, also induces activation of RhoA and ROCK. Both PKC and ROCK activate CPI-17 which binds to MLCP and inhibits its activity. ERK1/2 activation is mediated by the mitogen-activated protein kinases pathway involving Src homology 2 domain containing transforming protein (Sch), growth factor receptor-bound protein 2 (Grb-2), son of sevenless homolog protein (SOS),rat sarcoma protein (Ras), rapidly accelerated fibrosarcoma proto-oncogene protein kinase (Raf1), and mitogen-activated protein kinase kinases 1 and 2 (MEK1/2).The inhibition of the MLCP results in increased MLC_{20} phosphorylation for any given level of $[Ca^{2+}]_i$ and activity of MLCK. High glucose-induced upregulation of PKC leads to activation of the $iPLA_2\beta$/12/15-LOX pathway and upregulation of RhoA/ROCK. Also, PKC may mediate a high glucose-induced activation of RhoA/ROCKvia phosphorylation of RhoGDI or RhoGEF.

Both isoforms are highly homologous and are expressed in vascular tissues [44-46]. ROCK is mainly dispersed in the cytoplasm, but is partially translocated to the peripheral membrane upon activation [42,47].

ROCK is activated by monomeric G proteins (small GTPases) of the Rho (Ras homolog) protein family [39,42,48-50]. The Rho protein family consists of at least 20 members classified into 5 groups: the Rho-like, Rac-like, Cdc42-like, Rnd, and RhoBTB subfamilies, whereas RhoD, Rif, and RhoH/TTF do not fall into any obvious grouping [50]. Proteins of the Rho family regulate a wide range of fundamental cell functions such as contraction, motility, proliferation, and apoptosis [50-52]. RhoA, belonging to the Rho-like subfamily, is the main upstream activator of ROCK [50]. Binding of the active GTP-bound form of RhoA to the Rho-binding domain stimulates the phosphotransferase activity of ROCK by disrupting the interaction between the catalytic and the inhibitory C-terminal regions of the enzyme [49]. However, the stimulatory effect of RhoA on ROCK activity is limited [53]. RhoA functions as a molecular switch which cycles between an inactive GDP-bound and active GTP-bound conformations. Activated RhoA interacts with downstream targets leading to cellular responses. Upon activation RhoA is translocated from the cytosol to the cell membrane [54,55]. Activation of Rho GTPases is regulated by several mechanisms including the activation

of heterotrimeric G protein-coupled receptors [49]. Three types of regulators of Rho proteins have been identified. Rho guanine-nucleotide exchange factors (RhoGEF) promote the formation of the GTP-bound form of Rho proteins [50], whereas Rho GTPase activating protein (RhoGAP) accelerates the intrinsic GTPase activity resulting in the GDP-bound form [56]. Rho guanine nucleotide dissociation inhibitor (RhoGDI) binds to a subset of Rho proteins, inhibits nucleotide exchange and sequesters these proteins away from the membrane, where normally they would be active [50]. On the other hand, it has been shown that ROCK-1 activity can be inhibited by the Rho protein Rnd3/RhoE which binds to the N-terminal region of ROCK-1 and prevents RhoA binding to the Rho-binding domain [57]. Two other small G proteins of the Ras superfamily, Gem and Rad, have been shown to inhibit the ROCK function [58,59].

The Rho/ROCK signaling pathway has been implicated in the pathogenesis of diabetes. It has been demonstrated that ROCK activity is enhanced in vascular tissues in diabetes [31,44,48,60-63]. Recent publications demonstrated that diabetes type 1-induced vascular dysfunction in the rat aorta arises due to an upregulation of ROCK-2 isoform [64]. An increased level of ROCK-2 protein expression in corpus cavernosum of diabetic mice [65] and retinal vessels of type 1 model diabetic rats [66] has also been reported. ROCK-1 gene and protein upregulation has also been reported from different vessels in various diabetic animal models [61,62,67]. The mRNA and protein levels of RhoA is also increased in arteries of diabetic rats and mice [48,62,65,68-70,71]. The mechanism of RhoA and ROCK activation by high glucose levels remains to be defined. However ROS, thought to play a central role in the pathogenesis of vascular diabetic complications, have been shown to activate RhoA [72-74, 75]. In another study, the authors reported that glucose-induced calcium-independent phospholipase $A_2\beta$ ($iPLA_2\beta$) upregulation may activate the RhoA/ROCK via 12/15-lipoxygenases in vascular SMCs [76]. On the other hand, it has been suggested that activation of ROCK in DM and high glucose level can be mediated by ErbB2 and Ras/Raf/extracellular signal-regulated kinase 1/2 (ERK1/2) [77]. ErbB2 is a transmembrane tyrosine kinase receptor of the epidermal growth factor receptor (ErbB/EGFR) family which downstream effector in vascular SMCs is ERK1/2 [77] (Figure).

PKC-mediated SMCs Ca^{2+} sensitization in DM

While numerous studies have demonstrated that DM impairs vascular function by inhibiting endothelium-dependent vasodilatation [9,11-14], others have shown that DM enhances vascular contractility in an endothelium-independent manner [31,38,63,78-80]. A large number of studies suggest that vascular smooth muscle contractile responses are dramatically enhanced in diabetes [31,48,78,80-84]. Studies on different diabetic animal models have shown that α1-adrenomimetics-, serotonin-(or 5-hydroxytryptamine, 5-HT), and angiotensin II-induced SMC contraction is enhanced in arteries [31,37,48,67,77,79,81,84-88], and PKC-α,-ε, and –δ isozimes contribute to this process [31,48,63,76,78]. Similarly, endothelium-independent vasoconstriction mediated by prostaglandin E receptor 1 (EP1) and 3 (EP3) activation has been shown to be enhanced in type 2 diabetic rats' mesenteric arteries and highly sensitive to PKC-δ inhibition [83].

It is well known that vascular smooth muscle contractile responses are primarily triggered by increased intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). Both increased Ca^{2+} influx and release of Ca^{2+} from intracellular stores have been proposed to be involved in DM, contributing to the enhanced vascular reactivity [85,89]. There is evidence that stimulation of arteries with α1-adrenomimetics is associated with increased Ca^{2+} influx in diabetic vessels over normal ones [82,85,90,91]. Other authors have also reported increased intracellular Ca^{2+} to be involved in the diabetic vascular hyperreactivity [82,89]. However, there is a controversy here with reports of inhibition of voltage- and store-operated Ca^{2+} entry channels in vascular SMC in DM [92,93]. Alternatively, Ca^{2+} sensitization of the contractile proteins has been proposed as a more general mechanism of the elevated DM-associated vascular contractility [48,68,78,80]. One striking observation supporting this view is the enhanced contractile response to α1-adrenomimetics by arteries from diabetic rats and mice which is not associated with $[Ca^{2+}]_I$ changes [31,86]. Similarly, under high glucose conditions, thromboxane A_2-induced aortic smooth muscle contraction has also been shown to increase independently from intracellular calcium concentration [37].

Phosphorylation and dephosphorylation of the 20-kDa myosin light chain (MLC) are the major regulatory mechanisms of smooth muscle contractility. Increased $[Ca^{2+}]_I$ activates calmodulin-dependent MLC kinase (MLCK) which catalyzes the phosphorylation of MLC at Thr18 and Ser19 leading to actin-myosin interaction and vascular smooth muscles contraction [94,95]. On top of this primary regulatory pathway, several modulatory pathways exist in smooth muscles that can alter the magnitude of the force that is developed at any given $[Ca^{2+}]_I$ [94,95]. Both the PKC-mediated [96,97] and ROCK-mediated [39,49,97] mechanisms have been shown to be involved in elevated myofilament sensitivity to Ca^{2+}.

These two pathways converge to phosphorylate an inhibitory protein CPI-17 at Thr38 [95,96,98,99] (see Figure). The CPI-17 is the smooth muscle myosin light chain phosphatase (MLCP) PKC-dependent protein phosphatase-1 (PP1) inhibitor of 17 kDa [95,96,98,99]. The MLCP holoenzyme of smooth muscles is a heterotrimer consisting of three subunits: the130-kDa, 38-kDa, and 21-kDa subunit [100-102]. The 38-kDa subunit is identified as PKC-dependent protein phosphatase-1 (PP1) catalytic subunit (PP1cδ). The 130-kDa subunit is a regulatory and myosin-bound MLCP targeting subunit 1 (MYPT1) [100-103]. The 21-kDa subunit's function of MLCP is unclear [104]. Phosphorylated CPI-17 directly binds to MLCP and inhibits its activity [105]. Inhibition of MLCP results in a higher level of MLC phosphorylation for any given level of $[Ca^{2+}]_i$ and activity of MLCK [106]. This phenomenon, known as Ca^{2+} sensitization of smooth muscles, could thereby maintain vascular contraction [49,94].

Mueed and coauthors have reported that enhanced contractile responses of mesenteric arteries from streptozotocin (STZ)-induced diabetic rats upon stimulation of α_1-adrenoceptors are accompanied by elevated levels of PKC-α and -ε, and PKC-dependent elevation in CPI-17 phosphorylation in vascular SMCs [77]. We have recently shown that elevated vascular SMC contractility in STZ-diabetic rats is mediated by Ca^{2+} sensitization, and PKC is contributing to this process [80]. It has also been shown recently that PKC-δ mediates

Ca^{2+} sensitization in the intrarenal interlobar artery of type 2 diabetic *ob/ob* mice [31].

ROCK-mediated SMCs Ca^{2+} sensitization in DM

Numerous studies have demonstrated that enhanced contractile responses to α-adrenomimetics, angiotensin II, and 5-THin vessels from animal models of both types of DM [13,31,48,67,77,82,84,107] as well as in pre-diabetic obese Zucker rats [108] are mediated by ROCK.

For increase in myofilament Ca^{2+} sensitivity in the DMROCK-mediated mechanism have been shown [39,48,49,80,98,103,108,109]. In this mechanism, ROCK phosphorylates CPI-17 at Thr38 [95,96,98,99,110-114] which then directly binds to PP1cδ and inhibits the activity of MLCP [105, 115-117] (see Figure). In addition, ROCK directly phosphorylates the MYPT1 subunit of MLCP at Thr855 and/or Thr850, Thr695, Thr696, and Thr697 [39,49,98,103,106,109,114,118-121] that also consequently inhibits the phosphatase activity.

In smooth muscle cells from STZ-induced model of type 1 diabetes rats, phosphorylation levels of MYPT1 in aorta has been shown to be significantly elevated [64]. Similar results have been obtained from the saphenous vein of patients with DM by Matsuo and coauthors, which have shown that the hyper reactivity to 5-HT in smooth muscles of these vessels is due to higher phosphorylation of MLCP, evident from elevation of the P(Thr696)-MYPT1 to total MYPT1 ratio [121].

Our research group has shown that elevated vascular SMC contractility in rats with STZ-induced DM is associated with enhanced Ca^{2+} sensitivity of contractile myofilaments and both ROCK and PKC are clearly contribute to this process in diabetic vasculature [80]. ROCK-mediated calcium sensitization has been shown to be responsible for hypertension development in type 2 diabetes as well [31,68]. It has also been suggested recently that both PKC-δ and ROCK mediate Ca^{2+} sensitization in smooth muscles of the intrarenal interlobar artery of type 2 diabetic (*ob/ob*) mice [31]. It has been shown that a1-adrenoceptor-mediated vasoconstriction in penile [82] and gracilis arteries [107] from rat models of prediabetes/metabolic syndrome (obese Zucker rats) also involves ROCK-dependent Ca^{2+} sensitization. Other authors have demonstrated that ROCK-mediated CPI-17 phosphorylation increase in vascular SMCs of the type 2 diabetic *db/db* mice model and SMCs cultured in presence of high glucose concentration [48]. Both activation of the ROCK pathways that phosphorylates CPI-17 and increase in total CPI-17 protein level seem to be involved [48], and CPI-17 up-regulation/activation in type 2 diabetic *db/db* mice vasculature is associated with a significant blood pressure increase [122].

A number of papers have reported a link between the ROCK and PKC pathways in diabetes or under high glucose conditions. Some of them suggest that ROCK is upstream of PKC in diabetic bladder [123] and diabetic cardiomyocytes [124], whereas in diabetic vascular SMC ROCK has been reported to be downstream of PKC [48,77]. Xie and coauthors have shown that PKC in vascular SMC is required for high glucose-induced ROCK activation and consequently CPI-17 phosphorylation [48]. These authors also suggest that, although PKC can directly phosphorylate CPI-17 upon some agonist stimulation in the vascular smooth muscle tissue under physiological conditions, PKC is not the kinase that directly phosphorylates CPI-17 in the presence of high glucose [48]. It has been shown that ROCK mRNA and protein levels can be upregulated through PKC and it is luckily that under diabetic conditions RhoA/ROCK and CPI-17 are downstream effectors of PKC [48]. More recently, the same group of researchers has shown that high glucose-induced activation of PKC leading to activation of $iPLA_2\beta$ and up-regulation of RhoA/ROCK via 12/15-lipoxygenases, thereby contributes to diabetes-associated vascular smooth muscle Ca^{2+} sensitization and hypercontractility [76]. Products of the phospholipase A_2 enzymes group include arachidonic acid which can be rapidly metabolized to a variety of mediators by lipoxygenases and other oxygenases to a number of bioactive eicosanoids [125]. However, it remains unclear how the catalytic activity of the 12/15-lipoxygenase may activate RhoA/ROCK/CPI-17.It is possible to assume, that PKCmay mediate high glucose-induced activation of RhoA via phosphorylation of RhoGDI [126] or RhoGEF [127] (Figure).

Conclusion

The presented data suggest that PKC and ROCK in vascular SMC highly contribute to enhanced vascular tone and arterial hypercontractility in diabetes by mediating SMC Ca^{2+}-sensitization. Our review shows that PKC and ROCK are potential therapeutic targets for treating vascular diabetic complications. Developing innovative pharmacological approaches that could modulate PKC and ROCK activity is highly important for new strategies that might prove clinical relevancy in preventing the development and/or retarding the progression of diabetes-associated vascular complications.

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