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Nitric oxide attenuates endothelin-1-induced activation of ERK1/2, PKB, and Pyk2 in vascular smooth muscle cells by a cGMP-dependent pathway

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Bouallegue A, Daou GB, Srivastava AK. Nitric oxide attenuates endothelin-1-induced activation of ERK1/2, PKB, and Pyk2 in vascular smooth muscle cells by a cGMP-dependent pathway. Am J Physiol Heart Circ Physiol 293: H2072–H2079, 2007. First published July 20, 2007; doi:10.1152/ajpheart.01097.2006.—Nitric oxide (NO), in addition to its vasodilator action, has also been shown to antagonize the mitogenic and hypertrophic responses of growth factors and vasoactive peptides such as endothelin-1 (ET-1) in vascular smooth muscle cells (VSMCs). However, the mechanism by which NO exerts its antimitogenic and antihypertrophic effect remains unknown. Therefore, the aim of this study was to determine whether NO generation would modify ET-1-induced signaling pathways involved in cellular growth, proliferation, and hypertrophy in A-10 VSMCs. Treatment of A-10 VSMCs with S-nitroso-N-acetylpenicillamine (SNAP) or sodium nitroprusside (SNP), two NO donors, attenuated ET-1-induced phosphorylation of several key components of growth-promoting and hypertrophic signaling pathways such as ERK1/2, PKB, and Pyk2. On the other hand, inhibition of the endogenous NO generation with Nω-nitro-arginine methyl ester, a nitric oxide synthase inhibitor, increased the ET-1-induced phosphorylation of these signaling components. Since NO mediates its effect principally through a cGMP-soluble guanylyl cyclase (sGC) pathway, we investigated the role of these molecules in NO action. 8-Bromo-guanosine 3′,5′-cyclic monophosphate, a nonmetabolizable and cell-permeant analog of cGMP, exhibited a similar effect to that of SNAP and SNP. Furthermore, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an inhibitor of sGC, reversed the inhibitory effect of NO on ET-1-induced responses. SNAP treatment also decreased the protein synthesis induced by ET-1. Together, these data demonstrate that NO, in a cGMP-dependent manner, attenuated ET-1-induced phosphorylation of ERK1/2, PKB, and Pyk2 and also antagonized the hypertrophic effects of ET-1. It may be suggested that NO-induced generation of cGMP contributes to the inhibition of ET-1-induced mitogenic and hypertrophic responses in VSMCs.

endothelin-1 (ET-1) is a 21-amino acid peptide and is considered a potent vasoconstrictor (47). It also exhibits mitogenic activity in vascular smooth muscle cells (VSMCs) (5, 23, 24), suggesting a possible role for ET-1 in the pathogenesis of many diseases, such as atherosclerosis (29), hypertension (18), and restenosis after angioplasty (11).

ET-1 exerts its effects through a heteromeric G protein-coupled receptor that is linked to multiple signaling pathways that include phospholipases C and D (13), Ca2+ (32), mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun NH2-terminal kinase (JNK), and p38mapk (7, 8, 39, 50, 51), and phosphatidylinositol 3-kinase 3-kinase (8, 17). Activation of receptor and nonreceptor protein tyrosine kinases (PTKs) in transducing ET-1-induced signaling responses has also been suggested (16, 26, 27, 39, 49). PTKs activated by ET-1 include epidermal growth factor (EGF) (27), c-Src (16, 26, 38), and a Ca2+-dependent PTK, Pyk2 (26, 39). Of particular interest, ET-1 mediates Pyk2 activation, which contributes to ERK1/2 (26) and JNK (27) signaling in cardiomyocytes and p38mapk (39) in mesangial cells.

Nitric oxide (NO) is a free radical that has been suggested to play an important role in cardiovascular function (38). NO mediates relaxation principally through the stimulation of soluble guanylyl cyclase (sGC), leading to enhanced production of intracellular cGMP, which in turn, activates cGMP-dependent protein kinase (PKG) (30). NO can also influence cellular events by a PKG-independent mechanism (14, 22) and is also able to react with superoxide anion to form the reactive peroxynitrite radical (25), a potent oxidant with the potential to disrupt protein structures by nitrating the tyrosine residues in protein (48). In addition to its vasodilating effect, NO has been suggested to antagonize the physiological and pathophysiological effects of several growth factors such as EGF (52), angiotensin II (ANG II) (46), as well as ET-1 (1). This is probably achieved by inhibiting one or more serine/threonine/tyrosine kinases implicated in the signaling events induced by these factors. Several studies using ANG II have shown that NO suppressed the activation of ERK1/2, p38mapk, and JNK (45) as well as Pyk2 (46) in cardiac fibroblasts. It was also recently reported that in rat neonatal pulmonary VSMCs a NO donor inhibited ET-1-induced ERK1/2 phosphorylation (3). However, to our knowledge, a possible contribution of NO to ET-1-induced activation of other signaling events has not been investigated in VSMCs. Therefore, in the present studies we have examined the effect of NO on ET-1-stimulated phosphorylation of ERK1/2, PKB, and Pyk2, the key mediators of growth-promoting, proliferative, hypertrophic survival responses. In addition, we have also examined whether NO acts via a cGMP-dependent mechanism in eliciting these responses.

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REGULATION OF ET-1 SIGNALING BY NO

RESULTS

Both SNAP and SNP Inhibited ET-1-Induced Phosphorylation of ERK1/2, PKB, and Pyk2 in A-10 VSMCs

To determine whether the antimitogenic and antiproliferative effects of NO are mediated by its ability to attenuate growth-promoting signaling pathway in VSMCs, we examined the effect of SNAP, which spontaneously generates NO, on ET-1-induced phosphorylation of ERK1/2, PKB, and Pyk2. As shown in Fig. 1, pretreatment of A-10 VSMCs with SNAP for 15 min dose-dependently attenuated ET-1-induced phosphorylation of all of these protein kinases. Among the kinases, PKB appeared to be more sensitive to the inhibitory effect of SNAP and exhibited almost complete attenuation of ET-1-stimulated phosphorylation at 10 μM (Fig. 1B). In contrast, ET-1-enhanced phosphorylation of ERK1/2 and Pyk2 was inhibited significantly only at 300 μM SNAP.

In addition, SNP, another NO donor, also exhibited a similar effect and attenuated ET-1-induced ERK1/2, PKB, and Pyk2 phosphorylation to varying degrees (Fig. 2).

l-NAME Potentiated ET-1-Induced Phosphorylation of ERK1/2, PKB, and Pyk2 in A-10 VSMCs

To examine whether decreasing the endogenous NO production by inhibition of NOS activity would modify the effect of ET-1 on various signaling components, we investigated the effect of pretreatment of A-10 VSMC with l-NAME, a specific inhibitor of NOS, on ET-1-induced phosphorylation of ERK1/2, PKB, and Pyk2. As shown in Fig. 3, l-NAME treatment, at both doses used, potentiated the response of ET-1 in all three signaling components examined. l-NAME (100 μM) potentiated ET-1-induced ERK1/2 phosphorylation by fourfold (Fig. 3A), whereas only twofold potentiation in PKB and Pyk2 phosphorylation was observed under these conditions (Fig. 3, B and C).

Although it is generally believed that VSMCs are devoid of NOS, the ability of l-NAME to potentiate ET-1-induced signaling suggested the presence of NOS in A-10 VSMCs. This possibility was evaluated by subjecting the total cellular lysates of A-10 VSMCs or HUVECs to Western blotting using specific antibodies against eNOS and iNOS. As shown in Fig. 4A, A-10 cells exhibited a significant expression of eNOS in the basal state. However, compared with HUVECs the expression level of eNOS in A-10 VSMCs was much less. Under these conditions iNOS could not be detected in A-10 VSMCs (data not shown). Furthermore, treatment of A-10 VSMCs with ET-1 or l-NAME did not alter the eNOS expression in these cells (Fig. 4B).

8-BrcGMP Inhibited ET-1-Induced Phosphorylation of ERK1/2, PKB, and Pyk2 in A-10 VSMCs

Since SNAP-induced production of NO would cause an elevation in cGMP, we evaluated the possibility that the effect of SNAP on ET-1-induced responses was mediated by a mechanism involving cGMP. We tested this by pretreating the cells with 8-BrcGMP, a nonmetabolizable and cell-permeant analog of cGMP. As shown in Fig. 5, treatment of cells with 8-BrcGMP decreased ET-1-induced ERK1/2, PKB, and Pyk2 phosphorylation. 8-BrcGMP (100 μM) inhibited ET-1-induced ERK1/2 and Pyk2 phosphorylation almost completely,
Fig. 1. Dose-dependent effect of the nitric oxide (NO) donor S-nitroso-N-acetylpenicillamine (SNAP) on endothelin-1 (ET-1)-induced extracellular signal-regulated kinases (ERK)1/2, PKB, and Pyk2 phosphorylation in A-10 vascular smooth muscle cells (VSMCs). Serum-starved quiescent A-10 cells were pretreated without or with the indicated SNAP concentrations for 15 min, followed by 10 nM ET-1 for 5 min. Cell lysates were immunoblotted by phospho-specific-Tyr204-ERK1/2 antibodies (A), phospho-specific-Ser473-PKB antibodies (B), and phospho-specific-Tyr402-Pyk2 antibodies (C) (top). Blots were also analyzed for total ERK1/2, PKB, and Pyk2 (middle). Bottom: average data quantified by densitometric scanning of immunoblots. Values are means ± SE of at least 3 independent experiments and are expressed as % phosphorylation, where phosphorylation observed with ET-1 alone is defined as 100%. A: *P < 0.0001 vs. control; †P < 0.0001 vs. ET-1. B: *P < 0.0001 vs. control; †P < 0.0001 vs. ET-1. C: *P < 0.002 vs. control; †P < 0.0003 vs. ET-1.

Fig. 2. Dose-dependent effect of the NO donor sodium nitroprusside (SNP) on ET-1-induced ERK1/2, PKB, and Pyk2 phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated without or with the indicated SNP concentrations for 15 min, followed by 10 nM ET-1 for 5 min. Cell lysates were immunoblotted by phospho-specific-Tyr204-ERK1/2 antibodies (A), phospho-specific-Ser473-PKB antibodies (B), and phospho-specific-Tyr402-Pyk2 antibodies (C) (top). Blots were also analyzed for total ERK1/2, PKB, and Pyk2 (middle). Bottom: average data quantified by densitometric scanning of immunoblots. Values are means ± SE of at least 3 independent experiments and are expressed as % phosphorylation, where phosphorylation observed with ET-1 alone is defined as 100%. A: *P < 0.0003 vs. control; †P < 0.0001 vs. ET-1. B: *P < 0.001 vs. control; †P < 0.0006 vs. ET-1. C: *P < 0.0003 vs. control; †P < 0.0002 vs. ET-1.
Here we have provided evidence showing that NO generation induced by SNAP and SNP significantly attenuated ET-1-enhanced phosphorylation of ERK1/2, PKB, and Pyk2 in VSMCs. We have also demonstrated that SNAP treatment was able to antagonize ET-1-induced total protein synthesis, an index of hypertrophy, in VSMCs. Since both ERK1/2 and PKB pathways play critical roles in mediating hypertrophic and cell
survival responses (8), it is reasonable to suggest that the ability of NO donors to inhibit ET-1-induced activation of these pathways is responsible for the antihypertrophic and vascular protective effect of NO. Although NO donors have been found to attenuate EGF (52)-, platelet-derived growth factor (35)-, and ANG II (44)-stimulated proliferation of VSMCs and cardiac fibroblasts, the studies reported here are the first to demonstrate an effect of NO on ET-1-induced phosphorylation of ERK1/2, PKB, and Pyk2 in A-10 VSMCs. These results are similar to those of studies in neonatal pulmonary VSMCs, in which SNP treatment was found to inhibit ET-1-induced ERK1/2 phosphorylation (3), and in rat cardiac fibroblasts, in which ANG II-induced phosphorylation of ERK1/2 and Pyk2 was blocked by SNAP (45, 46). However, our work represents the first study demonstrating that NO antagonizes ET-1-induced PKB and Pyk2 activation as well as ET-1-induced protein synthesis in VSMCs.

The demonstration that pharmacological inhibition of basal NO production with L-NAME augmented ET-1 responses on ERK1/2, PKB, and Pyk2 phosphorylation supports an inhibitory role of NO on ET-1-induced signaling events in A-10 VSMCs. A similar increase in ET-1-induced phosphorylation of ERK1/2 in L-NAME-treated pulmonary artery VSMCs has also been demonstrated (3). It is generally believed that VSMCs are devoid of NOS activity; however, recently, both iNOS and eNOS immunoreactivity as well as NOS activities have been detected in isolated VSMCs (9, 10, 34). Our results showing that A-10 VSMCs express eNOS in the basal state further support the presence of eNOS in VSMCs. Thus it is possible that L-NAME-induced inhibition of eNOS by decreasing NO bioavailability potentiates ET-1-induced signaling events in these cells.

NO is believed to exert its physiological effect through activation of sGC, a heme-containing protein (20). Binding of NO to the heme iron leads to allosteric modification of sGC, resulting in its enhanced catalytic activity to produce cGMP (21). cGMP, thus generated, elicits its downstream responses by interacting with its target proteins such as PKG (37). Additional non-sGC/cGMP-dependent mechanisms of NO action have also been suggested, which include ONOO$^-$/catalyzed posttranslational modification of protein via nitration of tyrosine residues (6). However, our results showing that 8-BrcGMP mimicked the effect of SNAP and SNP in decreasing ET-1-induced ERK1/2, PKB, and Pyk2 phosphorylation suggest an intermediary role of cGMP in exerting this inhibitory response. Further proof for the involvement of sGC in this process has been provided by the use of ODQ, a specific inhibitor of sGC that can block SNAP-induced elevations in cGMP levels in rat aortic VSMCs (28), A-10 VSMCs (4), endothelial cells (19), and cardiomyocytes (36). We found that ODQ treatment of A-10 VSMCs was able to significantly reverse the inhibitory effect of SNAP on ET-1-induced ERK1/2, PKB, and Pyk2 phosphorylation. A similar involvement of cGMP/PKG pathway in NO-induced inhibition of ERK1/2 phosphorylation by ET-1 in cardiomyocytes and in pulmonary artery VSMCs has also been reported (3, 12). The fact that 8-BrcGMP caused only partial inhibition of ET-1-induced PKB phosphorylation and ODQ was not able to completely reverse the SNAP-induced responses in our studies suggests a partial contribution of non-cGMP-dependent events in mediating the effect of NO donors in ET-1-induced re-

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**Fig. 5.** Effect of a stable analog of cGMP, 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP), on ET-1-induced ERK1/2, PKB, and Pyk2 phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated without or with the indicated 8-Br-cGMP concentrations for 15 min, followed by 10 nM ET-1 for 5 min. Cell lysates were immunoblotted by phospho-specific-Tyr204-ERK1/2 antibodies (A), phospho-specific-Ser473-PKB antibodies (B), and phospho-specific-Tyr402-Pyk2 antibodies (C) (top). Blots were also analyzed for total ERK1/2, PKB, and Pyk2 (middle). Bottom: average data quantified by densitometric scanning of immunoblots. Values are means ± SE of at least 3 independent experiments and are expressed as % phosphorylation, where phosphorylation observed with ET-1 alone is defined as 100%. A: *P < 0.003 vs. control; †P < 0.005 vs. ET-1. B: *P < 0.0001 vs. control; †P < 0.0003 vs. ET-1. C: *P < 0.001 vs. control; †P < 0.005 vs. ET-1.
responses. These cGMP-independent mechanisms include nitration of some upstream signaling components resulting in attenuation of their catalytic activity. The existence of a cGMP-independent mechanism in mediating the antiproliferative effects of NO has also been suggested from other studies in which ODQ, despite lowering NO-induced cGMP levels, failed to reverse the antiproliferative effect of NO donors in pulmonary microvascular smooth muscle cells (41) or in human endothelial cells (19). In these studies, however, the effect of ODQ on signaling pathways linked to proliferative responses was not investigated.

The precise mechanism by which cGMP inhibits ERK1/2 signaling remains elusive; however, the ability of PKG, the downstream effector of cGMP action, to phosphorylate c-Raf kinase on Ser43 and the resulting uncoupling between Ras-Raf might contribute to this effect (43). Since the upstream elements leading the PKB phosphorylation are different from those of ERK1/2 (8), the precise mechanism by which the cGMP/PKG system attenuates PKB phosphorylation remains undefined.

Pyk2 is a Ca$^{2+}$-dependent proline-rich nonreceptor PTK that plays an essential role in ANG II-induced ERK1/2 signaling and hypertrophy in VSMCs (33) Pyk2 is activated by auto-phosphorylation in Tyr402 located in its catalytic domain (2).
thus may also be possible that SNAP/cGMP-induced decrease in Pyk2 phosphorylation observed in our studies contributed to the attenuating effect of SNAP on ET-1-induced signaling in A-10 VSMCs. NO generation has been shown to attenuate IGF-1- and insulin-induced elevation in H$_2$O$_2$ levels through a cGMP-dependent event in VSMCs (53). ET-1-induced ERK1/2 and PKB signaling is known to require activation of the NADPH-oxidase system and resultant H$_2$O$_2$ generation (15). Thus it is possible that a NO/cGMP-induced reduction in H$_2$O$_2$ generation contributes to the decrease in ET-1 response observed in our studies.

In summary, we have demonstrated that SNAP and SNP, NO donors, inhibit ET-1-stimulated increase of ERK1/2, PKB, and Pyk2 phosphorylation through a cGMP/sGC-dependent mechanism in A-10 VSMCs. We have also provided evidence showing that ET-1-stimulated protein synthesis, a hallmark of hypertrophic response, is also attenuated by the NO donor SNAP in A-10 VSMCs. Since ERK1/2, PKB, and Pyk2 play a crucial role in mediating VSMC growth and hypertrophy, it may be suggested that the ability of NO to attenuate these pathways may serve as a potential mechanism by which NO counteracts the growth-promoting and hypertrophic responses of ET-1 in VSMCs.

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