Induction of B$_1$-Kinin Receptors in Vascular Smooth Muscle Cells: Cellular Mechanisms of MAP Kinase Activation

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Abstract—Vascular smooth muscle cell (VSMC) proliferation is a prominent feature of the atherosclerotic process that occurs after endothelial injury. Although a vascular wall kallikrein-kinin system has been described, its contribution to vascular disease remains undefined. Because the B$_1$-kinin receptor subtype (B1KR) is induced in VSMCs only in response to injury, we hypothesize that this receptor may be mediating critical events in the progression of vascular disease. In the present study, we provide evidence that des-Arg$^9$-bradykinin (dABK) (10$^{-8}$ M), acting through B1KR, stimulates the phosphorylation of mitogen-activated protein kinase (MAPK) (p42mapk and p44mapk). Activation of MAPK by dABK is mediated via a chola toxin–sensitive pathway and appears to involve protein kinase C, Src kinase, and MAPK kinase. These findings demonstrate that the activation of B1KR in VSMCs leads to the generation of second messengers that converge to activate MAPK and provide a rationale to investigate the mitogenic actions of dABK in vascular injury. (Hypertension. 2001;38[part 2]:602-605.)

Key Words: receptors, kinin, B$_1$, protein kinases, muscle, smooth, vascular

K

inins are small peptide vasodilators generated by kal-
lkreins from their precursor kininogens. Binding of

kinins to their cell surface receptors produces a number of physiological effects, including vasodilatation, smooth muscle cell relaxation and contraction, and pain production. Two

receptors mediate the effects of kinins B$_1$ and B$_2$. The B$_2$-kinin receptor (B2KR) is responsive to intact kinins such as bradykinin (BK), whereas the B$_1$-kinin receptor (B1KR) has a higher affinity for des-Arg$^9$-bradykinin (dABK). B2KR is constitutively expressed in a wide range of tissues and mediates most effects assigned to kinins. B1KR is generally absent in normal tissues but is rapidly induced after injury through the effects of cytokines and growth factors. Recent reports, however, have shown that under certain conditions, rat aortic smooth muscle cells in culture do express functional B$_1$ receptors. B1KR is a member of the seven transmembrane G protein–coupled receptor superfamily. On binding to its receptor, dABK activates phospholipase C via a heterotrimeric GTP-binding protein and induces a marked increase in d-myo-inositol 1,4,5-tris-phosphate, leading to an increase in intracellular calcium.

Vascular injury is considered to be a primary event in the evolution of atherosclerotic vascular disease. The prevailing view of atherogenesis is the response-to-injury hypothesis, which proposes that injury to the endothelium is the initiating event. Because B1KR is expressed only in response to injury, we hypothesize that it could play a critical role in the development of vascular disease. Therefore, it is crucial to understand the signaling pathways through which B1KR evokes its response and to determine whether these signaling pathways parallel those of B2KR in vascular smooth muscle cells (VSMCs). In the present study, we explored the role of dABK in the activation of MAPK and elucidated the signal transduction pathway through which dABK stimulates MAPK phosphorylation in VSMCs. Here we provide evidence that dABK, via activation of B$_1$-kinin receptors, induces phosphorylation of p42mapk and p44mapk. The activation of MAPK by dABK is mediated through a chola toxin–sensitive (CTx) pathway and involves both protein kinase C and cytoplasmic tyrosine kinases.

Methods

VSMC Culture

Rat aortic VSMCs from male Sprague-Dawley rats were isolated as described in detail previously. Cells were incubated at 37°C in a humidified atmosphere of 95% oxygen/5% CO$_2$. Medium was changed every 3 to 4 days, and cells were passaged every 6 to 8 days by harvesting with trypsin-EDTA. VSMCs isolated by this procedure are homogenous and were used in all studies between passages 2 to 6.

Western Blotting

Cells were washed twice in PBS containing 2 mmol/L sodium orthovanadate and were subsequently scraped and lysed in 100 μL of SDS/sample buffer. Western immunoblots were performed as previously described. Membranes were incubated with antibodies p42mapk or p44mapk (1:6000) or total MAPK (1:6000) (New England Biolabs); Densitometric analysis was performed using the National Institutes of Health (NIH) image program.
Reverse Transcriptase–Polymerase Chain Reaction

RNA was extracted from cells using Tri-Reagent (Molecular Research Center). The RNA was then converted to cDNA using AMV (Promega) at 42°C for 1 hour. The polymerase chain reaction (PCR) was carried out in 25 μL total volume containing 1× PCR buffer, 200 μM dNTPs, 2 ng/μL each primer, 5 μL first-strand cDNA, and 1 U Taq (Qiagen). Primers used for amplification were 5′-AAGACAAGCTCACCTGCT-3′ (exon 1) and 5′-GACACCACCACCACGGGA-3′ (exon 2) for the rat B1-receptor, and 5′-GAACCCTAGGCCAACCGTG-3′ and 5′-TGGCATAGAGGCCTTTAAGG-3′ for β-actin. PCR were visualized on a 1% agarose gel, photographs were taken, and densitometric analysis was performed using the NIH IMAGE program.

Statistical Analysis

All data are expressed as mean±SE and were analyzed by ANOVA or Student’s t test for unpaired analysis. Values were considered significant if P<0.05.

Results

Induction of B1KR by Interleukin-1β

To examine whether interleukin-1β (IL-1β) would induce B1KR expression, quiescent VSMCs were treated with IL-1β (10 ng/μL) for 24 hours. RNA was isolated from the cells, and reverse transcriptase (RT)–PCR was performed using rat B1KR-specific primers. IL-1β–treated cells showed a 2-fold increase in B1KR compared with that of untreated control cells (Figure 1). These results demonstrate that IL-1β is a potent inducer of B1KR in VSMCs.

Activation of p42MAPK and p44MAPK by dABK

To understand the signal transduction events mediated by dABK in VSMCs, we examined its effects on the activation of MAPK. Quiescent VSMCs were treated with IL-1β (10 ng/μL) for 24 hours to induce B1KR, followed by stimulation with dABK (10−8 M) for 5 minutes. This treatment resulted in an increase in tyrosine phosphorylation of both p42MAPK and p44MAPK, whereas minimal phosphorylation was detected in unstimulated control cells or cells treated with either dABK or IL-1β alone (Figure 2A).

To establish that B1KR was the receptor through which dABK stimulates MAPK phosphorylation, VSMCs pretreated with IL-1β were then treated for 30 minutes with either the B1KR-specific antagonist des-Arg9-Leu8-BK (Leu8) (10−8 M) or the B2KR-specific antagonist HOE 140 (10−6 M), followed by stimulation with dABK (10−8 M) for 5 minutes. Again, treatment of VSMCs with IL-1β and dABK produced a 2-fold increase in p42MAPK and p44MAPK phosphorylation compared with that of unstimulated cells (Figure 2B). Addition of Leu8 to VSMCs completely inhibited the dABK-induced phosphorylation of MAPK (Figure 2B), whereas HOE 140 had no effect on the dABK-induced phosphorylation (data not shown). These findings demonstrate that dABK stimulates MAPK activation via the inducible B1-kinin receptor subtype.

Effects of Pertussis Toxin and/or Cholera Toxin on dABK-Induced MAPK Phosphorylation

To determine through which G protein dABK signals to stimulate MAPK phosphorylation, we studied the effect of pertussis toxin (PTx), an inhibitor of Gi, and CTx, which downregulates Gβγ, on dABK-induced MAPK activation in VSMCs. Treatment of VSMCs with PTx (100 ng/mL) for 24 hours did not affect dABK-induced MAPK phosphorylation, whereas treatment with CTx (5 μg/mL) for 24 hours did significantly block the dABK-induced MAPK phosphorylation, indicating that dABK activates MAPK via a CTx-sensitive G protein (Figure 3). This result demonstrates that B1KR is mediating its effects via a Gi pathway.

Role of Cytoplasmic Kinases in dABK-Induced MAPK Phosphorylation

To determine the role of cytoplasmic kinases on dABK-induced MAPK phosphorylation, we investigated the effects of cell-permeable kinase inhibitors bisindolylmaleimide (Bis) (Calbiochem), which inhibits PKC; PP1 (Biomol Research Laboratory Inc), which inhibits the Src family tyrosine kinases; and PD 098,059 (NEN Biolabs), which specifically inhibits MEK in this response. VSMCs, pretreated with IL-1β
through which IL-1β induces B1KR are not yet defined, it is known that one of the major transcription factors mediating IL-1β biological activity is NF-κB.9 In arterial smooth muscle cells, constitutive expression of NF-κB is necessary for smooth muscle cell proliferation in vitro,9 and basal activity transiently increases following arterial injury.10 In this regard, it is interesting to note that an NF-κB–binding site has recently been identified within the promoter region of the B1KR gene.7 It is known that IL-1β stimulation leads to a rapid phosphorylation and degradation of IκBα, the most common NF-κB inhibitor. This reaction allows NF-κB to translocate to the nucleus, to bind DNA, and to activate the transcription of specific genes.11 Therefore, this is a possible mechanism through which IL-1β stimulates the induction of B1KR. To understand the possible participation of the B1KR in the development of vascular disease, it is necessary to elucidate the signaling pathways through which B1KR evokes its response. As previously indicated above, B1KR is a member of the G protein–coupled receptor family.2 Many second messengers are induced by these receptors depending on the G protein that is coupled to them. Our data show that the B1KR, unlike B2KR,5 is coupled to the Gq form; other G protein–coupled receptors shown to utilize this pathway include histamine and dopamine receptors.12 MAPKs belong to the group of serine/threonine kinases that are rapidly activated in response to growth factor stimulation. They integrate multiple signals from various second messengers, leading to cellular proliferation and differentiation.13 We have previously shown that bradykinin (10−8 M for 5 minutes), through its B2KR, can stimulate phosphorylation of MAPK consistently;5 therefore, we chose to use the same conditions for dABK. When we studied the activation of MAPK by the B1KR, we observed a similar response to the one described for the B2KR. By the use of specific antagonists to the different kinin receptor,14 we confirmed that the signal was mediated by the B1KR. Many intracellular reactions that mediate MAPK activation require protein phosphorylation. We studied the participation of 3 kinases on this pathway. Our results demonstrate that PKC, Src kinase, and MEK are needed to transmit the signal to MAPK. In fibroblasts, it has been shown that inhibition of both MAP kinases p42<sup>mit</sup> and p44<sup>mit</sup> suppresses cell growth15 and that both are rapidly translocated to the nucleus in response to strong mitogens, whereas their common activator MEK remains in the cytoplasm.16 Once in the nucleus, MAPK can phosphorylate and activate transcription factors such as TCF/ELK-1, resulting in the regulation of expression of transcription factors such as c-fos,17 and the formation of complexes such as AP-1.18 Recently it has been shown that MAPK activity is transiently activated following vessel wall injury using a balloon catheter.19 In this regard, it is possible that certain proteins, such as B1KR, that are differentially expressed following vascular injury may be playing a role in this increased activity of MAPK.

In summary, the present study identifies several second messenger systems that are generated on induction and activation of the B1-kinin receptor in response to dABK stimulation. Our findings demonstrate that dABK can induce the phosphorylation of p42<sup>mit</sup> and p44<sup>mit</sup> in VSMCs. This

**Discussion**

It has been previously shown that tissue isolation and exposure in vitro to proinflammatory cytokines induces B1KR expression via a mechanism that requires protein synthesis,6 and that cytokines, such as IL-1β, play an important role in the induction of B1KR.3,7 Although the cellular mechanisms

![Figure 3. Effects of PTx and CTx on dABK-induced MAPK phosphorylation. VSMCs were treated with IL-1β (10 ng/μL) and dABK (10−8 M) in the presence or absence of either PTx (100 ng/ml) or CTx (5 μg/ml), and PYMAPK or TMAPK was measured by Western blot analysis (n=4). *P<0.05 vs C, #P<0.05 vs IL+dABK. Bar graphs, Mean±SE of the intensities of the bands expressed as percentage of control.](image)

![Figure 4. Role of cytoplasmic kinases in dABK-induced phosphorylation of MAPK. VSMCs were stimulated with IL (10 ng/μL) plus dABK (10−8 M) in the presence of absence of (A) a PKC inhibitor (Bis; 2 μmol/L), (B) a Src inhibitor (PP1; 10 μmol/L), or (C) a MEK inhibitor (PD 098,059; 40 μmol/L). PYMAPK or TMAPK was measured by Western blot analysis (n=4). *P<0.05 vs C, #P<0.05 vs IL+dABK. Bar graphs, Mean±SE of the intensities of the bands expressed as percentage of control.](image)
phosphorylation of MAPK appears to be CTx sensitive and involve the activation of PKC, Src kinase, and MEK. These findings provide evidence of early mitogenic signals induced by dABK through B1KR in VSMCs. It remains to be seen whether or not the downstream effects of B1KR activation include cellular proliferation and/or cellular matrix production which are characteristic features of vascular injury.

Acknowledgments
This work was supported by NIH grants DK-46543 and HL-55782, a research award from the American Diabetes Association (A.A.J.) and FONDECYT grant 1000660 (V.V.). Dr Christopher is supported by NIH training fellowship grant HL-07260.

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Hypertension. 2001;38:602-605
doi: 10.1161/01.HYP.38.3.602

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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