Effects of ANG II on bradykinin receptor gene expression in cardiomyocytes and vascular smooth muscle cells

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Kintsurashvili, Ekaterina, Irena Duka, Irene Gavras, Conrado Johns, Dimitrios Farmakiotis, and Haralambos Gavras. Effects of ANG II on bradykinin receptor gene expression in cardiomyocytes and vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 281: H1778–H1783, 2001.—Bradykinin has vasodilatory and tissue-protective effects exerted via its B2 type receptor, whereas the B1 receptor is constitutively absent but inducible by inflammation and toxins. In previous studies, we found that B2 receptor gene knockout mice exhibit overexpression of the B1 receptor, which assumes a vasodilatory function and is further upgraded in renovascular hyperten-

sion. The present study was designed to explore the effects of excess angiotensin II (ANG II) on B1 receptor and B2 receptor gene expression in mouse cardiomyocytes and rat vascular smooth muscle cells (VSMC) in vivo (after a 3-day infusion of 30 ng/min ANG II in 11 wild-type and in 13 genetically engineered mice with deleted B2 receptor gene) and in vitro (ANG II added in rat VSMC culture in the presence or absence of AT1 or AT2 receptor antagonist). Expression of B1 and B2 receptor mRNA was assessed by reverse transcriptase-polymerase chain reaction. ANG II infusion caused up-regulation by 30% of the already significantly overexpressed B1 receptors in cardiomyocytes of the B2 receptor gene knock-out mice, but in the wild-type mice it upregulated only the B2 receptor mRNA by 47%. The addition of ANG II in VSMC culture produced a time-dependent induction of B1 and up-regulation of B2 receptor gene expression, maximal at 3 h (by fivefold), declining almost to baseline by 24 h. The addition of losartan completely blocked this effect, whereas the AT2 blocker PD-123319 made no difference, indicating that this is an AT1-mediated effect of ANG II. The data indicate that excess ANG II in supressor doses in vivo upregulates expression of the B2 receptor, but in its absence, the already overexpressed B1 receptor is further upregulated, evidently assuming a counterregulatory response; in vitro, it transiently upregulates both bradykinin receptors.

The Kallikrein-Kinin System contributes to many aspects of cardiovascular and metabolic homeostasis. Kinins, cleaved by kallikrein from the substrate kininogen, are vasodilatory peptides that act as local hormones causing release of endothelial mediators such as nitric oxide and prostaglandins (4, 29). They also participate in other biological processes, including insulin-dependent glucose transport, pain, tissue inflammation, and local regulation of tissue perfusion. Of particular interest to clinicians was the discovery that kininase II, which degrades kinins, is identical to the angiotensin-converting enzyme (ACE) (31), raising the possibility that the effects of ACE inhibition might be partly attributable to potentiation of kinins.

Kinins exert their actions via the G protein-coupled kinin receptors bradykinin (BK) B1 and B2 (2, 7, 23). The BK B2 receptors are constitutively expressed in a variety of tissues and account for most of the vascular and metabolic actions of BK (2, 7, 23), either acting directly or by interacting with prostaglandins and other autacoids (22). In contrast, the BK B1 receptors are not present in significant amounts in normal tissues, but their expression is inducible by cytokines and growth factors, bacterial lipopolysaccharides, or vascular injury (1, 6, 20, 24).

Experimental studies using specific BK B2 receptor antagonists have now established that BK contributes to the antihypertensive effect of ACE inhibitors (3), and has tissue-protective properties that enhance regional blood flow in vital organs (30) and vulnerable tissues (25), suppress neointimal proliferation after endothelial injury (10), decrease infarct size after ischemia-reperfusion (19), and improve left ventricular function in heart failure models (26). In a recent series of experiments using genetically engineered mice with deleted BK B2 receptor gene, we found that in the absence of the B2 receptor, the B1 receptor becomes upregulated and appears to take over some of the hemodynamic functions of the B2 receptor (9). Moreover, renal artery constriction used to produce hypertension was also found to upregulate the expression of both the B2 and the B1 receptor in normal mice and to further upregulate the already overexpressed B1 receptor in the B2 gene knockout mice (9).

Because acute renovascular hypertension is characterized by excessive stimulation of renin/angiotensin (ANG), we hypothesized that this effect on BK recep-

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tors may be attributable to ANG II, which is not only a potent vasoconstrictor, but also a powerful mitogen, causing growth and proliferation of vascular smooth muscle cells (VSMC) and cardiomyocytes (13, 15). The present study was designed to investigate whether the BK B1 and B2 receptors in VSMC and cardiomyocytes can be upregulated by ANG II, and whether hemodynamic changes contribute to the response. To ascertain that the results would not be species specific, we chose mice for the in vivo studies because they are suitable for genetic engineering, and rat cell cultures for the in vitro studies because they are readily available and widely used for such experiments. We evaluated changes in the expression of the BK receptors after exogenous infusion of ANG II in BK B2 gene knockouts and wild-type mice in vivo, and changes in expression of these receptors when ANG II was introduced in rat cell cultures in vitro to eliminate the influence of hemodynamic factors.

METHODS

Animals. BK B2 receptor gene knockout mice (BK B2 receptor –/–) and their wild-type controls (BK B2 receptor +/+ ) were used in this study. BK B2 receptor –/– mice were derived from a breeding pair of homozygous knockout mice generated by gene targeting and homologous recombination mice (15) that were provided to us by the Jackson Laboratories (Bar Harbor, ME) and were bred in our mouse facility. Wild-type B6129Sv mice were obtained from Jackson Laboratories and served as controls. The animals, 8–12 wk old and weighing 24–31 g, were housed in the animal quarters with a 12:12-h light-dark cycle and were provided food (Purina Rodent Chow 5002) and distilled water ad libitum. All experiments were conducted in accordance with the “Guidelines for the Care and Use of Animals” approved by the Boston University Medical Center.

Surgical procedure. Surgery was performed under anesthesia with intraperitoneal pentobarbital sodium (50 mg/kg). A modified polyethylene (PE-50) catheter was introduced in the right iliac artery for blood pressure (BP) recording, and Silastic tubing (PE-10) was placed in the right iliac vein for drug administration, as described elsewhere (17). Both lines were exteriorized at the back of the neck and sealed. After surgery, the animals were returned to their cages and allowed to recover.

Experimental protocol. After 3 days of recovery, the animals were housed in metabolic cages. The two lines were unsealed and attached to a swivel. The arterial line was connected to a BP transducer, and mean BP was recorded with the use of a computerized data-acquisition system (Power Lab/400, AD Instruments; Castle Hill, Australia). The venous line was connected to an infusion pump (Harvard Apparatus; Holliston, MA) for drug infusion. The baseline BP was continuously recorded until it became stable. At this point, infusion of ANG II (30 ng/min) was started and continued for 3 days. BP was continuously recorded for 8 h every day.

Tissue preparation. At the end of the experiment, the mice were euthanized with pentobarbital sodium. The heart was then excised, washed with saline, weighed, cleaned, and prepared for tissue receptor expression studies. Hearts from four mice were used for each tissue receptor study. Two additional groups of knockout and wild-type mice (n = 4 each) were used for baseline tissue receptor studies, i.e., without prior ANG II infusion.

Cell culture. VSMC were isolated from the rat thoracic aorta of 8-wk-old male Wistar rats by an explant method and cultured in Dulbecco's modified Eagle's medium/F-12 that was supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in humidified atmosphere of 95% O2–5% CO2. VSMC passages 8–15 were grown to 70–80% confluence, incubated for 24 h in serum-free medium, and then incubated with 0.1 µM ANG II for different times (3, 6, and 24 h). Cells were incubated with 0.1 µM ANG II for 3 h after a 30-min preincubation period with either 10 µM losartan or 10 µM PD-123319. For purposes of comparison, a similarly treated VSMC culture was incubated for 3 h with 1 µM norepinephrine.

Materials. Dulbecco's modified Eagle's medium/F-12, fetal calf serum, and the penicillin-streptomycin mixture were purchased from Life Technologies (Grand Island, NY). PD-123319 was purchased from Calbiochem (San Diego, CA). Losartan was generously provided by DuPont (Mississauga, Ontario, Canada). ANG II was purchased from Sigma (St. Louis, MO).

Preparation of RNA. Total RNA was prepared from rat VSMC and from heart tissues of BK B2 receptor knockout and wild-type mice at baseline and end points, with the use of TRIZol (GIBCO-BRL; Gaithersburg, MD). To increase the purity of the RNA samples, we added an additional step, a DNase digestion procedure to eliminate DNA with the use of a total RNA isolation kit (SNAP, Invitrogen; Carlsbad, CA).

Expression of BK receptor genes. The expression of BK B1 and B2 receptor genes in rat VSMC and in mouse heart tissues was examined by reverse transcriptase-polymerase chain reaction (RT-PCR) techniques. From each tissue, 1 µg of total RNA was converted to cDNA using a RNA PCR kit (Perkins-Elmer; Branchburg, NJ), and PCR was performed with oligonucleotide primers complementary to the mouse BK B1 and B2 receptor cDNA and 18S rRNA in the same tube. BK B1 receptor transcripts were amplified with primers 5’-TGCTCTCTCCCTTTGGTGCTG-3’ and 5’-ACGACCGAGGGAACGCAG-3’, producing a 391-base pair product. BK B2 receptor transcripts were amplified with the reverse primer 5’-CATCTCTGTGGTCCTCAGCA-3’ and forward primer 5’-ACGACGATCTGGCTCTCTGAT-3’, producing a 322-base pair fragment. For internal standard we chose 18S rRNA. The RT-PCR product from 18S rRNA-specific primers (Ambion; Austin, TX) produced a 488-base pair fragment. From rat VSMC, 1 µg of total RNA was converted to cDNA, and PCR was performed with oligonucleotide primers complementary to the rat BK B1 and B2 receptor cDNA and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) in the same tube. Rat BK B1 receptor transcripts were amplified with reverse primers 5’-CTCTTGAGCATCCCCACATT-3’ and forward primers 5’-CAGCTGTTGACAAAGGCAAT-3’, producing 400-base pair fragments. Rat BK B2 receptor primers are identical to those of mouse BK B2 receptor. We used G5PDH (Clonetech; Palo Alto, CA) as a standard for rat VSMC. To establish conditions that allow comparison of the amounts of cDNA produced by RT-PCR, we varied the number of cycles from 24 to 40; a cycle number of 30 was chosen to compare the different levels of expression of various mRNAs. PCR was performed with the use of the following conditions: 120 s at 95°C and annealing 60 s at 60°C, followed by 7 min at 72°C. Each of the PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The resulting gels were scanned with a Pdi scanner (model 420OE, Pdi; Huntington Station, NY) and analyzed with the use of NIH Image software.

Statistical analysis. All data are expressed as means ± SE. Two-way analysis of variance for repeated measures was
used to test for interaction between time and grouping factor. Differences within and between groups were determined with the use of paired and unpaired Student’s t-test, respectively. Tukey’s test was used for multiple comparisons. Differences at $P < 0.05$ were considered significant.

RESULTS

Hemodynamics. Eleven BK B2 receptor knockout mice and 13 wild-type controls were used in these experiments. At baseline, the BK B2 receptor knockout mice tended to have somewhat higher BP, but the differences did not attain statistical significance. Administration of 30 ng/min ANG II caused a transient increase in mean direct BP in both groups of mice during the first day of infusion, with the knockouts again showing a tendency to somewhat higher levels. However, this increase was not sustained, and by the end of the experiment the BP had returned to baseline (Fig. 1). No differences were found in body weight or heart and kidney weights between knockout animals and their wild-type controls.

Expression of BK B1 and B2 receptor mRNA in the heart. The data of BK B1 and B2 receptor mRNA expression in the heart of baseline and ANG II-infused mice are presented in Fig. 2. BK B1 receptors in the knockout mice were significantly upregulated relative to wild-type mice (Fig. 2A).

After the 3-day infusion of ANG II in the B2 receptor knockout mice, the B1 receptor mRNA expression in the heart was further increased by 30% compared with baseline. In contrast, in wild-type animals, ANG II did not change the level of BK B1 receptor expression, although it did increase significantly (by 47%) the level of expression of BK B2 receptor mRNA (Fig. 2B).

Effect of stimulation of VSMC with ANG II on BK B1 and B2 receptor mRNA expression. Figure 3 shows the BK B1 and B2 receptor expression in cultured rat VSMC stimulated by 0.1 μM ANG II. Figure 3A shows that BK B1 receptor expression was induced in ANG II-treated cells and reached maximum in 3 h, whereas untreated cells did not show BK B1 receptor expression. Under the same conditions, the BK B2 receptors in VSMC were significantly expressed and at 3 h reached maximum upregulation (by fivefold). After 6 h of treatment with ANG II, expression of both receptor types decreased by one-half and after 24 h to one-third.

Figure 1. Time course of mean blood pressure changes during infusion of 30 ng/min angiotensin II (ANG II) in bradykinin (BK) B2 receptor gene knockout mice (−/−, dashed line) and wild-type (+/+ , solid line) mice.

Fig. 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA expression for B1 (A) and B2 receptors (R) (B) in hearts of BK B2R+/+ and BK B2R−/− mice at baseline (B) and after a 3-day infusion of 30 ng/min ANG II. Top: representative RT-PCR. Bottom: mean densitometric data expressed as a ratio of 18S mRNA (n = 4 in each group). *$P < 0.05$, between baseline (B) and end point (3 days after ANG II infusion); $P < 0.05$, between knockout and wild-type animals at baseline.
fourth compared with 3 h of treatment. Thus there was a time-dependent change in BK B1 and B2 receptor mRNA.

In contrast, addition of 1 μM norepinephrine to the culture medium produced no change in BK B1 or B2 expression from baseline (data not shown).

BK B1 and B2 receptor regulation by ANG II. After a 3-h incubation of VSMC with 0.1 μM ANG II, there was induction of BK B1 and upregulation of BK B2 receptor mRNA. The addition of the specific AT1 receptor antagonist losartan (10 μM) completely blocked this effect (see Fig. 4), whereas addition of the AT2 receptor antagonist PD-123319 did not. However, PD-123319 and losartan given alone had no effect on BK B1 and B2 receptor mRNA expression. This indicates that ANG II exerts this effect through the AT1 receptor.

**DISCUSSION**

The vasodilatory and tissue-protective properties of BK are well established (3, 10, 19, 25, 26, 30) and are generally attributed to local humoral mediators (such as nitric oxide and prostaglandins) released after activation of the B2 receptor (4, 22, 29). The B1 receptor, as mentioned earlier, is normally absent, but its expression is readily inducible in a variety of cell types, including VSMC, endothelial cells, and fibroblasts (1, 6, 20, 24). Bacterial lipopolysaccharides, toxins, and inflammatory mediators have long been known to induce B1 receptor expression (1, 6, 20, 24), but recently we (9) found that deletion of the B2 receptor gene and experimental hypertensive maneuvers can also produce this effect.
In the present study, we demonstrate that in hearts of normal animals, the B2 receptor expression, but not the B1 receptor, is upregulated by exogenous ANG II infusion in vivo, even in the absence of hypertension. However, in the heart of B2 receptor gene knockout animals, the already overexpressed B1 receptor becomes further upregulated by the subpressor doses of ANG II, suggesting that in the absence of B2 receptor, the B1 receptor assumes this presumably counterregulatory response. It has been shown in the past that B1 receptor activation can elicit cellular responses involving release of arachidonic acid and prostaglandins (21), which might be beneficial to regional tissue perfusion, as well as to local trophic and metabolic function.

The in vitro addition of ANG II in rat VSMC culture also produced the induction of B1 receptor and upregulation of B2 receptor expression in these cells. This stimulation of VSMC by ANG II was transient and time dependent, which could be attributed to either decreasing response to the stimulus of ANG II or gradual degradation of ANG II concentrations. It could be completely inhibited by losartan, but not by PD-123319, indicating that this effect is exerted through specific AT1 receptor-mediated signal pathways. Obviously, there would be no hemodynamic stimulus to trigger this response. However, in addition to being a potent vasoconstrictor, ANG II is known to have a number of tissue-specific AT1 receptor-mediated actions, including mitogenic, oxidative, prothrombotic, and ion channel altering properties (11), all of which tend to produce damage in vulnerable tissues. In contrast, BK, via its B2 receptor that stimulates a number of downstream tissue autacoids, has generally the opposite effects, including antimitototropic/antiproliferative, antioxidant, antithrombotic, and antiarrhythmic effects (12, 23). It would therefore appear that the insult of excess ANG II triggers a counterregulatory response in the form of upgraded B1 and B2 receptor expression and function. It is believed that the local trophic and proliferative effects of AT1 receptor stimulation are exerted through autocrine-paracrine mediators, such as protooncogenes (16), transforming growth factor-β (27), endothelin-1 (14), and others (8), but there has been no evidence so far that AT1 receptors interact with the kinin system. In contrast, it has been suggested that the AT2 receptor may counteract actions of the AT1 receptor by interacting with BK (18, 28) and thus activating its downstream mediators. Yet blockade of the AT1 receptor, not the AT2 receptor, was found to alter the ANG II-induced overexpression of BK receptors in cultured VSMC. It is difficult at this time to reconcile these findings and their pathophysiological significance. The lack of effect of norepinephrine in vitro on the expression of BK receptors would not be unexpected because no direct interaction is known between catecholamines and kinins. It does, however, eliminate the possibility that the response to ANG II might have been a nonspecific reaction to any biological stimulus. Norepinephrine would have activated both β1- and β2-adrenergic receptors on VSMC membranes but this stimulus would not be expected to trigger changes in BK receptor expression.

It is possible that the upregulation of the B2 receptor (and, in its absence, of the B1 receptor) by ANG II, independently of hemodynamic influences, is teleologically meant to ensure better tissue protection. Because kinins are involved in a variety of cardiovascular diseases and inflammatory processes, the inducibility of the B2 receptor suggests that it might be considered as a potential target to therapeutic interventions in such pathological conditions (21).

In conclusion, our data demonstrate that subpressor doses of ANG II can induce and upregulate the expression of both the B1 and B2 receptors of BK, in vivo and in vitro, indicating that this is not a response to hemodynamic challenge. Because this response could be inhibited by losartan but not by an AT2 receptor antagonist, and because it could not be elicited by norepinephrine, indicate that it is a specific result of AT1 receptor activation, evidently involving selective release of local tissue mediators.

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