Effects of Superoxide on Signaling Pathways in Smooth Muscle Cells From Rats

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Abstract—The effects of hypoxanthine and xanthine oxidase–induced superoxide anion were evaluated on various signal transduction pathways in aortic smooth muscle cells (SMCs) from spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). Superoxide increased inositol 1,4,5-tris-phosphate (IP₃) formation in a concentration- and time-dependent manner in both strains but more markedly in SMCs from SHR. Various antioxidants significantly decreased the superoxide-induced IP₃ formation in both strains. In addition, tyrosine kinase inhibitors, genistein and tyrphostin A25, inhibited the superoxide-induced IP₃ formation more markedly in SHR than in WKY. Moreover, superoxide decreased the basal level of cGMP to a greater extent in SHR and also suppressed the rise in cGMP induced by S-nitroso-N-acetylpenicillamine. In addition, the superoxide-induced increase in IP₃ formation was significantly inhibited by guanylyl cyclase stimulator S-nitroso-N-acetylpenicillamine but was potentiated by ODQ (a guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) and KT5823 (a cGMP-dependent protein kinase inhibitor), with a greater effect in SHR. Finally, the superoxide-enhanced IP₃ formation was not accompanied by simultaneous changes in cAMP levels, and inhibition of the adenylyl cyclase pathway did not modify the superoxide-induced IP₃ formation. Our results thus demonstrate a stimulatory effect of superoxide on IP₃ formation, mediated by the tyrosine kinase–coupled phospholipase C₇ activity, and an inhibitory effect of superoxide on cGMP formation in vascular SMCs. The increased reactivity of the phospholipase C pathway and the decreased cross inhibition of the IP₃ pathway by cGMP in the presence of superoxide may underlie the altered functions of vascular SMCs in SHR.

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Key Words: rats, spontaneously hypertensive ■ oxidative stress ■ anions ■ signal transduction

The signal transduction pathway linked to inositol 1,4,5-tris-phosphate (IP₃) plays an important role in the regulation of cardiovascular functions under physiological and pathological conditions. The specific ligand-receptor interaction at the plasma membrane surface activates phospholipase C (PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP₂) to form IP₃ and diacylglycerol. Three PLC isozymes (β, γ, and δ) have been identified in mammalian tissues: PLCβ is found in rat vascular smooth muscle cells (SMCs), PLCγ and PLCδ are found in human aortic tissue, and PLCδ is found in rat aorta. PLCβ is activated by the receptor-coupled Gq, G11 proteins, whereas PLCγ is activated when its tyrosine residues are phosphorylated by tyrosine kinase. It is clear that the activation of either PLCβ or PLCγ leads to the generation of IP₃, although the relation between the activation of PLCβ and the IP₃ production is still unclear.

A study by Nishizuka and our previous study have shown that certain oxidants, or oxidant-derived products, could result in the hydrolysis of phosphatidylinositol (PIP). It was found in our study that the norepinephrine-induced vasoconstriction and formation of inositol phosphates (IPs) in rat aortic SMCs were partially inhibited by superoxide dismutase (SOD), but not by catalase; therefore, the norepinephrine-induced increase in IP formation might be partially related to the auto-oxidation of catecholamine, leading to the generation of superoxide. In addition, our recent studies have demonstrated that the superoxide-enhanced formation of IPs in vascular SMCs of mesenteric arteries from Sprague-Dawley rats is mediated by an enhanced activity of the tyrosine kinase pathway. However, whether activation of the tyrosine kinase pathway is a common mechanism for the cellular effect of superoxide and whether its effect on the IP₃ formation is altered in hypertension are presently unsettled.

Previous studies have suggested that the production of IP₃ can be modulated by cGMP or cAMP pathways. It has been reported that the IP₃ response of arterial endothelial cells to thrombin can be inhibited by cGMP or by the guanylyl cyclase stimulator sodium nitroprusside. We have also reported that the phenylephrine-induced IP formation in rat aortic SMCs is inhibited by cAMP or by the adenylly cyclase stimulator forskolin (FSK). Interestingly, recent studies have provided some evidence that H₂O₂ either stimulated or had no effect on soluble guanylyl cyclase in different cell prepara-
tions, whereas superoxide either inhibited or had no effect on soluble guanylyl cyclase.10,11 These studies raised many intriguing and important issues concerning the cross interactions between the PLC pathway and other signaling pathways. Whether superoxide alters cGMP and/or cAMP metabolism in vascular SMCs and whether the effect of superoxide on IP₃ formation is modulated by simultaneous changes in cGMP or cAMP levels needs to be addressed.

An overproduction of superoxide was observed in aortas of spontaneously hypertensive rats (SHR).12 In addition, the xanthine–xanthine oxidase (XO) reaction–induced contraction of endothelium-free aortic rings was reported to be twice greater in SHR than in Wistar-Kyoto rats (WKY).13 All those studies suggest that the production of superoxide and the reactivity of SMCs to superoxide are increased in hypertension. Therefore, a better knowledge of the effects of superoxide on different signaling pathways may provide a better understanding of the mechanisms responsible for the abnormal functions of vascular SMCs in hypertension. The purpose of the present study was to determine the modulatory role of superoxide on the activities of different signaling pathways in vascular SMCs and to investigate whether the reactivities of different signaling pathways to superoxide are altered in hypertension. The cellular levels of IP₃ were measured in the absence or presence of superoxide in SMCs from SHR and WKY, and various putative mechanisms underlying the effect of superoxide on IP₃ formation were explored in both strains. More specifically, the superoxide-induced IP₃ formation was determined after blockade of the tyrosine kinase–mediated signaling pathway by directly inhibiting tyrosine kinase. The effects of superoxide on the basal level of cGMP or cAMP were also studied and compared with the effects of specific modulators of cGMP or cAMP metabolism. Moreover, the superoxide-induced IP₃ formation was determined after the blockade of cAMP or cGMP pathways in order to evaluate the cross-talk interactions between the superoxide-induced IP₃ formation and other signal transduction pathways in both strains.

Methods

Cell Culture

Single aortic SMCs were isolated and identified as described previously.9 Cells between passages 2 and 10 were used. There was no significant difference in the superoxide-induced increase in IP₃ formation among cultured SMCs from passages 2 to 10.

Measurement of IP₃ Formation

SMCs were incubated for 24 hours in the serum-free and inositol-free DMEM, to which 5 μCi/mL myo-[2-3H]inositol (Du Pont Canada Inc) was added.6–9 The cells were then washed with 1× Hanks balanced salt solution minus glucose and incubated in fresh Hanks balanced salt solution with 100 μmol/L hypoxanthine and 15 μU/mL xanthine–xanthine oxidase (XO) in different experiments for various periods, and the reaction was terminated by adding 0.9 mL methanol:chloroform:HCl (40:20:1). The tritiated IP₃ pool of the aqueous phase composed of inositol 4-phosphate (IP₁), inositol 4,5-biphosphate (IP₂), and IP₃, was eluted consecutively by ion-exchange chromatography (AG1-X8 resin, Bio-Rad Laboratories). The lipid phase was counted to determine the PIP lipid pool. IP₃ was expressed as a relative value of [(IP₃/PIP)×10³] (arbitrary units) to correct for the variation in the labeling of the lipid pool.

Quantitative Determination of cAMP and cGMP Levels

The level of cAMP in cultured SMCs was determined by a protein-binding assay (cAMP [3H] assay system, Amersham Corp). The quantitative determination of cGMP was performed with a cGMP [32P] assay system in the presence of 100 μmol/L 3-isobutyl-1-methylxanthine (cGMP [32P] assay system, Amersham Corp).

Chemicals and Data Analysis

HX, XO, SOD, and FSK were purchased from Sigma Chemical Co. Genistein, tyrphostin A25, N-acetylcysteine (NAC), α-lipoic acid (LA), KT5720, KT5823, S-nitroso-N-acetylpenicillamine (SNAP), and SQ 22536 were from Calbiochem. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was from Tocris Cookson Inc. Unless otherwise specified, the HX-XO treatment indicated that 150 μmol/L HX and 15 μmol/L XO were applied to the cells. Data are expressed as mean±SEM and analyzed by the Student t test or ANOVA in conjunction with the Newman-Keuls test where applicable. Differences between groups were considered statistically significant at P<0.05.

Results

Effect of Superoxide on IP₃ Formation

Superoxide generated from the HX-XO reaction significantly increased IP₃ formation in a concentration-dependent manner in SMCs from both strains. The increases in IP₃ formation were significantly greater in SHR than in WKY at all concentrations tested (Figure 1A), suggesting an enhanced sensitivity of the IP₃ pathway to superoxide in SMCs from SHR. The effect of superoxide on IP₃ formation was also time dependent and reached a plateau at 60 minutes in SMCs from both strains (Figure 1B). After the cells were preincubated with an antioxidant NAC (0.3 to 10 mmol/L) for 20 minutes, the HX-XO–induced IP₃ formation was significantly inhibited in both strains (Figure 2A). In addition, the antioxidants SOD and LA had a greater inhibitory effect on the superoxide-induced IP₃ formation in SMCs from SHR than those from WKY. As shown in Figure 2B, the increased IP₃ formation induced by HX-XO was significantly inhibited by 22±3% in WKY and 45±4.5% in SHR (P<0.05) after 20-minute pretreatment of cells with SOD (120 U/mL), whereas IP₃ formation was inhibited by 36±4.5% in WKY and 61±4.0% in SHR after 20-minute pretreatment with LA (P<0.05). Whether H₂O₂ could affect IP₃ formation was further examined. It was found that H₂O₂ at 10 μmol/L, which is within the physiological concentration range,14 had no effect on IP₃ formation in both strains (P>0.05, n=4 for each group). A similar result was also observed in mesenteric artery SMCs from Sprague-Dawley rats; only at a high concentration (1 mmol/L) did H₂O₂ slightly increase (30%) IP₃ formation (P<0.05, n=4). This increase was completely inhibited by catalase (data not shown).

Effect of Superoxide on Tyrosine Kinase Pathway

To explore the possibility that the superoxide-induced IP₃ formation may be secondary to the activation of the tyrosine kinase pathway, the superoxide-induced IP₃ formation in SMCs was examined in the presence of tyrosine kinase inhibitors. Figure 3 shows that the HX-XO–induced IP₃ formation was significantly inhibited by genistein or tyrphostin A25 (20-minute incubation) in a concentration-dependent
manner (5 to 50 \( \text{mol/L} \)). The inhibitory effects of both tyrosine kinase inhibitors were more potent in SMCs from SHR than in SMCs from WKY. These data suggest that the superoxide-induced IP\(_3\) formation is at least in part mediated by an increase in the activity of the tyrosine kinase signaling pathway.

**Effect of Superoxide on cGMP Pathway**

The basal levels of cGMP were significantly higher in SMCs from SHR than SMCs from WKY \((P<0.05, n=5\) for each group). The cGMP levels were significantly increased 30 minutes after incubation of SMCs with SNAP (100 \( \text{mol/L} \)), a stimulator of soluble guanylyl cyclase (Figure 4A). This effect of SNAP was greater in SHR than in WKY \((P<0.05)\). One-hour treatment of cells with HX-XO significantly decreased basal cGMP levels by \(58\pm2.5\%\) in WKY \((P<0.05, n=5)\) and \(73\pm2.7\%\) in SHR \((P<0.05, n=5)\), indicating a greater sensitivity of the cGMP pathway to superoxide in SHR. In addition, the SNAP-induced increase in cGMP level was significantly inhibited by pretreatment of the cells with HX-XO in both strains \((P<0.05, n=5\) for each group).

**Effect of Superoxide on the Cross Inhibition of IP\(_3\) Formation by cGMP**

Because superoxide simultaneously increased IP\(_3\) formation and decreased cGMP formation, it was speculated that the superoxide-increased IP\(_3\) formation in vascular SMCs might also be under the influence of the activity of cGMP pathway. Therefore, the superoxide-increased IP\(_3\) formation was evaluated after stimulating or inhibiting the soluble guanylyl cyclase. Figure 4B shows that the HX-XO–induced IP\(_3\) formation was significantly inhibited by ODQ \((20\text{-minute pretreatment at 100 } \text{mol/L})\). Both the inhibitory effect of SNAP and the stimulatory effect of ODQ on the superoxide-induced IP\(_3\) formation were significantly greater in the cells from SHR than cells from WKY \((P<0.05)\). In addition, SNAP \((100 \text{ mol/L})\) decreased the basal IP\(_3\) formation by \(16\pm4\%\)
WKY (P<0.05, n=4) and by 20±3.5% in SHR (P<0.05, n=4), but ODQ (100 μmol/L) had no significant effect on basal levels of IP3 in either strain (P>0.05, n=4 for each group (data not shown)). We further examined the interaction of the superoxide-induced IP3 formation with the cGMP-dependent protein kinases (PKG). It was observed that the superoxide-induced increase in IP3 formation was significantly potentiated by the PKG inhibitor KT5823 (Figure 4C). The effect of KT5823 was also dose dependent (0.1 to 30 μmol/L) and more potent in SMCs from SHR than in SMCs from WKY.

**Effect of Superoxide on cAMP Pathway**

Similar basal levels of cAMP were observed in SMCs from SHR and WKY. FSK treatment (1 hour at 10 μmol/L) also induced similar increases in cAMP formation in both strains (n=8 for each group) (Figure 5A). Unlike the cGMP response to superoxide, HX-XO had no effect on the basal levels of cAMP. The FSK-enhanced cAMP levels were unaffected by the HX-XO treatment. Moreover, the HX-XO–induced increase in IP3 formation was not altered either by 20-minute pretreatment of cells with the adenyl cyclase inhibitor SQ 22536 (500 μmol/L) or with the selective cAMP-dependent protein kinase (PKA) inhibitor KT5720 (10 μmol/L) in both SHR and WKY (Figure 5B). In addition, both SQ 22536 and KT5720 had no effect on the basal levels of IP3 in SMCs from both strains (data not shown). In agreement with our previous findings,⁹ FSK (10 μmol/L) induced an inhibition of the basal IP3 levels (data not shown) or the HX-XO–stimulated IP3 formation in SMCs from both strains (P<0.05, n=4 for each group; Figure 5B). Overall, these data suggest that the superoxide-induced increase in IP3 formation is not associated with simultaneous changes in the activity of cAMP signaling pathway.

**Discussion**

Growing evidence suggests the importance of superoxide anion in the regulation of cardiovascular functions. In hypertension, an abnormal production of superoxide in vascular...
our understanding of the signaling role of superoxide under physiological conditions and in hypertension in the following aspects: Our study suggests that tyrosine kinases are the most likely targets of superoxide in aortic SMCs, whereas the activation of PLC by superoxide is less likely. This conclusion is based on the fact that the application of tyrosine kinase inhibitors (genistein or tyrphostin A25) significantly reduced or even completely abolished the superoxide-induced IP₃ formation in aortic SMCs in both strains, with a more potent inhibition observed in SHR (Figure 3). The specificity of the inhibitory effects of genistein or tyrphostin A25 on tyrosine kinases has been demonstrated with platelet-derived growth factor (PDGF) stimulation of rat vascular SMCs. PDGF is well known for its stimulatory effect on tyrosine kinases in vascular SMCs, and we have found that the PDGF-induced increase in IP₃ formation in rat vascular SMCs is significantly inhibited by tyrphostin A25 or genistein. Therefore, the regulation of the tyrosine kinase-PLC-IP₃ axis by superoxide may represent a novel signal transduction mechanism. Through this mechanism, superoxide can activate the tyrosine kinase-PLC-IP₃ pathway in a membrane receptor-independent fashion.

The interaction between the IP₃ signaling pathway and the cGMP pathway has been reported previously. It has also been shown that elevated cGMP levels, through the activation of PKG, can inhibit PLC and lower the basal levels of IP₃ or attenuate the agonist-stimulated IP₃ formation. It is consequently hypothesized that the superoxide-induced IP₃ formation is in part modulated by the activity of the cGMP signaling pathway. The present findings showed that superoxide inhibited cGMP formation and that this effect was greater in aortic SMCs from SHR than aortic SMCs from WKY. Moreover, it was found that superoxide not only decreased the basal cGMP levels but also suppressed the cGMP response to SNAP stimulation in aortic SMCs (Figure 4A). This effect of superoxide could be ascribed to a direct inhibitory effect of superoxide on soluble guanylly cyclase. Alternatively, superoxide might scavenge nitric oxide generated from SNAP or from endogenous sources, leading to a decreased stimulation of soluble guanyly cyclase.

Our data showed that superoxide increased IP₃ formation directly and also indirectly by lowering cGMP levels for the following reasons: First, superoxide effectively decreased the basal cGMP level (Figure 4A). Second, the increased cGMP formation reduced IP₃ levels. Third, the superoxide-induced IP₃ formation was significantly potentiated by the guanylyl cyclase inhibitor ODQ (Figure 4B) and by the PKG inhibitor KT5823 (Figure 4C) but inhibited by the guanylyl cyclase stimulator SNAP. Therefore, it is rationalized that the inhibitory effect of superoxide on cGMP formation probably contributes to the activation of IP₃ formation induced by superoxide by lifting the negative feedback exerted by cGMP on the PLC pathway(s). Because the basal levels of cGMP in aortic SMCs are higher in SHR than in WKY (Figure 4A), the interaction of cGMP and IP₃ may be more important in SHR in view of the fact that higher cGMP levels, through the activation of PKG, can inhibit PLC and lower the basal levels of IP₃ or attenuate the agonist-stimulated IP₃ formation. Alternatively, superoxide might scavenge nitric oxide generated from SNAP or from endogenous sources, leading to a decreased stimulation of soluble guanyly cyclase.

SMCs and an altered responsiveness of SMCs to superoxide have been postulated. An overproduction of superoxide was shown in aortas of SHR, and the intravenous administration of SOD was found to reduce arterial blood pressure in SHR. However, the cellular responsiveness to superoxide has not yet been explored in SHR regarding the effects of superoxide on different signal transduction pathways, including IP₃, cGMP, and cAMP pathways, in vascular SMCs.

In 1989, Auch-Schwelk et al reported that the contraction of endothelium-free aortic rings induced by xanthine plus XO reaction, which is presumably due to superoxide production, was twice greater in SHR than in WKY. Our previous studies also demonstrated that both the vasoconstriction and IP formation induced by norepinephrine were greater in aortic SMCs from SHR than aortic SMCs from WKY and that those enhanced responses were partially inhibited by SOD, but not by catalase. In addition, we observed the stimulatory effect of superoxide on IP formation in aortic SMCs, which confirmed the earlier observation that some oxidants or oxidant-derived products contribute to the hydrolysis of phospholipids such as PIP. The present study has increased...
paired by an overproduction of superoxide in SHR, demonstrated by several lines of evidence. (1) The increase or decrease in cGMP levels induced by SNAP or superoxide was greater in SMCs from SHR than in SMCs from WKY. Our findings are in agreement with the previous observation that the sodium nitroprusside–stimulated cGMP levels were higher in aortic SMCs from SHR than aortic SMCs from WKY. Those observations suggest the existence of a hypersensitivity of the cGMP pathway to superoxide or nitric oxide–related stimulation in SMCs from SHR. (2) The IP₃ responsiveness to superoxide was markedly decreased in the presence of SNAP in SMCs from SHR and WKY, but it was more significantly enhanced in the presence of ODQ or the PKG inhibitor KT5823 in aortic SMCs from SHR than aortic SMCs from WKY (Figure 4). These findings indicate that the inhibition of the cGMP-mediated pathway by superoxide contributes to the stimulatory effect of superoxide on the IP₃ pathway and that the cross inhibition of the IP₃ pathway by the cGMP pathway is more suppressed by superoxide in SHR than in WKY.

An increase in either cGMP or cAMP concentrations in vascular SMCs results in vasorelaxation. This phenomenon has been partially interpreted as the consequence of the cross activation of PKG by both nucleotides. Because PKG can be cross-activated by an increase in cAMP, it is possible that cAMP could also participate indirectly in the regulation of IP₃ levels by the activation of PKG. Previous studies from our laboratory as well as those of others have shown the existence of a complex cross-talk interaction between IP₃ and cAMP pathways whereby an activation of the cAMP pathway resulted in an inhibition of the phenylephrine-induced IP₃ formation in aortic SMCs from SHR and WKY. The present data confirm our previous observation by showing that the elevated IP₃ level induced by superoxide is reduced by FSK treatment (Figure 5B). However, it has been clearly demonstrated from our results that superoxide does not cause any changes in cAMP levels in vascular SMCs from SHR or WKY, whereas it significantly affects IP₃ and cGMP levels. This conclusion is further supported by the finding that FSK increases cAMP concentration in vascular SMCs to similar levels in the absence or presence of superoxide (Figure 5A). Thus, the superoxide-induced increase in IP₃ formation in SMCs from both SHR and WKY is associated with a coupled decrease in intracellular cGMP levels. This selective modulation of superoxide on IP₃ and cGMP signal transduction pathways may represent a novel mechanism by which superoxide could be actively involved in the functional regulation of vascular SMCs. More important, it was also observed that the simultaneous increase in IP₃ formation and the decrease in cGMP level induced by superoxide were significantly enhanced in vascular SMCs from SHR compared with WKY. Therefore, the increased IP₃ levels in vascular SMCs from SHR could result from a direct stimulatory effect of the overproduction of superoxide on a hypersensitive tyrosine kinase pathway and from an indirect inhibitory effect of superoxide on a hypersensitive cGMP pathway. Our results not only emphasize the complexity of interactions among different signal transduction pathways but also reveal an important signaling role of superoxide in vascular SMCs in SHR. The present study may unveil a new mechanism to explain the development of alterations in vascular function in the genesis or maintenance of hypertension. Moreover, the finding of novel signaling effects of superoxide in vascular SMCs and their alterations in hypertension may provide avenues for the development of new strategies in the prevention and treatment of hypertension.

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