British Journal of Pharmacology (2002) 136, 484–491
www.nature.com/bjp

AT_2 receptor-dependent vasodilation is mediated by activation of vascular kinin generation under flow conditions

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1 Physiological roles of angiotensin II type 2 receptor (AT_2) are not well defined. This study was designed to investigate the mechanisms of AT_2-dependent vascular relaxation by studying vasodilation in pressurized and perfused rat mesenteric arterial segments.

2 Perfusion of angiotensin II in the presence of AT_1 antagonist elicited vascular relaxation, which was completely dependent on AT_2 receptors on endothelium. FR173657 (>1 μM), a bradykinin (BK) B_2-specific antagonist, significantly suppressed AT_2-dependent vasodilation (maximum inhibition: 68.5% at 10 μM).

3 Kininogen-deficient Brown Norway Katholiek rats showed a significant reduction in AT_2-mediated vasodilatory response compared with normal wild-type Brown Norway rats.

4 Indomethacin (>1 μM), aprotinin (10 μM) and soybean trypsin inhibitor (10 μM) also reduced AT_2-dependent vasodilation.

5 Our results demonstrated that stimulation of AT_2 receptors caused a significant vasodilation through local production of BK in resistant arteries of rat mesentery in a flow-dependent manner. Such vasodilation counterbalances AT_1-dependent vasoconstriction to regulate the vascular tone.
British Journal of Pharmacology (2002) **136,** 484–491

Keywords: Angiotensin II; bradykinin; kallikrein; mesenteric artery; B_2 receptor

Abbreviations: Ang II, angiotensin II; AT_1, angiotensin II type 1 receptor; AT_2 angiotensin II type 2 receptor; B_2, bradykinin B_2 receptor; BK, bradykinin; BN-Ka, Brown Norway Katholiek; BN-Ki, Brown Norway Kitasato; EDHF, endothelium-derived hyperpolarizing factor; EIA, enzyme immunoassay; FR173657, (E)-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2-4-dichloro-3-[(2-methyl-8-quinolinyl)oxymethyl]phenyl]-*N*-methylaminocarbonyl -methyl]acrylamide); NE, norepinephrine; NO, nitric oxide; SBTI, soybean trypsin inhibitor

Introduction

Angiotensin II (Ang II), a vasoactive peptide with pleiotropic roles, is implicated in the regulation of cardiovascular and renal systems (Stroth & Unger, 1999). At present, at least two Ang II receptors, AT_1 and AT_2, have been identified (Inagami *et al*., 1994; Matsubara & Inada, 1998). Most of the Ang II-induced vasoconstrictive actions are mediated by AT_1 subtype, while little is known about the physiological roles of AT_2. Although both types of receptors are expressed in blood vessels, their distribution is somewhat different. AT_2 is expressed relatively weakly in large vessels such as the aorta while a high level expression is often observed in resistant microvessels (Viswanathan *et al*., 1991; Song *et al*., 1995; Nora *et al*., 1998). Recent studies using knockout mice have shown that disruption of the AT_2 gene is associated with hypertension and enhanced pressor action of Ang II (Ichiki *et al*., 1995; Hein *et al*., 1995), suggesting that AT_2 plays a significant role in the regulation of blood pressure, although the functional role of AT_2 is not well defined.

Recent studies have investigated the role of nitric oxide (NO) and bradykinin (BK) in AT_2-mediated vasodilatory response to Ang II in various vessels including the aorta (Gohlke *et al*., 1998), canine coronary microvessels (Seyedi *et al*., 1995) and pig heart (Jalowy *et al*., 1998). These studies suggest that the AT_2 receptor is coupled to the kinin-NO-cyclic GMP pathway. Several reports, however, have concluded that AT_1 receptor also mediates the generation of NO (Thorup *et al*., 1999). Thus, regulation of the vascular tone by Ang II and signalling pathways is complex and may vary with species, blood vessel diameters and anatomic origin of blood vessels used in these studies. On the other hand, in small resistant arteries, mechanical stimuli such as tensile stress and shear stress are important factors implicated in vascular responses. Pressure (tensile stress) influences myogenic tone, while flow (shear stress) induces vasodilation (Kuo *et al*., 1991; Bevan & Laher, 1991; Koller & Huang, 1994; D'Angelo & Meininger, 1994). In particular, shear stress induces the release of endothelium-derived vasoactive agents (Koller & Huang, 1994; Koller *et al*., 1993; Friebel *et al*., 1995; Matrougui *et al*., 1997). The AT_2 receptor is also involved in flow-induced dilation of resistant arteries in rats (Matrougui *et al*., 1999).

In the present study, we investigated the underlying mechanisms of AT_2 receptor-mediated dilation in rat resistant mesenteric arteries in the presence of continuous intraluminal flow. The aim of the present study was to evaluate the contribution of locally produced bradykinin to the vasodilatory effects of Ang II under pressure and flow conditions.

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Methods

Animals

Male SD rats (Japan SLC, Hamamatsu, Japan) weighing 250–300 g were used for the experiments. Brown Norway rats weighing 350–450 g were used in experiments designed to examine the significance of kininogens in angiotensin-dependent vasodilatory response. As previously described, Brown Norway Katholiek (BN-Ka) rats are genetically kininogen-deficient and Brown Norway Kitasato (BN-Ki) rats are their wild-type strain (Oh-Ishi *et al*., 1982). We confirmed that the plasma levels of high molecular weight kininogen and low molecular weight kininogen in BN-Ki rats used for the present study were 15.4 ± 0.5 ng BK equivalent mg^{-1} plasma protein ($n=6$) and 9.5 ± 0.3 ng BK equivalent mg^{-1} plasma protein ($n=6$), respectively. Both of those in BN-Ka rats were less than 0.5 ng BK equivalent mg^{-1} plasma protein ($n=6$). BN-Ka rats were originally obtained from Katholiek Universiteit and kept at Kitasato University School of Medicine. The rats were housed in an air-conditioned room with a 12-h light/12-h dark cycle. They received standard laboratory rat chow and tap water. All experimental procedures were approved by the Animal Care Committee of Kitasato University of Medicine and conformed with the international guidelines.

Materials

Angiotensin II (Ang II), norepinephrine (NE), indomethacin, HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethane-sulfonic acid), PD123319 and tetraethylammonium (TEA) were purchased from Sigma (St. Louis, MO, U.S.A.). Bradykinin (BK) was purchased from Peptide Institute, Inc. (Osaka, Japan). Soybean trypsin inhibitor (Worthington Biochemical Co., Cleveland, OH, U.S.A.) and aprotinin (Wako Pure Chemical Industries, Osaka, Japan) were also used. FR173657, a specific antagonist for BK B_2 receptor (Asano *et al*., 1997), was a kind gift from Fujisawa Pharmaceutical (Osaka, Japan). An enzyme immunoassay (EIA) kit for BK determination (cat. no. MA001) was purchased from Dainippon Pharmaceuticals (Osaka, Japan). A B_1 receptor antagonist, desArg^9-D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8] bradykinin (desArg^{10}-[Hoe140]), was obtained from Peninsula Laboratories (San Carlos, CA, U.S.A.).

Vessel preparation

The rats were sacrificed by decapitation while under light ether anaesthesia. A portion of the small intestine was excised and pinned on a rubber sheet placed in a Petri dish filled with cold HEPES-Tyrode solution (mM): NaCl 141.8, KCl 4.7, $MgSO_4$ 1.7, EDTA 0.4, $CaCl_2$ 2.8, KH_2PO_4 1.2, HEPES 10 and glucose 5, pH 7.4. Under stereoscopic microscope, third-order arterial branches arising from the superior mesenteric artery were dissected free of surrounding fat and connective tissue. The internal diameter of the arterial segments at the resting state was approximately 100–150 μm. The arterial segment was cannulated at both ends and mounted in a video monitored perfusion system (Living System Instrumentation Inc., Burlington, VT, U.S.A.) (Halpern *et al*., 1984). The artery was bathed in a 5-ml organ bath containing HEPES-Tyrode solution at 37°C and superfused at a rate of 4 ml min^{-1}. The artery was also perfused with the same physiological solution at an approximate rate of 50 μl min^{-1}. The pressure in the proximal end of the artery segment was monitored by a pressure transducer and controlled by a servofusion system. Arterial lumen diameter was measured by use of a video electronic dimension analyser. During the constant perfusion (50 μl min^{-1}), the lumen diameters ranged from 108 to 196 μm and averaged 154.6 ± 21.5 μm (mean ± s.d.). In some experiments, the endothelium was removed by passing air bubbles through the lumen of the arterial segments for 10 min. We confirmed that endothelium-denuded vessels responded normally to NE.

Experimental protocol

After dissection and cannulation, the arterial segments were pressurized to 50 mmHg and a constant flow rate (50 μl min^{-1}) was established by changing the inflow and outflow pressures while maintaining intravascular pressure at a constant level. After perfusion for 30 min, 0.5 μM norepinephrine solution was perfused for 10 min to induce vasoconstriction. The arterial segments that failed to respond to norepinephrine were not used in the following experiments. The contraction state was stable after termination of NE perfusion over at least 20 min. After perfusion with HEPES-Tyrode solution for washout of NE, arterial segments were stimulated with 1 nM Ang II. The internal diameter of the arterial segments at the end of perfusion with norepinephrine was defined as the initial diameter, which ranged from 48 to 113 μm and averaged 92.1 ± 19.7 μm (mean ± s.d.). The steady-state diameter after stimulation with Ang II was measured and expressed as per cent relaxation of the initial diameter. Perfusion of inhibitors or antagonists commenced 10 min before application of Ang II and continued during perfusion of Ang II. Perfusion of inhibitors or antagonists (SBTI, aprotinin and FR173657) did not affect vessel diameter. The arterial segments were not reused after stimulation with Ang II except in experiments where effect of repetitive applications of Ang II was examined. For repetitive applications, Ang II was washed out by perfusion of HEPES-Tyrode solution for 30 min. All agents including NE, Ang II, inhibitors, and antagonists, such as FR173657, were administered by intraluminal perfusion.

To check responsiveness of the arterial segments used here, we examined the effects of several vasoactive agents on the internal diameter of the arterial segments. Bradykinin (10^{-7} M) and acetylcholine (10^{-7} M) induced vasodilation of arterial segments pretreated with NE, thus increasing the diameter by 152.3 ± 8.2% (140.3 ± 7.6 μm, mean ± s.d., $n=6$) and 159.6 ± 6.5% (147.0 ± 6.0 μm, mean ± s.d., $n=4$), respectively, relative to the initial diameter. The BK- and acetylcholine-induced vasodilation completely disappeared in segments with denuded endothelium. Instead, treatment with BK (10^{-7} M) and acetylcholine (10^{-7} M) induced contraction of the denuded preparations, and thus reduced the internal diameter to 65.4 ± 4.4 and 55.7 ± 6.7% (mean ± s.d., $n=4$), respectively, relative to the initial value. In vessels with or without endothelium, NE (0.5 μM) caused vasoconstriction; the internal diameter decreased from 154.6 ± 21.5 to 92.1 ± 19.7 μm in intact vessels and from 138.3 ± 16.2 to 79.3 ± 6.8 μm in denuded vessels.

The perfusate was collected over 2 min (2–4 min after the commencement of the Ang II perfusion) and stored at −80°C. Perfusate samples were used for determination of BK concentrations by EIA, which is highly specific for BK and does not react non-specifically with kallidin, kininogens and angiotensins (as reported by the supplier). The detection limit of the assay is about 0.1 pg ml^{-1}.

Statistical analysis

Results were expressed as mean ± s.e.mean. The Student's *t*-test was used to analyse paired data while one-way analysis of variance (ANOVA) and Dunnett's *post hoc* test or Bonferroni's test were used for analysis of more than two groups. *P* values less than 0.05 were considered significant.

Results

Responses to Ang II

Perfusion of preconstricted arterial segments with 1 nM Ang II solution resulted in further reduction in the vascular diameter (Figure 1). The latency period was very short; the response was induced rapidly after application of the perfusate, and immediately reached a plateau level. In contrast, in the presence of 1 μM losartan, a specific antagonist of AT_1 receptor, 1 nM Ang II induced a vasodilatory response (Figure 1). This relaxation was relatively slow compared with the contractile response to Ang II and the maximal response was obtained a few minutes after commencement of Ang II perfusion. As shown in Figure 2, in intact arterial segments, the relative internal diameter of the arterial segments in response to 1 nM Ang II in the presence and absence of losartan were 43.2 ± 5.1 and 160.8 ± 6.8% of the initial diameter, respectively. In the presence of PD123319, a selective antagonist of AT_2 receptor, the contractile response to Ang II was augmented ($P < 0.05$ vs vehicle control). By contrast, blockade of AT_2 receptors with PD123319 significantly attenuated the vasodilative response induced by Ang II in the presence of losartan. In endothelium-denuded arterioles (−EC in Figure 2), vasodilation caused by Ang II was nearly completely abolished whereas the vasoconstrictive action observed in the absence of losartan was significantly increased ($P < 0.05$).

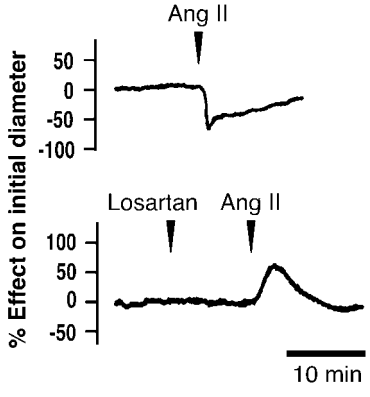

Figure 1 Representative tracings showing Ang II-induced responses in mesenteric arterial segments with or without blockade of AT_1 receptor. The arterial segments, preconstricted by NE, were perfused with 1 nM Ang II solution in the absence (upper panel) or presence (lower panel) of 1 μM losartan. The abscissa represents the internal diameter expressed as per cent of the preconditioning initial diameter.

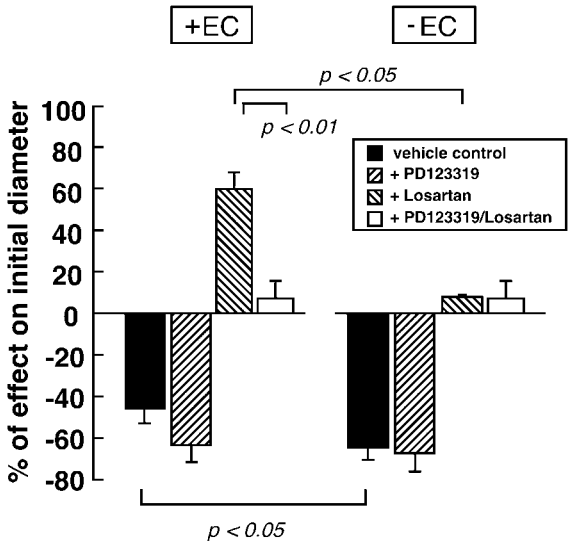

Figure 2 Response of arterial segments to intraluminal perfusion of Ang II. Arterial segments, preconstricted by norepinephrine, were perfused with 1 nM Ang II solution in the absence or presence of 1 μM losartan and/or 1 μM PD123319. The internal diameter of arterial segments at the end of perfusion with norepinephrine was defined as the initial diameter and the steady-state diameter after stimulation with Ang II was expressed as per cent vasodilation of the preconstricted initial diameter. Negative values represent the vasoconstriction response. +EC represents intact arterial segments. −EC represents arterial segments with denuded endothelium. Results are expressed as mean ± s.e.mean of five experiments.

Bradykinin concentrations in the perfusate of each group were also determined. In the absence of Ang II, perfusate BK levels were below the detection limit. In vehicle control group (with Ang II treatment in the absence of AT_1/AT_2 inhibitors), BK concentration in the perfusate was 5.6 ± 0.45 pg ml^{-1} ($n = 5$). Blockade of AT1 receptor with losartan did not affect BK concentration (5.2 ± 0.61 pg ml^{-1}, $n = 5$), while it decreased in the presence of PD123319 to 0.17 ± 0.12 pg ml^{-1} ($n = 5$). In endothelium-denuded vessels, only small amounts of BK were detected in the perfusate (0.11 ± 0.08 and 0.08 ± 0.13 pg ml^{-1} for vehicle and losartan-treated vessels, respectively).

Effects of BK B_2 receptor blockade on Ang II-induced vasodilation

It has been reported that bradykinin, a potent vasodilator, is generated after stimulation of AT_2 in coronary arteries or myocardium. Therefore, we examined the role of in Ang II-induced vasodilation in mesenteric arterial segments by using FR 173657, a selective antagonist of B_2 receptor. As shown in Figure 3A, Ang II-induced dilation of the arterial segments was significantly abolished by co-perfusion of FR 173657 (0.1–10 μM). FR 173657-induced inhibition of the vasodilatory effect of Ang II was dose-dependent, but a complete inhibition could not be observed at the highest dose examined (per cent inhibition was 68.5 ± 6.3% at 10 μM). A B_1 receptor

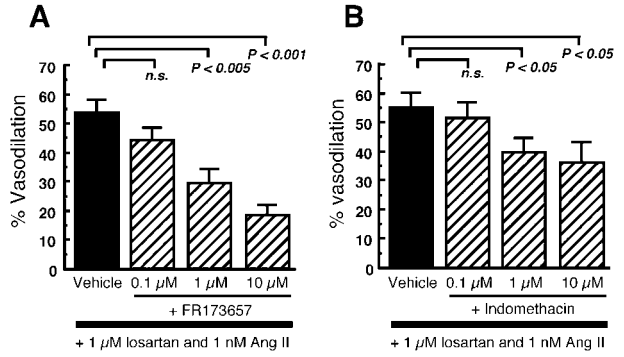

Figure 3 Effects of FR173657 or indomethacin on Ang II-induced vasodilatory response in rat mesentery arterial segments. (A) Arterial segments, preconstricted by norepinephrine, were perfused with 1 nM Ang II in the presence of 1 μM losartan and FR173657, a specific BK B2 receptor antagonist, at various concentrations. (B) Arterial segments, preconstricted by norepinephrine, were perfused with 1 nM Ang II in the presence of 1 μM losartan and indomethacin, an inhibitor of cyclo-oxygenases, at various concentrations. Results are expressed as mean$\pm$s.e.mean of five experiments.

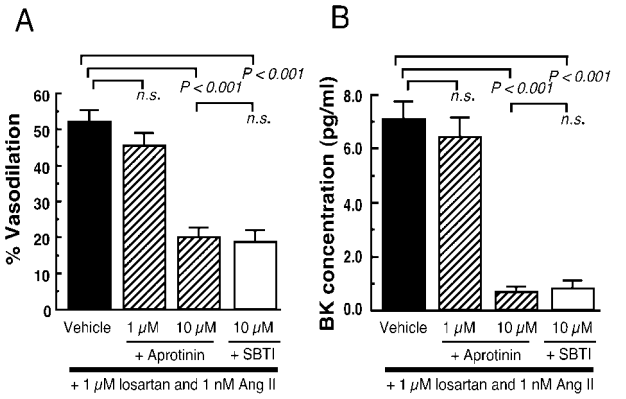

Figure 4 Effects of serine protease inhibitors on Ang II-induced vasodilatory responses and BK production in rat mesenteric arterial segments. Arterial segments, preconstricted by NE, were perfused with 1 nM Ang II in the presence of 1 μM losartan and serine protease inhibitors, aprotinin or SBTI. Effect on per cent vasodilation (A) and BK concentrations (B) in the perfusates were determined. Results are expressed as mean$\pm$s.e.mean of five experiments.

antagonist, desArg^{10}–Hoe140, did not affect the vasodilative response at 10 μM (data not shown). Bradykinin concentrations in the perfusates were not altered by treatment with FR173657 (5.9$\pm$0.54 pg ml^{-1} in vehicle control, 5.5$\pm$0.41 pg ml^{-1} at 0.1 μM, 5.6$\pm$0.63 pg ml^{-1} at 1 μM and 5.9$\pm$0.62 pg ml^{-1} at 10 μM; mean$\pm$s.e.mean, $P>0.05$).

Effects of indomethacin on Ang II-induced vasodilation

Results of the first series of experiments indicated that BK mediated Ang II-induced arterial relaxation but also suggested that BK was not the sole mediator. To elucidate the involvement of prostanoids such as prostacyclin in Ang II-induced vasodilation, we examined the effects of indomethacin, an inhibitor of cyclo-oxygenases. In arterial segments pretreated by perfusion of various concentrations of indomethacin solution (0.1–10 μM) for 10 min, dilation in response to Ang II was reduced in a dose-dependent manner and the maximal inhibition (per cent inhibition: 35.8$\pm$4.8%) was obtained at 10 μM (Figure 3B). The BK concentrations in the perfusates were not affected by treatment with indomethacin (5.5$\pm$0.36 pg ml^{-1} in vehicle control, 5.4$\pm$0.63 pg ml^{-1} at 0.1 μM, 5.4$\pm$0.54 pg ml^{-1} at 1 μM and 5.6$\pm$0.45 pg ml^{-1} at 10 μM; mean$\pm$s.e.mean, $P>0.05$). The combined application of FR173657 (10 μM) and indomethacin (10 μM) almost completely eliminated Ang II-induced dilation (per cent inhibition: 96.9$\pm$3.4%).

Preincubation of arterial segments with kallikrein inhibitors

In the next series of experiments, arterial segments were perfused with 1 or 10 μM aprotinin for 10 min to block the action of kallikreins, then stimulated with Ang II in the presence of Iosartan and aprotinin. As shown in Figure 4A, treatment with aprotinin resulted in a significant reduction in Ang II vasodilatory reaction, particularly at 10 μM, which resulted in per cent inhibition of 49.5$\pm$4.8%. Treatment with 10 μM soybean trypsin inhibitor also reduced Ang II-induced vasodilation (61.7$\pm$5.2%). Ten μM aprotinin and 10 μM SBTI resulted in a similar inhibition (64.1$\pm$6.4%, $P=0.75$, Figure 4A). Treatment with aprotinin and SBTI resulted in the reduction of the BK concentrations in the perfusates (Figure 4B). The reduction of the BK concentrations was almost parallel with the inhibition of the vasodilative responses.

Repetitive stimulation of BK-mediated Ang II-induced vasodilation

To examine the reproducibility of BK-mediated Ang II-induced vasodilatory response, arterial segments were repetitively stimulated with 1 nM Ang II in the presence of losartan and indomethacin. As shown in Figure 5, the response to the same concentration of Ang II diminished as the number of applications increased. Bradykinin concentrations in the perfusate during the application of Ang II were determined by EIA. Bradykinin concentrations in the perfusate also significantly decreased with the number of applications.

Effects of flow rates on BK-mediated Ang II-induced vasodilation

Shear stress is one of the important factors that control vascular tone *in vivo*. To examine the effect of shear rate on BK-mediated Ang II-induced vasodilation, the arterial segments were perfused at three different flow rates (10, 33 and 100 μl min^{-1}) while intraluminal pressure was kept constant (50 mmHg). As shown in Figure 6, in the presence of indomethacin, BK-mediated Ang II-induced vasodilation was influenced by the flow rates. Per cent relaxation at 100 μl min^{-1} was significantly higher than that at 10 μl min^{-1}. In contrast, Ang II-induced vasodilation measured in the presence of FR173657, was not dependent on the flow rate. These results indicate that the contribution of bradykinin to Ang II-induced vasodilation was greater at higher flow rates.

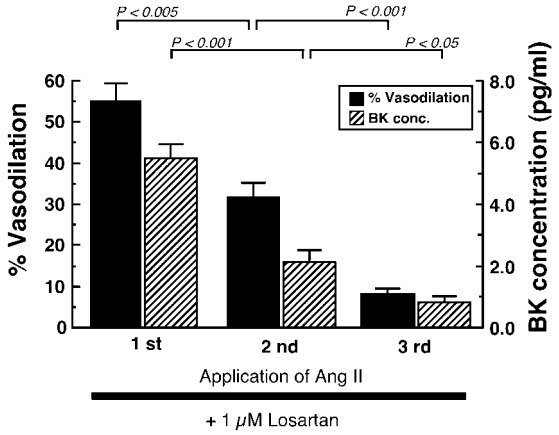

Figure 5 Reduced responsiveness to repetitive applications of Ang II in rat mesenteric arterial segments. Arterial segments, preconstricted by NE, were perfused with 1 nM Ang II in the presence of 1 μM losartan for 10 min. After a 30 min-perfusion with HEPES-Tyrode buffer for washout of Ang II and recovery, the segments were preconstricted with NE, followed by a second application of Ang II. The perfusates were collected after the first 3 min of commencement of each Ang II perfusion and concentrations of BK in each perfusate was determined by EIA. Closed column: mean per cent vasodilation upon each application of Ang II. Hatched column: BK concentrations. Data are expressed as mean$\pm$s.e.mean of five experiments.

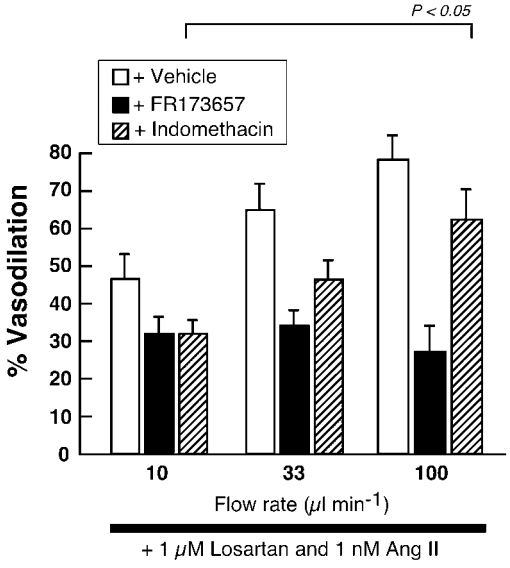

Figure 6 Effects of flow rates on Ang II-induced vasodilatory response. Arterial segments, preconstricted by NE, were pressurized to 50 mmHg and the flow rate (10, 33 and 100 μl min^{-1}) was established by changing the inflow and outflow pressures while holding the intravascular pressure constant. Vasodilatory responses were induced by perfusion of 1 nM Ang II in the presence of 1 μM losartan and either vehicle (open bars), 10 μM FR173657 (solid bars) or 10 μM indomethacin (hatched bars). Results are expressed as mean$\pm$s.e.mean of five experiments.

Bradykinin concentrations in the perfusate of the vessels treated with Ang II and losartan (in the absence of FR173657 and indomethacin) also increased with increased flow rates (4.7 ± 0.51 pg ml^{-1} at 10 μl min^{-1} and 6.4 ± 0.43 pg ml^{-1} at 100 μl min^{-1}).

Ang II-induced vasodilation in kininogen-deficient rats

We also confirmed the role of bradykinin in Ang II-induced vasodilation by using Brown Norway Katholiek rats (BN-Ka rats), which are genetically devoid of kininogens and fail to generate BK. In the presence of losartan, mesenteric arterial segments isolated from wild-type Brown Norway Kitasato rats (BN-Ki rats) exhibited vasodilation in response to Ang II, similar to SD rats (Figure 7A). Application of 1 nM Ang II increased the luminal diameter by $56.9\pm5.5\%$. In contrast, the dilation response to Ang II was significantly diminished in BN-Ka rats compared with BN-Ki rats ($22.4\pm4.1\%$, $P<0.05$). Ang II-induced vasodilation in BN-Ki rats was significantly attenuated in the presence of 10 μM FR173657 (Figure 7B), resulting in the same per cent relaxation as that obtained in BN-Ka rats ($P=0.93$). In contrast, further reduction in the Ang II-induced vasodilation in BN-Ka rats was not observed with treatment with 10 μM FR173657 ($P=0.66$). Determination of the BK concentration in the perfusates revealed that significant BK production (6.3 ± 0.72 pg ml^{-1}) occurred by treatment with Ang II in the arterial segments from BN-Ki rats, whereas BK was not detected by EIA in the perfusates of arterial segments from BN-Ka rats.

Discussion

The major new findings of the present study were that stimulation of AT_2 receptor caused a significant vasodilation in resistant arteries of the rat mesentery under perfusion conditions. These vasodilator actions of Ang II were AT_2-dependent and primarily mediated by locally formed BK through B_2 receptors because vasodilation was abolished by blockade of AT_2 receptors with PD123319 or BK B_2 receptors with FR173657. This BK-mediated vasodilation was flow-dependent and augmented with increased perfusion flow rates. Similar results were reported recently by Bergaya

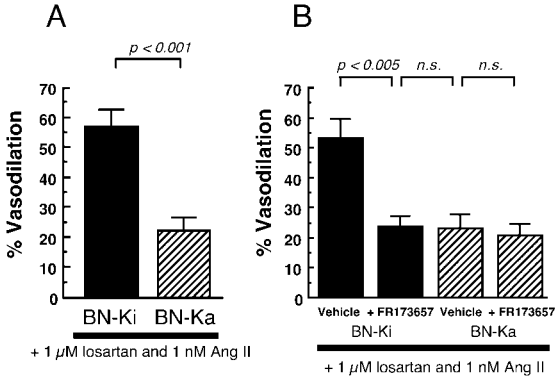

Figure 7 Reduced vasodilatory response to Ang II in kininogen-deficient rats. Arterial segments were prepared from either wild-type BN-Ki rats or kininogen-deficient BN-Ka rats. After constriction by norepinephrine, segments were perfused with 1 nM Ang II in the presence of 1 μM losartan and per cent vasodilation was determined (A). In B, effects of F173657 on per cent vasodilation were examined in both strains. Results are expressed as mean$\pm$s.e.mean of five experiments.

et al. (2001), showing the involvement of AT_2 receptors in flow-dependent dilation mediated by endogenous kinins.

Perfusion of Ang II without AT_1 blockade caused vasoconstriction, which was probably mediated by AT_1 receptor on smooth muscle cells. As shown in Figure 2, removal of endothelium almost completely abrogated Ang II-induced vasodilation observed in the presence of losartan, suggesting that the vasodilation was endothelial cell-dependent and probably mediated through AT_2 receptor on endothelial cells. Because Ang II-induced vasoconstriction was enhanced by removal of endothelial cells, it seems that AT_1-dependent constriction is counterbalanced by AT_2-dependent vasodilation in the intact mesenteric artery. It is likely that these counterbalancing systems are important for the regulation of vascular tone.

Several reports have shown that losartan interacts not only with AT_1 receptors but also with TXA_2 receptors. To confirm that our results using losartan were not secondary to blockade of TXA_2 receptors, we examined the effects of candesartan, another selective AT_1 antagonist with no action on TXA_2 receptor (Fukuhara *et al*., 2001). Because Ang II-dependent vasodilation responses observed in the presence of 1 μM losartan and 1 μM candesartan were not different (data not shown), we concluded that blockade of TXA_2 receptor by losartan is negligible, if any, in this study.

Our results showed that BK is a primary mediator of Ang II-induced vasodilation in rat mesenteric arteries since blockage of B_2 receptors resulted in reduction in the dilatory response by about 70% (Figure 3A). The remainder, which did not respond to a B_2 antagonist, was indomethacin-sensitive (Figure 3B), suggesting the involvement of prostanoids such as prostacyclin. The involvement of BK in vasodilation induced by AT_2 stimulation has been reported in previous studies in murine aortas (Gohlke *et al*., 1998; Tsutsumi *et al*., 1999). These studies, however, have also suggested that the NO/cGMP system is stimulated by BK. In contrast, significant roles of endothelium-derived hyperpolarizing factor (EDHF) in promotion of vasodilation by BK have also been suggested. It is likely that the discrepancy between vasodilatory mediators is due to differences in anatomic origin of the blood vessels used. Larger contribution of EDHF to endothelium-dependent relaxation by BK has also been reported in microvessels (Urakami-Harasawa *et al*., 1997; Honing *et al*., 2000), suggesting the significance of EDHF as a vasodilation mediator especially in resistant arteries.

The present results demonstrated that both aprotinin and SBTI inhibited Ang II-induced vasodilation. Treatment with aprotinin and/or SBTI also diminished the BK concentration in the perfusate. These results strongly suggest that BK is generated enzymatically from kininogens upon stimulation with Ang II and the primary enzyme responsible for the generation is probably plasma kallikrein-like enzyme. Because the arterial segments were extensively washed by perfusion, it is unlikely that sufficient amount of plasma kallikrein remained in the lumen as a contaminant. Recently, Dedio *et al*. (2001) showed that human umbilical vein endothelial cells and bovine aortic endothelial cells express significant amounts of tissue kallikrein, which likely serves in the local generation of vasoactive kinins. Production of tissue kallikrein or kallikrein-like enzymes by endothelial cells has been also reported by others (Madeddu *et al*., 1993; Okamoto *et al*., 1998; Wolf *et al*., 1999). Other studies have also suggested the binding of plasma kallikrein to endothelial cells (Seyedi *et al*., 1995; Schmaier *et al*., 1999). As for kininogens, production by endothelial cells (Okamoto *et al*., 1998; Oza *et al*., 1990) or binding to endothelial cells (Schmaier *et al*., 1988; van Iwaarden *et al*., 1988; Oza *et al*., 1990) has been reported. Considered together with the present findings, the above results suggest that stimulation of endothelial cells with Ang II *via* AT_2 would induce local release of BK, in which endothelial cell-derived kallikreins and kininogens are implicated.

Recent studies have focused on the physiological roles of the inhibitory and vasodilatory actions of AT_2. For example, it was demonstrated in normal subjects that AT_2 receptors do not play a significant role in blood flow regulation (Phoon & Howes, 2001). In contrast, Siragy and colleagues (Siragy & Carey, 1999; Siragy *et al*., 1999a, b) demonstrated that blockade or disruption of AT_2 receptor resulted in the development of hypertension, suggesting the pivotal role of AT_2 receptors in regulation of blood pressure. However, the significance of AT_2 receptor under physiological conditions is still unclear. Many recent studies have suggested that AT_2 receptor is perhaps more important in cardiovascular diseases. For example, it has been shown that expression of AT_2 is significantly upregulated in vessels of young spontaneously hypertensive rats (SHR) (Touyz *et al*., 1998) and that long-term blockade of AT_1 in SHR enhances AT_2 receptors expression (Savoia *et al*., 2001). Moreover, the AT_2-mediated vasodilation of coronary artery is enhanced in infarcted animals (Schuijt *et al*., 2001). Thus, it is highly plausible that AT_2 expression becomes upregulated under pathological conditions, and under the same conditions, enhanced AT_2 dependent-vasodilation may counteract AT_1-dependent vasoconstriction to relieve the diseased state.

Repetitive stimulation of the same vascular preparation by Ang II resulted in a gradual decrease in the amplitude of the responses (Figure 5). Bradykinin concentrations in the perfusates also decreased in parallel with the decreased responses, suggesting that depletion of vascular kininogens occurs in response to repetitive stimulation. It is also plausible that tachyphylaxis of AT_2 and/or B_2 receptor signalling is involved in the diminished response, although there are few reports that have investigated the phenomenon of tachyphylaxis of AT_2 receptor or B_2 receptor in the vascular tissues.

The present results also demonstrated that flow (shear stress) is an important factor in Ang II-induced vasodilation. As shown in Figure 8, AT_2-dependent vasodilation increased with increasing perfusion flow rates. Measurement of BK concentrations in the perfusates also revealed that these concentrations were also increased in a flow rate-dependent manner, although the increment in BK concentrations was not as marked as in vasodilative responses. Therefore increased Ang II-induced vasodialtion observed at higher flow rates can be explained at least in part by enhanced BK release.

In conclusion, we demonstrated in the present study that stimulation of AT_2 receptors caused a significant vasodilation of resistant arteries of the rat mesentery under flow conditions. This vasodilation was mediated primarily by BK locally generated by vascular tissues and was flow-dependent.

This counterbalancing vasodilatory system, probably regulated by hemodynamics of microcirculation, could play an important role in pathogenesis of cardiovascular diseases.

We are grateful to Fujisawa Pharmaceutical for kindly providing FR173657. This work was supported by The Ministry of Education, Science, Sports and Culture in Japan.

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(Received January 14, 2002
Revised March 12, 2002
Accepted March 22, 2002)