Angiotensin AT_2 Receptors Directly Stimulate Renal Nitric Oxide in Bradykinin B_2-Receptor–Null Mice

Peter M. Abadir, Robert M. Carey, Helmy M. Siragy

Abstract—Both bradykinin B_2 and angiotensin II type 2 (AT_2) receptors are known to stimulate renal production of nitric oxide (NO). To evaluate the individual contributions of AT_2 and B_2 receptors to renal NO production, we monitored renal interstitial, stable NO metabolites and cGMP by a microdialysis technique in conscious, bradykinin B_2–null and wild-type mice (n=8 in each group) during low sodium intake alone or with the angiotensin AT_1 or AT_2 receptor blockers, valsartan (0.5 μg/min) or PD123319 (0.15 μg/min), or both. During normal salt intake, renal interstitial fluid NO and cGMP levels in B_2-null mice were not different from those of wild-type mice. Low sodium intake increased NO and cGMP in wild-type mice but not in B_2-null mice. Valsartan increased NO and cGMP in both wild-type and B_2-null mice but to a significantly greater degree in the wild-type than in B_2-null mice. PD123319 decreased NO and cGMP in both wild-type and B_2-null mice, but there was no significant difference during combined treatment from their levels after administration of PD123319 alone. Our results indicate that during ingestion of a low-salt diet, production of NO is mediated mainly via the AT_2-B_2 receptor cascade. Blockade of the AT_1 receptor enhances the production of NO via the AT_2 receptor in both wild-type and B_2-null mice. We conclude that NO can be produced by 2 alternative pathways: directly through the AT_2 receptor or indirectly from AT_2 receptor stimulation of bradykinin via the B_2 receptor. (Hypertension. 2003;42:600-604.)

Key Words: nitric oxide ■ receptors, angiotensin II ■ animals, transgenic ■ receptors, bradykinin ■ angiotensin II ■ cyclic GMP

Previous studies demonstrated that angiotensin II type 1 (AT_1) receptor blockade increases renal nitric oxide (NO) and that this increase is abolished by angiotensin II type 2 (AT_2) receptor blockade, proving that the AT_2 receptor is responsible for the increase in NO.1,2 Similarly, activation of the AT_2 receptor results in an increase in renal cGMP.3 Because cGMP functions as a second messenger for NO, its increase in response to AT_2 receptor stimulation is thought to be mediated by NO.1 Furthermore, AT_2 receptor stimulation increases renal bradykinin (BK),4,5 which in turn increases NO production.6 Thus, previous studies demonstrated that the AT_2 receptor mediates a vasodilator cascade that includes BK, NO, and cGMP. These observations raise the question as to whether BK is an obligatory intermediate in the putative BK-NO-cGMP signaling cascade mediated by the AT_2 receptor.

BK, the major effector hormone of the kallikrein-kinin system, acts mainly through the BK B_2-subtype (B_2) receptor to mediate the majority of its cardiovascular and renal actions.7 Studies in mice that lack the B_2 receptor (B_2^{−/−}) have reported normal development, blood pressure, and renal function.8–10 It is not known how B_2^{−/−} mice produce NO to maintain their normal blood pressures.

In the present study, we investigated whether the AT_2 receptor can directly stimulate renal NO production independently of BK. Using a microdialysis technique in B_2^{−/−} and B_2^{+/+} mice, we monitored changes in the renal interstitial fluid (RIF) NO stable end products, nitrate and nitrite (NOX), and cGMP levels during low sodium intake alone and with AT_1 or AT_2 receptor blockade, individually or combined.

Methods

Animals
B_2^{−/−} mice (n=8) were derived from a breeding pair of homozygous mice on a 129Sv genetic background, as described elsewhere.11 Wild-type, B6SvF2129 mice (B_2^{+/+}), purchased from Jackson Laboratory (Bar Harbor, Me), served as controls (n=8). Animals received a normal-sodium (0.25%, BioServe Biotechnologies) or a low-sodium (0.03%) diet. All experiments were conducted in mice aged 12 to 16 weeks whose weight averaged 28 to 32 g. All studies were approved by the University of Virginia Animal Research Committee.

In Vivo Microdialysis Technique
For the determination of RIF cGMP and NOX, we constructed a microdialysis probe as previously described.2,3

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In Vitro Microdialysis

In vitro best recoveries for renal NOX and cGMP were observed with a perfusion rate of 3 μL/min and were 70% for both cGMP and NOX.1,3

Blood Pressure Measurements

Systolic blood pressure (SBP) was measured in the tail artery in B2−/− and B2+/+ mice under restraint by using an automated sphygmomanometer.

Animal Preparation

With mice under general anesthesia, the left kidney was exposed through a left flank incision. A microdialysis probe was inserted into the outer renal cortex, as previously described,4 ~1 mm deep from the outer renal surface. The inflow and outflow tubes of the dialysis probe were burrowed subcutaneously and were exited near the intrascapular region. The external portions of the tubes were placed in a stainless steel spring. While the mice were still under general anesthesia, an indwelling renal interstitial infusion catheter, constructed as described previously,5 was implanted into the left renal cortex through a small hole made with a 26-gauge needle. Mice were housed under controlled conditions. Experiments were initiated at the same time each day. For collection of RIF, the inflow tube was connected to a gas-tight syringe that was filled with lactated Ringer's solution and perfused at 3 μL/min.

Analytical Methods

RIF nitrate/nitrite (NOX) and cGMP levels in dialysate samples were measured with an enzyme immunoassay kit.1 The sensitivity was 2.0 μmol/L and 0.09 pmol/mL for NOX and cGMP,1 respectively, and the specificity was 100% for both.

Effects of Sodium Depletion and Angiotensin Receptor Blockade on RIF NOX and cGMP

RIF sample collections were performed 5 days after surgical insertion of the renal microdialysis probes. RIF samples were obtained for NOX and cGMP while the mice were consuming a normal-sodium diet. Mice were placed on a low-sodium diet for 10 days. On experimental days 6 to 10 while the animals were consuming the low-sodium diet, RIF samples were collected during renal cortical interstitial administration of 5% dextrose in water (D,W); the angiotensin AT1 receptor blocker valsartan (Novartis) at 0.5 μg/min; and the AT1 receptor blocker PD123319 (Parke-Davis) at 0.15 μg/min, individually or combined. Renal cortical interstitial infusion of each treatment was given at 3 μL/min for 4 hours on different experimental days.

Statistical Analysis of Data

Data are expressed as mean±SEM. Differences between mean values of multiple or 2 groups were analyzed by ANOVA, with subsequent Tukey honestly significant difference multiple-comparisons test. Differences of P<0.05 were considered significant.

Results

Blood Pressures in Response to Low Salt Intake, PD123319, and Valsartan, Individually or Combined

Baseline SBP was not different in the B2−/− and B2+/+ mice. The low-salt diet did not significantly alter SBP in either the B2−/− or B2+/+ mice. Heart rate was not significantly different between the normal-salt or low-salt diet in either B2−/− or B2+/+ mice. SBP was not significantly altered in either the B2−/− or B2+/+ mice in response to PD123319 or valsartan, individually or combined, during low salt intake (Table).

RIF NOX Response to Low Sodium Intake, PD123319, or Valsartan, Individually or Combined

During normal salt intake, there were no significant differences in RIF NOX levels between B2−/− (4.0±0.5 μmol/L) and B2+/+ (4.6±0.6 μmol/L) mice. The low salt intake increased RIF NOX levels in B2−/− mice to 7.0±0.3 μmol/L (P<0.05). However, low salt intake did not increase RIF NOX levels in B2+/+ mice (4.7±0.2 μmol/L; Figure 1).

AT1 receptor blockade with valsartan increased RIF NOX in B2−/− mice to 11.9±1.4 μmol/L (P<0.0001) and in B2+/+ mice to 8.34±0.5 μmol/L (P<0.0001), compared with RIF NOX levels during low salt intake. The increase in RIF NOX levels was more pronounced in B2−/− than in B2+/+ (P<0.01) mice in response to valsartan treatment. In contrast, AT1 receptor blockade with PD123319 caused a significant reduction in the RIF NOX level in the B2−/− mice, to 3.3±0.3 μmol/L (P<0.01), and in B2+/+ mice to 2.0±0.16 μmol/L (P<0.0001). Combined infusion of PD123319 and valsartan

Statistical Analysis of Data

Data are expressed as mean±SEM. Differences between mean values of multiple or 2 groups were analyzed by ANOVA, with subsequent Tukey honestly significant difference multiple-comparisons test. Differences of P<0.05 were considered significant.

Table 1. Vital Signs of Experimental Mice during Low-Salt Intake, PD123319, and Valsartan, Individually or Combined

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SBP, mm Hg</th>
<th>HR, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>111.3±0.8</td>
<td>523.1±6.9</td>
</tr>
<tr>
<td>PD123319</td>
<td>110.3±1.2</td>
<td>512.4±8.3</td>
</tr>
<tr>
<td>Valsartan</td>
<td>110.6±0.8</td>
<td>522.3±13.5</td>
</tr>
<tr>
<td>PD123319</td>
<td>112.1±1.0</td>
<td>523.5±3.66</td>
</tr>
<tr>
<td>PD123319</td>
<td>112.4±1.0</td>
<td>514.9±10.9</td>
</tr>
</tbody>
</table>

Figure 1. RIF NOX in conscious B2−/− null mice (B2−/−; n=8; open bars) and wild-type mice (B2+/−; n=8; solid bars) in response to low sodium intake (LS), valsartan (Val), and PD123319 (PD), individually or combined. **P<0.05 vs normal salt; ***P<0.01,
**P<0.0001, ***P<0.0001 vs LS; #P<0.05 vs B2+/− under the same treatment.
significantly decreased RIF NOX levels in both B2+/+ and B2−/− mice, to 2.3±0.2 μmol/L (P<0.0001) and 2.1±0.16 μmol/L (P<0.0001), respectively. There were no significant differences between RIF NOX levels during administration of PD123319 alone or during administration of combined PD123319 and valsartan.

**RIF cGMP Responses to Low Sodium Intake, PD123319, and Valsartan, Individually or Combined**

RIF cGMP levels increased in response to low sodium intake, from 0.31±0.07 to 0.81±0.1 pmol/mL, in B2+/+ mice (P<0.01) but did not change significantly in B2−/− mice (Figure 2). RIF cGMP values increased by >3-fold in response to administration of valsartan in the B2+/+ mice, to 2.64±0.6 pmol/mL (P<0.01), and in B2−/− mice, from 0.28±0.06 to 0.85±0.1 pmol/mL (P<0.0001). AT1 receptor blockade with valsartan caused a larger increase in RIF cGMP in B2+/+ than in B2−/− mice.

In contrast, AT2 receptor blockade with PD123319 significantly decreased RIF cGMP values in B2+/+ mice to 0.24±0.08 pmol/mL (P<0.001) and in B2−/− mice to 0.16±0.04 pmol/mL (P<0.0001). Combined infusion of PD12331 and valsartan significantly decreased RIF cGMP in both B2+/+ and B2−/− mice, to 0.22±0.05 (P<0.05) and 0.16±0.03 pmol/mL (P<0.0001), respectively. There were no significant differences between RIF cGMP levels during administration of PD alone or during combined PD123319 and valsartan treatment.

**Contribution of AT1, AT2, and B2 Receptors, Individually or Combined, to Renal NOX During Low Sodium Intake**

The contributions of AT1, AT2, and B2 receptors to renal levels of NO measured as the percent change from the condition in which AT1, AT2, and B2 receptors were simultaneously active in B2+/+ mice under low-salt conditions are shown in Figure 3. Data were calculated as the percentage of increase or decrease in the level of NOX for each mouse under each condition or treatment from the NOX level in wild-type mice during low salt intake. Data were averaged for each treatment to reflect the effect of blockade of each specific receptor on the renal production of NOX. AT1 receptor blockade caused a 70.3% increase in the level of NOX. Whereas solitary blockade of the AT2 receptor caused a 53% decrease in the level of NOX, the combination of AT1 and AT2 blockade caused a greater decrease, leading to a 66% decrease in the level of NOX. In contrast to blockade of the B2 receptor (ie, in B2−/− mice), which decreased NOX by 31.6%, combined blockade of AT1 and B2 receptors increased NOX by 18%. The combination of AT2 and B2 receptor blockade (PD123319 in B2−/− mice) decreased NOX production by 71%, which did not change on addition of AT1 receptor blockade to the combination of AT2 and B2 receptor blockade (69%).

**Discussion**

This study demonstrates renal production of NO by the angiotensin AT1 receptor in the absence of the BK B2 receptor. The study also elucidates the relative roles of AT1, AT2, and B2 receptors in NO production.

Previous studies1-5 demonstrated that angiotensin II, via the AT1 receptor, mediates renal production of BK, NO, and cGMP. Gohlke et al13 have shown that angiotensin II stimu-
lates aortic cGMP in stroke-prone hypertensive rats by stimulating BK via the AT\textsubscript{2} receptor. Tsutsumi et al\textsuperscript{14} confirmed these results by indicating that the AT\textsubscript{2} receptor stimulates the BK-NO-cGMP cascade. However, it was unclear whether the B\textsubscript{2} receptor is necessary for AT\textsubscript{2} receptor-mediated production of NO or whether the AT\textsubscript{2} receptor can stimulate NO production directly.

In the present study, low salt intake, a stimulus for both the renin-angiotensin system and the kallikrein-kinin system,\textsuperscript{15} failed to increase NO production in the absence of the B\textsubscript{2} receptor. These data demonstrate the importance of B\textsubscript{2} receptor interaction with angiotensin receptors\textsuperscript{16} during this physiologic maneuver. The interaction between the AT\textsubscript{1} and AT\textsubscript{2} receptors\textsuperscript{16} or the formation of a stable AT\textsubscript{1}-B\textsubscript{2} receptor heterodimer\textsuperscript{17} might necessitate the presence of the B\textsubscript{2} receptor to maintain the level of NO production in response to sodium restriction. During AT\textsubscript{2} receptor blockade, angiotensin synthesis is increased because of inhibition of the short, negative-feedback loop on renin secretion, which allows for further stimulation of the AT\textsubscript{2} receptor.\textsuperscript{18}

In the current study, in the absence of the B\textsubscript{2} receptor, direct production of NO via the AT\textsubscript{2} receptor was exaggerated on blockade of the AT\textsubscript{2} receptor, providing evidence that NO can be produced directly via the AT\textsubscript{2} receptor. The contribution of AT\textsubscript{2} receptors to the production of NO under dietary sodium restriction became evident when the AT\textsubscript{2} receptor was blocked, leading to a significant reduction in renal tissue NO. The reductions in NO and cGMP during AT\textsubscript{2} receptor blockade suggest that in the presence of low salt intake, the majority of NO and cGMP production is mediated via an AT\textsubscript{2} receptor pathway.

One possibility to explain these findings is that NOX production might theoretically be stimulated by the B\textsubscript{1} receptor in B\textsubscript{2}\textsuperscript{-/-} mice. Metabolites of the kinin system might stimulate NO and cGMP production via the B\textsubscript{1} receptor.\textsuperscript{19} However, the B\textsubscript{1} receptor either is not expressed or is expressed only at very low levels in tissues under normal conditions, although it is induced under pathologic conditions. The B\textsubscript{1} receptor is induced by cytokines such as interleukin-1\textbeta, bacterial lipopolysaccharides, or vascular injury.\textsuperscript{20–22} In addition, BK though potent, is short-lived and is degraded quickly in the system.\textsuperscript{23}

Using a potent and specific BK B\textsubscript{2} receptor antagonist, icatibant,\textsuperscript{24} to evaluate the role of intrarenal BK in the regulation of renal NO under conditions of a low-salt diet, we have shown that blocking the B\textsubscript{2} receptor increases BK and decreases NO and cGMP. This dissociation elucidates that the B\textsubscript{2} receptor does influence the production of NO\textsuperscript{24–26} in normal kidney. The combination of AT\textsubscript{1} and AT\textsubscript{2} receptor blockade decreased NO and cGMP. However, the levels of NO and cGMP were not significantly different from their levels during AT\textsubscript{2} receptor blockade alone, suggesting that the AT\textsubscript{1} receptor does not influence the production of NO and cGMP in the absence of AT\textsubscript{1} and B\textsubscript{2} receptors.

Our data suggest that when AT\textsubscript{1} and B\textsubscript{2} receptors were blocked and inactive, respectively, there was an 18% increase in renal NOX. The role of the AT\textsubscript{2} receptor in this increase became evident when we added AT\textsubscript{2} receptor blockade, leading to an \textasciitilde 88% decrease in the production of NO compared with that when only AT\textsubscript{1} and B\textsubscript{2} receptors were blocked or inactive. This finding suggests that NO is produced directly via the AT\textsubscript{2} receptor. This observation is consistent with our previous study,\textsuperscript{2} which showed that combined administration of valsartan and PD123319 caused a greater decrease in renal tissue levels of NO and cGMP than did combined treatment with valsartan and icatibant. These results suggest that the AT\textsubscript{2} receptor can directly stimulate NO in addition to its effects through the kinin system.

When the AT\textsubscript{2} receptor was blocked, we observed a decrease in NOX production by 53.4%, and this decrease was magnified by additional blockade of the AT\textsubscript{1} receptor (66%). This observation is consistent with previous reports\textsuperscript{17,25} that have demonstrated the interaction between AT\textsubscript{1} and B\textsubscript{2} receptors as an example of signal enhancement triggered by heterodimerization of 2 different, vasoactive hormone receptors. The blockade of B\textsubscript{2} receptors led to a 31% decrease in the level of NOX. This decrease was reversed, and the level of NOX increased by 50% with the addition of AT\textsubscript{2} blockers. These results suggest tonic inhibitory effects of AT\textsubscript{1} on the AT\textsubscript{2} receptor.

The maximum increase in NO production was observed on blocking the AT\textsubscript{1} receptor, leading to a 70% increase in the level of NO, and this result suggests potentiation between AT\textsubscript{2} and B\textsubscript{2}. Lack of this potentiation might explain the diminished cardioprotective response to inhibition of the AT\textsubscript{1} receptor in B\textsubscript{2}\textsuperscript{-/-} mice.\textsuperscript{26} The present study indicates that the AT\textsubscript{2} receptor is capable of stimulating NO production by 2 alternative pathways: through the BK B\textsubscript{2} receptor and by direct stimulation of NO and cGMP. These findings suggest a potential role for the AT\textsubscript{2} receptor in the pathophysiology and management of cardiovascular diseases.

Perspectives

The primary findings of this study are as follows: (1) During ingestion of a low-salt diet, production of NO is mediated mainly through the AT\textsubscript{2}-B\textsubscript{2} receptor cascade. (2) Blockade of the AT\textsubscript{1} receptor enhances the production of NO through the AT\textsubscript{2} receptor in both wild-type and B\textsubscript{2}-null mice. Our study demonstrates that NO can be produced directly through AT\textsubscript{2} receptor stimulation. Understanding the mechanism of interaction among different receptors in the production of NO might lead to new insights into the pathophysiologic and treatment of various cardiovascular disorders, such as hypertension and congestive heart failure. Human studies are required for a better understanding of the interrelations among AT\textsubscript{1}, AT\textsubscript{2}, and B\textsubscript{2} receptors in patients with cardiovascular disorders.

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References


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