Role of Reactive Oxygen Species in Bradykinin-Induced Mitogen-Activated Protein Kinase and c-fos Induction in Vascular Cells

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Abstract—Bradykinin stimulates proliferation of aortic vascular smooth muscle cells (VSMCs). We investigated the action of bradykinin on the phosphorylation state of the mitogen-activated protein kinases p42mapk and p44mapk in VSMCs and tested the hypothesis that reactive oxygen species (ROS) might be involved in the signal transduction pathway linking bradykinin activation of nuclear transcription factors to the phosphorylation of p42mapk and p44mapk. Bradykinin (10^{-8} mol/L) rapidly increased (4- to 5-fold) the phosphorylation of p42mapk and p44mapk in VSMCs. Preincubation of VSMCs with either N-acetyl-L-cysteine and/or α-lipoic acid significantly decreased bradykinin-induced cytosolic and nuclear phosphorylation of p42mapk and p44mapk. In addition, the induction c-fos mRNA levels by bradykinin was completely abolished by N-acetyl-L-cysteine and α-lipoic acid. Using the cell-permeable fluorescent dye dichlorofluorescein diacetate, we determined that bradykinin (10^{-8} mol/L) rapidly increased the generation of ROS in VSMCs. The NADPH oxidase inhibitor diphenylene iodonium (DPI) blocked bradykinin-induced c-fos mRNA expression and p42mapk and p44mapk activation, implicating NADPH oxidase as the source for the generation of ROS. These findings demonstrate that the phosphorylation of cytosolic and nuclear p42mapk and p44mapk and the expression of c-fos mRNA in VSMCs in response to bradykinin are mediated via the generation of ROS and implicate ROS as important mediators in the signal transduction pathway through which bradykinin promotes VSMC proliferation in states of vascular injury. (Hypertension. 2000;35:942-947.)

Key Words: bradykinin ■ kinases ■ proto-oncogenes ■ reactive oxygen species

The vasoactive nonapeptide bradykinin (BK) is the principal effector of the kallikrein-kinin system and has been implicated in the regulation of renal and cardiovascular function and vascular tone.1,2 BK can be generated both systemically and locally within the vascular wall.3–5 Thus, kinins could act as a paracrine and autocrine manner to influence vascular function. The physiological effects of BK are mediated via generation of second messengers, such as nitric oxide and eicosanoids.6,7

In vascular smooth muscle cells (VSMCs), activation of the B2-kinin receptor by BK stimulates phospholipase C activity via a heterotrimeric GTP-binding protein leading to the generation of inositol phosphate and diacylglycerol, both of which are involved in intracellular calcium mobilization and the activation of protein kinase C.8 Through activation of its B2 receptor, BK has also been shown to increase proliferation, stimulate mitogen-activated protein kinase (MAPK) activation and nuclear translocation, and induce the expression of proto-oncogenes c-fos and c-jun and the formation of the activator protein-1 complex.9,10 The cellular mechanism through which BK stimulates MAPK activation and c-fos mRNA expression in VSMCs involves the activation of a calcium/calmodulin pathway, src kinase, protein kinase C, and MAPK kinase.11,12

MAPKs, also known as extracellular signal-regulated kinases, belong to the group of serine/threonine kinases that are rapidly activated in response to growth factor stimulation. Recent studies have implicated MAPK in the pathogenesis of cardiovascular disease.13,14 The MAPKs p42mapk and p44mapk are activated by dual-specificity (threonine and tyrosine residues) protein kinases called MAPKK/MEK. When phosphorylated, p42mapk and p44mapk are capable, in turn, of phosphorylating a variety of diverse targets, such as effector kinases and transcription factors, thereby regulating the expression of different genes associated with cellular proliferation/fibrosis.

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO·), are produced by a variety of cell types and have important physiological and pathophysiological effects.15 The generation of ROS is increased in blood vessels and myocardium in response to a variety of injury-related conditions,
such as ischemia, thrombosis, reperfusion, and angioplasty. More recently, it has been shown that ROS can act as second messengers in pathways leading to cellular proliferation. In this regard, ROS have been shown to induce cell growth, DNA synthesis, and proto-oncogene mRNA expression in VSMCs.

Therefore, the present studies were designed to explore the role of ROS in BK-induced MAPK activation and c-fos mRNA expression in VSMCs. We observed that BK stimulation leads to a rapid generation of ROS in VSMCs. Furthermore, the activation of MAPK and the induction of c-fos mRNA expression by BK are mediated via generation of ROS. These findings point to an important function of oxidative stress and the generation of ROS on the cellular responses mediated by BK in VSMCs.

Methods

Cell Culture

Rat aortic VSMCs from 75- to 150-g male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) were prepared by a modification of the method of Majack and Clowes. A 2-cm segment of artery cleaned of fat and adventitia was incubated in 1 mg/mL collagenase for 3 hours at room temperature. The artery was then cut into small sections, fixed to a culture flask for explantation in MEM (GIBCO-BRL) supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were identified as VSMCs by their characteristic morphology and contractile properties. Cells were used between passages 2 and 6 in all experiments. Quiescence was achieved by transferring the 60% to 70% confluent cell cultures to MEM without serum for 24 to 48 hours. Quiescence was confirmed by transferring the 60% to 70% confluent cell cultures to MEM without serum for 24 to 48 hours. This method (Renaissance, New England Biolabs) with the use of Kodak X-LS film. Immunoreactive bands were visualized by a chemiluminescent method (Optic Co) at an excitation wavelength of 485 nm; emission was measured at a wavelength of 530 nm.

Cytosolic and Nuclear Extraction of Proteins

Cytosolic and nuclear proteins were extracted from VSMCs by the technique of Dignam et al. Quiescent VSMCs, grown in 15-cm dishes, were stimulated with BK (10⁻⁸ mol/L) for 5 minutes. The cells were suspended in 400 μL of cold cell lysis buffer (10 mmol/L HEPES [pH 7.9], 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, and 0.2 mmol/L phenylmethylsulfonyl fluoride), incubated on ice for 10 minutes, and centrifuged at 13 000g for 10 seconds. The supernatant was harvested as the cytosolic fraction. The pellet fraction was resuspended in 500 μL of cold nuclear lysis buffer (20 mmol/L HEPES [pH 7.9], 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 0.5 mmol/L phenylmethylsulfonyl fluoride), incubated on ice for 20 minutes, and centrifuged at maximum speed for 15 minutes. The protein concentration in nuclear and cytosolic fractions was determined by the method of Lowry et al.

Phospho-MAPK Immunoblots

To measure MAPK activity in the cytosol and nuclear fractions, soluble proteins (20 to 25 μg) obtained as described above were subjected to SDS-PAGE. The separated proteins in the gel were transferred to polyvinylidine difluoride membranes and immunoblotted with rabbit polyclonal phospho-specific MAPK antibodies that specifically recognize Tyr204-phosphorylated (but not nonphosphorylated) p42mapk and p44 mapk (New England Biolabs). The phospho-MAPK antibody was used at 1:6000 dilution, whereas the control antibody, which recognizes total MAPK, was used at 1:4000 dilution. The membranes were incubated overnight with the antibodies in antibody buffer (Tris-buffered saline, 0.05% Tween 20, and 1% BSA), washed, and exposed to goat anti-rabbit horseradish peroxidase–conjugated IgG (1:5000) in antibody buffer for 1 hour. Immunoreactive bands were visualized by a chemiluminescent method (Renaisance, New England Biolabs) with the use of Kodak X-LS film.

RNA Extraction and Northern Blotting

Total RNA from VSMCs was extracted with Tri-reagent (Molecular Research Center Inc) according to the manufacturer’s instructions. This method is based on the method of Chomczynski and Sacchi. Total RNA (20 μg) was denatured at 65°C and electrophoresed on a 1.5% agarose gel. Gels were stained with ethidium bromide to determine the position in each lane of the 28S and 18S ribosomal RNA and to demonstrate that similar amounts of intact RNA were
used for each sample. The RNA was subsequently transferred to Nytran membrane filters (Schleicher & Schuell), prehybridized for 3 hours, and then hybridized for 18 hours at 60°C with 32P-labeled c-fos and GAPDH cDNA probes. Membranes were washed and exposed to Kodak X-Omat AR film; this procedure was followed by densitometric analysis of the intensities of bands observed in the autoradiographs.

**Thymidine Incorporation**

DNA synthesis was assessed by measuring the incorporation of [3H]thymidine into DNA fragments. Quiescent VSMCs grown in 12-well plates were stimulated with BK (10⁻⁴ mol/L) in the presence and absence of N-acetyl-l-cysteine (NAC, 1 mmol/L) and then pulsed for 4 hours with 1 μCi/mL per well of [3H]thymidine. The experiment was terminated by the addition of 0.5 mL of 0.3 mol/L perchloric acid for 30 seconds, followed by a cold saline wash. Cells were solubilized in 0.1% SDS/0.1N NaOH, and incorporated radioactivity was quantified.

**Statistical Analysis**

Data are expressed as mean±SEM and were analyzed by ANOVA for repeated measures and by using the Student t test for unpaired 2-tailed analyses. Differences were considered significant at P<0.05.

**Results**

**Generation of ROS by BK in VSMCs**

To explore whether BK induces ROS generation, quiescent VSMCs were pretreated with DCF-DA for 30 minutes, followed by BK (10⁻⁴ mol/L) stimulation for various times (0 to 15 minutes). Relative fluorescence intensity and microscopic fluorescence images of VSMCs were obtained from 1 to 15 minutes after the exposure to BK with laser confocal scanning microscopy. Our initial studies indicated that the increase in fluorescence in response to BK peaked at 5 minutes and persisted at similar levels over the following 10 minutes. The results shown in Figure 1 demonstrate that BK induced a marked increase in fluorescence intensity 5 minutes after stimulation, reflecting an increase in ROS production. Quantitative analysis of the confocal images indicated that the increase in fluorescence intensity was ∼3 times higher in VSMCs treated with BK than in control cells. No fluorescence was observed when VSMCs were stimulated with BK alone (Figure 1).

To determine the receptor subtype through which BK stimulates ROS generation, VSMCs were pretreated for 30 minutes with the B₂-kinin receptor antagonist HOE-140 (10⁻⁶ mol/L), followed by BK (10⁻⁴ mol/L) stimulation for 5 minutes. Addition of HOE-140 to VSMCs prevented the BK-induced increase in ROS generation that was seen with VSMCs treated with BK alone (Figure 1). The B₂-kinin receptor antagonist had no significant effect on the basal production of ROS in VSMCs.

**Antioxidants and NAD(P)H Oxidase Inhibitors Attenuate BK-Induced MAPK Phosphorylation**

To determine whether the generation of ROS by BK plays a role in p42mapk and p44mapk activation, quiescent VSMCs were pretreated with NAC (30 mmol/L, 45 minutes), a thiol-based antioxidant, or with α-lipoic acid (α-LA, 500 μmol/L, 120 minutes), a broad-range antioxidant, and/or with the NAD(P)H oxidase inhibitor diphenylene iodonium (DPI, 100 μmol/L, 30 minutes). BK produced a 5- to 6-fold increase in MAPK phosphorylation in cells not pretreated with antioxidants compared with unstimulated cells (Figure 2). However, pretreatment of VSMCs with NAC, α-LA, or DPI significantly reduced BK-induced MAPK phosphorylation (Figure 2). NAC inhibited BK-induced MAPK phosphorylation by 55%, α-LA inhibited BK-induced MAPK phosphorylation by 30%, and DPI inhibited BK-induced MAPK phosphorylation by 70%. These findings suggest that the generation of ROS by BK is upstream from the p42mapk and p44mapk pathway and participates in the signal transduction pathway through which BK stimulates MAPK activation in VSMCs.

**Figure 2. Antioxidants and NAD(P)H oxidase inhibitors attenuate BK-induced MAPK phosphorylation.** Pretreatment of quiescent VSMCs with DPI (100 μmol/L) for 30 minutes, α-LA (500 μmol/L) for 2 hours, or NAC (30 mmol/L) for 45 minutes was followed by BK (10⁻⁸ mol/L) stimulation for 5 minutes. MAPK phosphorylation in the cell lysate was measured by immunoblots with the use of anti–phosphotyrosine-MAPK antibodies (P-MAPK). Total MAPK was measured in the same immunoblot by stripping the membrane and reincubating with anti–total MAPK antibodies (T-MAPK). Blots are representative of 5 experiments. The bar graph represents the intensities of both p42mapk and p44mapk bands measured in a densitometer relative to total MAPK and expressed as percent phosphorylation relative to control. *P<0.05 vs control; †P<0.05 vs BK alone.

**Nuclear Phosphorylation of MAPK by BK Is ROS Dependent**

The nuclear targets for p42mapk and p44mapk include the phosphorylation of Elk1/TCF, which, in turn, leads to transcriptional activation at serum response elements and induction of c-fos mRNA levels. For MAPKs to influence gene expression, activation and translocation to the nucleus are required. To assess the role of ROS in BK-induced nuclear
phosphorylation of p42mapk and p44mapk, we measured the tyrosine phosphorylation of p42mapk and p44mapk in the cytosol and in nuclear extracts of VSMCs treated with BK (10^{-8} mol/L) in the presence and absence of the antioxidants NAC, α-LA, and DPI. Compared with unstimulated cells, VSMCs treated with BK for 5 minutes showed a marked increase in tyrosine phosphorylation of p42mapk and p44mapk in both cytosolic and nuclear fractions (Figure 3). Pretreatment of VSMCs with NAC, α-LA, or DPI significantly decreased both cytosolic and nuclear MAPK phosphorylation in response to BK (Figure 3). Nuclear phosphorylation of p42mapk and p44mapk in response to BK was reduced by 50%, 25%, and 80% in VSMCs pretreated with NAC, α-LA, and DPI, respectively, compared with VSMCs treated with BK alone (Figure 3).

BK-Induced c-fos mRNA Expression Is Modulated by ROS

To evaluate whether the induction of c-fos mRNA levels by BK is ROS dependent, we measured c-fos mRNA levels in VSMCs pretreated with NAC, α-LA, or DPI, followed by BK (10^{-8} mol/L) stimulation for 30 minutes. As shown in Figure 4, c-fos mRNA levels, which were expressed relative to GAPDH mRNA levels, were almost undetectable in untreated control cells but were markedly induced within 30 minutes of BK stimulation. However, in the presence of NAC, α-LA, or DPI, the induction of c-fos mRNA by BK was significantly suppressed by the antioxidants (Figure 4). DPI produced the most inhibition, followed by NAC and α-LA. GAPDH mRNA levels measured in the same cells were not altered by any of the treatments (Figure 4).

NAC Inhibits BK-Induced VSMC Proliferation

The mitogenic effect of BK in VSMCs was evaluated by measuring DNA synthesis. There was a significant increase in [3H]thymidine incorporation in BK-treated cells compared with unstimulated cells (1254±83 cpm versus 792±68 cpm for BK versus control, respectively; \[P < 0.02, n = 4\] experiments). This effect of BK was blocked by the antioxidant NAC (1254±83 cpm versus 913±151 cpm for BK versus BK+NAC, respectively; \[P < 0.05, n = 3\] experiments). NAC alone had no significant effect on [3H]thymidine incorporation (852±89 cpm versus 792±68 cpm for NAC versus control, respectively). These findings implicate a role for ROS in the mitogenic actions of BK in VSMCs.

Discussion

The present study demonstrates that BK induces the generation of ROS in VSMCs and that this induction of ROS is mediated via activation of the B_{2}-kinin receptor. In addition, we have shown that ROS act as second messengers to modulate the actions of BK to stimulate cytosolic and nuclear phosphorylation of p42mapk and p44mapk, to promote induction of early response genes (such as c-fos), and to stimulate DNA synthesis. These findings provide the first indication that ROS play a key role in mediating the early mitogenic signals initiated by BK in VSMCs.

Recently, several groups have reported that ROS can act as second messengers in signal transduction to activate transcription factors and to stimulate cellular growth, differentiation, and apoptosis. In this regard, treatment of VSMCs with H_{2}O_{2} or xanthine oxidase increased DNA synthesis and...
stimulate cell proliferation. 

Downstream signaling events of these growth factors to ROS in a variety of cell systems, which, in turn, mediate the proto-oncogene expression. Furthermore, several growth factors, such as platelet-derived growth factor, angiotensin II, and oleic acid, have been shown to stimulate the generation of ROS in a variety of cell systems, which, in turn, mediate the downstream signaling events of these growth factors to stimulate cell proliferation. 

A role for ROS as second messengers for BK signaling in VSMCs has not been explored. Therefore, to investigate whether BK can generate ROS in VSMCs, we used the peroxide-sensitive fluoroprobe DCF-DA to measure the generation of intracellular ROS by laser confocal scanning microscope. Our findings demonstrate that treatment of VSMCs with BK results in the generation of ROS, as evidenced by the intense fluorescence. The fact that BK induced an increase in DCF fluorescence and that the B2 kinin receptor antagonist HOE-140 diminished the intensity of fluorescence indicates that BK generates ROS via activation of its receptor. Although the cellular mechanisms through which BK increases ROS generation are not fully defined, cellular production of ROS can be generated via several sources. These include the mitochondria electron transport system, the cytosolic enzyme xanthine oxidase, cyclooxygenase, nitric oxide synthase, and flavin-containing oxidases. In addition, membrane-bound flavin-containing oxidases have been identified in VSMCs. It is possible that BK can increase the activity of one or more of enzymes that are implicated in the generation of ROS or decrease the activity of antioxidant enzymes, such as superoxide dismutase and catalase, that scavenge O$_2^{-}$ and H$_2$O$_2$, respectively.

The MAPK pathway has been implicated as a key player through which ROS promote cellular proliferation. In this regard, VSMCs treated with H$_2$O$_2$ or with growth factors that generate ROS stimulated cell proliferation and DNA synthesis via activation of the p42mapk and p44mapk pathway. In the present study, we have shown that the increase in ROS evoked by BK is upstream from MAPK. Support for this comes from the findings that antioxidants, such as NAC and α-LA, significantly reduced cytosolic as well as nuclear phosphorylation of MAPK in response to BK stimulation. NAC serves as an antioxidant directly, by protecting sulphydryl groups from oxidation, or indirectly, by serving as a precursor for the synthesis of glutathione, an abundant endogenous cellular reducing antioxidant, and by the recycling of other antioxidants. α-LA is a scavenger of hydroxyl radicals, singlet oxygen, and hypochlorous acid and may exert antioxidant effects by chelation of transition metals. In addition, DPI, a compound that inhibits NAD(P)H oxidase (a major enzyme involved in the generation of cellular oxygen-derived free radicals by binding to the flavin site), also reduced BK-induced cytosolic and nuclear phosphorylation of MAPK. Taken together, these findings demonstrate that ROS are essential requirements in the signal transduction pathway through which BK activates MAPK.

Our results indicate that one of the nuclear targets for BK is the induction of c-fos, which binds with c-jun to form the activator protein-1 complex transcription factor, thereby regulating the expression of genes containing this element. In this regard, a recent study by El Dahr et al showed that the tyrosyl phosphorylation of Elk-1 in response to BK is mediated via MAPK activation in mesangial cells. The present study shows that treatment of VSMCs with antioxidants significantly reduced the increase in c-fos mRNA expression that was observed in response to BK. Although we did not inhibit other ROS-producing enzymes, we were able to block c-fos mRNA expression with DPI, suggesting that one of the main enzymes involved in BK-induced ROS production is the flavin-containing NAD(P)H oxidase. This is the first indication that BK stimulates c-fos induction via the generation of ROS.

Under normal physiological conditions, BK is well known as a potent stimulator of nitric oxide release from endothelial cells, which, in turn, results in the vasodilatation of the vessel wall and relaxation of the underlying VSMCs. In contrast, when the endothelial layer is denuded or dysfunctional, as is observed in disease states, such as hypertension or diabetes, BK can directly act on VSMCs to induce vasoconstriction in a manner similar to other vasoconstrictors, such as angiotensin II. Indeed, we have shown that BK can activate multiple signaling pathways in VSMCs similar to those activated by angiotensin II, ultimately resulting in cellular proliferation. Although there are still many steps to be defined in the signal transduction pathway through which BK alters VSMC function, the findings of the present study implicate a role for ROS as mediators of BK signaling. These results are of particular significance because of our interest in diabetes, a
condition in which oxidative stress plays a key role in the development of diabetic vascular disease, and are relevant to our recent findings indicating that hyperglycemia induces the expression of B₂-kinin receptors in VSMCs. Further studies involving the role of ROS in BK signaling will contribute significantly to our understanding of the functional diversity of signals through which BK alters VSMC function.

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