Flow-Dependent Dilation Mediated by Endogenous Kinins Requires Angiotensin AT$_2$ Receptors

Sonia Bergaya, Rob H.P. Hilgers, Pierre Meneton, You Dong, May Bloch-Faure, Tadashi Inagami, François Alhenc-Gelas, Bernard I. Lévy, Chantal M. Boulanger

Abstract—The vascular kallikrein-kinin system contributes to about one third of flow-dependent dilation in mouse carotid arteries, by activating bradykinin B$_2$ receptors coupled to endothelial nitric oxide (NO) release. Because the bradykinin/NO pathway may mediate some of the effects of angiotensin II AT$_2$ receptors, we examined the possible contribution of AT$_2$ receptors to the kinin-dependent response to flow. Changes in outer diameter after increases in flow rate were evaluated in perfused arteries from wild-type animals (TK$^{+/+}$) and in tissue kallikrein-deficient mice (TK$^{-/-}$) in which the presence of AT$_2$ receptor expression was verified. Saralasin, a nonselective angiotensin II receptor antagonist, impaired significantly flow-induced dilation in TK$^{+/+}$, whereas it had no effect in TK$^{-/-}$ mice. In both groups, blockade of AT$_1$ receptors with losartan or candesartan did not affect the response to flow. Inhibition of AT$_2$ receptors with PD123319 reduced significantly flow-induced dilation in TK$^{+/+}$ mice, but had no significant effect in TK$^{-/-}$ mice. Combining PD123319 with the bradykinin B$_2$ receptor antagonist HOE-140 had no additional effect to AT$_2$ receptor blockade alone in TK$^{+/+}$ arteries. Flow-dependent dilation was also impaired in AT$_2$ receptor deficient mice (AT$_2^{-/-}$) when compared with wild-type littermates. Furthermore, HOE-140 significantly reduced the response to flow in the AT$_2^{+/+}$, but not in AT$_2^{-/-}$ mice. In conclusion, this study demonstrates that the presence of functional AT$_2$ receptors is necessary to observe the contribution of the vascular kinin-kallikrein system to flow-dependent dilation.

Key Words: kinins ■ angiotensin II ■ flow-dependent vasodilation ■ angiotensin AT$_2$ receptor ■ bradykinin B$_2$ receptor
receptor pathways during flow-induced dilation. Therefore, the purpose of the present study is to determine whether or not angiotensin AT₂ receptors contribute to flow-dependent dilation mediated by endogenously formed kinins using isolated carotid arteries from control mice and mice lacking the tissue kallikrein kklk1 gene.

Materials and Methods

Animal Groups
Littermate 12-week-old male wild-type (TK+/+), and tissue kallikrein null (TK−/−) mice were used as previously described. Twelve-week-old male AT₁ receptor–deficient mice (AT₁−/−) and their age-matched wild-type littermates (AT₁+/+) were produced as described. They were backcrossed 10 times in the C57BL/6j genetic background.

RNA Extraction and RT-PCR Analysis
For each group of animals, carotid arteries from 10 mice were pooled and total RNA was extracted according to the Trizol reagent protocol (Life Technologies). The quality of the RNA preparation was confirmed by ethidium bromide staining.

The single-strand cDNA synthesis was performed in 20 µL of reaction buffer, consisting of first strand buffer 5X ( GibcoBRL), RNase inhibitor (40 UI/µL), dNTPs triphosphate (25 µmol/L), DTT (100 µmol/L) (Amersham), and Reverse MMLV (200 UI/µL). The reverse transcriptase reaction was performed by incubating the reaction mixture for 90 minutes at 37°C followed by 10 minutes at 65°C, using a 3′ primer (5′-GGT TCTTCCAAAGGAAGGGG-TATGAG). The polymerase chain reaction included three steps of denaturation (94°C, 45 seconds), annealing (65°C, 45 seconds), and extension (72°C, 105 seconds) for 35 cycles using the previously mentioned 3′-oligonucleotide and the other 5′-oligonucleotide (5′-CATGCTTTGTCTTGCGGT CCTGTC). These primers were taken from the unique exon from position 2176 to 2749 of the angiotensin II AT₁ receptor gene, localized in chromosome X, thus, producing a 0.573-kb fragment of the AT₁ receptor cDNA. The cDNA was amplified using 5 IU/µL of TaqDNA polymerase (Life Technologies) and 20 µmol/L of each set of primers in 50 µL of buffer 10X (22 µmol/L Tris- HCl; pH 8.4; 55 µmol/L KCl), MgCl2 (1.65 mmol/L), dNTPs triphosphate (25 µmol/L) and 10 µL of loading dye (0.02% of red cresol and 60% of sucrose). The PCR products were sequenced and corresponded to the mouse AT₁ receptor sequence.

In addition, expression of GAPDH was evaluated in parallel to that of AT₁ receptor. Briefly, total RNA was reverse transcribed as mentioned above using the 3′ primer (5′-CATGTAAGGCGCATTGAGTGCCACAC-3′). Then, the RT-product was amplified by 35 cycles as described for AT₁ receptor, using the 3′-primer and the 5′-oligonucleotide (5′-TGAAGGTCGTTGTTGAGGATTTTGGC-3′). The migration of both PCR products (AT₁ receptor and GAPDH) was then followed by electrophoresis on a 2% agarose gel and ethidium bromide staining.

In Vitro Measurement of the Arterial Diameter
Carotid arteries were carefully exposed and quickly excised. The procedure was in accordance with the European Community guidelines on the care and use of laboratory animals (Ministère de l’Agriculture, France, authorization n°7430). They were canulated at both extremities and then perfused continuously in vitro in a video-monitored perfusion system where flow and pressure can be modified independently, as previously described. Briefly, arteries were bathed in a physiological salt solution warmed (37°C) and gassed (95% O2/5% CO₂) throughout the experiments. The pressure was monitored by a servo-perfusion system. Intraluminal and extraluminal perfusions were provided by the mean of two perfusion pumps. The outer diameter, as well as proximal and distal pressures, was continuously recorded. The presence of the endothelium was ascertained by assessing the relaxation by acetylcholine (1 µmol/L) during phenylephrine-induced (1 µmol/L) contraction. Experiments were discarded when the relaxation by acetylcholine was smaller than 60% of that induced by sodium nitroprusside (0.1 µmol/L), because it indicated that the endothelial layer was damaged. At the end of each experiment, passive diameter was obtained after incubation of the artery (40 minutes) with a Ca²⁺-free control solution containing EGTA (2 mmol/L) and sodium nitroprusside (0.1 µmol/L), which abolished the smooth muscle tone. Phenylephrine and all the inhibitors or antagonists used in this study were delivered both in the intraluminal and extraluminal perfusions.

In Vitro Protocols for Mice Carotid Arteries
All experiments evaluating the response to increases in intraluminal flow rate were performed in presence of phenylephrine (1 µmol/L). When the contraction to phenylephrine was stable for at least 10 minutes, the intraluminal flow rate was increased in a stepwise manner from 10 to 800 µL/min. Each flow rate was applied for about 3 to 9 minutes, until the diameter reached a plateau, and then was augmented to the next level. Experiments were performed in the presence of either saralasin (a nonspecific AT₁ and AT₂ blocker; 1 µmol/L), losartan (an angiotensin AT₁ antagonist; 0.1 µmol/L), candesartan (an angiotensin AT₂ antagonist; 10 nmol/L), PD123319 (an angiotensin AT₂ antagonist; 1 µmol/L), or HOE-140 (a bradykinin B₂ receptor antagonist; 1 µmol/L). Tissues were preincubated for 40 minutes with each antagonist or inhibitor in intraluminal and extraluminal perfusions. Unless otherwise indicated, responses under control conditions were obtained on contralateral carotid arteries.

Some experiments were performed on isolated mice mesenteric arteries, which were mounted as described above for carotid arteries. Mesenteric artery basal diameters in AT₂+/+ and AT₂−/− mice were 177±12 (n=7) and 181±20 µm (n=6), respectively. The arteries were perfused (40 µL/min), pressurized (80 mm Hg), and dilation to increasing concentrations of exogenous bradykinin (0.1 µmol/L to 10 µmol/L; given extraluminally) was recorded during contractions induced by the thromboxane analog U46619 (1 to 10 µmol/L).

Drugs and Chemical Agents
The compounds used for in vitro studies were acetylcholine chloride, bradykinin diacetate salt, L-phenylephrine hydrochloride, PD 123319, and losartan (Sigma). HOE-140 was kindly provided by Drs H.J. Lang and B.A. Scholkmens (Hoechst-Marion-Roussel, Frankfurt, Germany). Candesartan was kindly provided by ASTRA-ZENECA.

Data Analysis and Statistics
Data are given as changes in diameter (microns) from the artery diameter obtained during contraction with phenylephrine. Results are expressed as mean±SEM of n experiments; n represents the number of animals used for each experimental protocol. pD₂ values represent the negative logarithm of the concentration of agonist, which causes 50% of its maximal response. Statistical evaluation was performed by use of ANOVA for factorial or repeated measurements, followed by Scheffe t test. Values of P<0.05 were considered to be statistically significant.

Results
Vasoactive Responses in Perfused TK+/+ and TK−/− Mouse Carotid Arteries
Exposure of perfused TK+/+ and TK−/− carotid arteries to phenylephrine, acetylcholine, or sodium nitroprusside caused similar changes in outer diameter for each set of experiments (Table). Step-increases in intraluminal flow rate augmented the carotid artery diameter in both TK+/+ and TK−/− animals. At high flow rates, this response to flow was significantly smaller in preparations from TK−/− mice when compared with TK+/+ mice, as previously observed (P=0.0003) (Figure 1).
Effect of Angiotensin II Receptor Antagonists on Flow-Dependent Response

In TK$^{+/+}$ mice, flow-dependent dilation was significantly impaired by the nonspecific angiotensin II receptors antagonist saralasin ($P=0.02$ between 200 and 800 $\mu$L/min) (Figure 1). Conversely, saralasin did not significantly modify the response to flow of TK$^{-/-}$ carotid arteries ($P=0.84$) (Figure 1). We then investigated the effect of preferential angiotensin AT$_1$ and AT$_2$ receptor antagonists on the response to flow. The response to flow was not affected by the AT$_1$ receptor antagonist losartan, in both TK$^{+/+}$ and TK$^{-/-}$ arteries ($P=0.46$ and $P=0.71$ respectively; Figure 2). The same conclusion was reached with candesartan ($P=0.99$ and $P=0.66$, respectively; data not shown).

Changes in Outer Diameter of Carotid Arteries From TK$^{+/+}$ and TK$^{-/-}$ Mice, in Response to Phenylephrine, Acetylcholine, and Sodium Nitroprusside

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<th>Losartan</th>
<th>Losartan+PD123319</th>
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PHE indicates phenylephrine; ACH, acetylcholine; SNP, sodium nitroprusside. The contractions to PHE and the relaxations to SNP are given as changes in $\mu$m from the basal diameter (positive and negative numbers indicate a gain and loss in diameter, respectively). Relaxations to ACH are expressed as percent inhibition of the contraction evoked by PHE. Statistical comparison between treatments was performed using ANOVA.

**Figure 1.** Changes in diameter ($\mu$m) after step-increases in flow rate in carotid arteries from TK$^{+/+}$ (top, closed symbols) (n=7) and TK$^{-/-}$ mice (bottom, open symbols) (n=6). Experiments were performed under control condition (○, □) or in the presence of saralasin (●, ▢). *Significant difference when compared with control conditions ($P<0.05$).
Interestingly, the AT₂ receptor antagonist PD123319 significantly impaired the response to high flow rates in TK⁺/⁻/⁺/⁻ arteries exposed to losartan (P=0.05 when compared with losartan alone, and P=0.01 when compared with control conditions) (Figure 2). However, PD123319 had no significant effect on the response to flow in TK⁻/⁻/⁺/⁻ arteries exposed to losartan (P=0.88, when compared with losartan alone) and P=0.74 when compared with control conditions) (Figure 2).

Expression of angiotensin II AT₂ receptors was examined in carotid arteries of both TK⁺/⁻/⁺/⁻ and TK⁻/⁻/⁺/⁻ mice by RT-PCR experiments. The AT₂ receptor mRNA was present in carotid arteries of both groups, as demonstrated by the band at 0.6 kb (Figure 3).

**Effect of AT₂ and B₂ Receptors Antagonists on Flow-Dependent Response**

In TK⁺/⁻/⁺/⁻ arteries, blockade of AT₂ receptors with PD123319 alone reduced significantly flow-dependent dilation (P=0.005 from 200 to 800 µL/min). Interestingly, the response to flow of TK⁺/⁻/⁺/⁻ arteries exposed to PD123319 was not different from that of TK⁻/⁻/⁺/⁻ arteries under control conditions (P=0.59) (Figure 4). Furthermore, PD123319 alone had no significant effect on the response to flow of TK⁻/⁻/⁺/⁻ arteries (P=0.19) (Figure 4).

The bradykinin B₂ receptor antagonist HOE-140 also decreased the response to flow in TK⁺/⁻/⁺/⁻ arteries, as previously shown. Combining PD123319 plus HOE-140 significantly altered the response to flow when compared with control conditions (P=0.002 from 400 to 800 µL/min) (Figure 4). However, the impairment of flow-dependent dilation observed in the presence of PD123319 plus HOE-140 was not different from that caused by PD123319 alone (P=0.84).

**Flow- and Bradykinin-Induced Dilation in AT₂-Deficient Mice**

Flow-dependent dilation was also significantly reduced in carotid arteries of AT₂⁻/⁻/⁻ mice when compared with their wild-type littermates AT₂⁺/⁺/⁻ (P=0.04) (Figure 5). In addition, HOE-140 significantly decreased the response to flow in wild-type mice, but had no significant effect in the AT₂⁻/⁻/⁻ animals (P=0.01 and P=0.80, respectively) (Figure 5). Dilatation to exogenous bradykinin was investigated in perfused AT₂⁻/⁻/⁺/⁻ and AT₂⁻/⁻/⁺/⁻ mesenteric arteries (n=6), where full-dose-response curves to the peptide could be obtained. There was no significant difference in pD₂ values (7.89±0.31 versus 8.18±0.33; P=0.54) and maximal responses (52±12 and 47±7%; P=0.63) to bradykinin between AT₂⁺/⁺/⁻ and AT₂⁻/⁻/⁻ mesenteric arteries, respectively.

**Discussion**

Tissue kallikrein activation contributes to one third of flow-mediated dilation by activating bradykinin B₂ receptors coupled to endothelial NO synthesis. In this study, we demonstrate that the angiotensin AT₂ receptor mediates the tissue-kallikrein–dependent dilation induced by flow in perfused murine carotid arteries.

Before investigating the possible contribution of AT₂ receptors in flow-induced dilation mediated by the endogenous kinin-kallikrein system, we verified the presence of AT₂ receptors in this preparation because these receptors are either absent or expressed at a low level in blood vessels from adult animals. RT-PCR experiments demonstrated the presence of angiotensin II AT₂ receptor mRNA in arteries from control mice and from mice lacking tissue kallikrein (TK⁻/⁻). In perfused carotid arteries, we observed that the response to an increase in flow rate was significantly smaller in TK⁻/⁻ when...
compared with TK\(^{+/+}\), thus confirming our previous study.\(^{10}\)

The first hint regarding the involvement of angiotensin II receptors in flow-induced dilation mediated by endogenous kinins came from experiments with saralasin, a nonspecific angiotensin II AT\(_1\)-AT\(_2\) receptor antagonist. Saralasin impaired the response to flow in TK\(^{+/+}\) arteries, but not in TK\(^{-/-}\) arteries, which lack the formation of endogenous kinins.\(^{10,26}\)

To determine further which of the angiotensin II receptor subtype is implicated in the response to flow mediated by the vascular kinin-kallikrein system, we investigated the effect on flow-induced dilation of preferential antagonists of either AT\(_1\) or AT\(_2\) receptors. Neither losartan nor candesartan modified flow-induced dilation in both TK\(^{+/+}\) and TK\(^{-/-}\) animals, demonstrating that AT\(_1\) receptors do not contribute to the kinin-mediated dilation induced by flow. This interpretation is in agreement with other studies showing the lack of effect of angiotensin II AT\(_1\) receptor antagonists in flow-mediated dilation,\(^{15}\) but may contrast with previous studies showing that angiotensin II increases cyclic GMP levels by activating an endothelial AT\(_1\) receptor.\(^{33,34}\) The apparent discrepancy between these interpretations might result from the absence of flow rate and the use of high concentrations of exogenous angiotensin II in earlier studies.

Unlike the blockade of angiotensin AT\(_1\) receptor alone, exposing arteries to the combination of an AT\(_1\) and AT\(_2\) receptor antagonist decreased the response to flow in TK\(^{+/+}\) arteries, thus implying that angiotensin AT\(_2\) but not AT\(_1\) receptors contribute to flow-induced dilation in wild-type arteries. The inhibitory effects of PD123319 on flow-induced dilation in TK\(^{-/-}\) arteries further reinforce this interpretation. Interestingly, PD123319 reduced flow-induced dilation in wild-type mice to reach comparable levels to those observed in TK\(^{-/-}\) mice under control conditions. The effect of AT\(_1\) and AT\(_2\) receptor antagonists was also examined in TK\(^{-/-}\) arteries. Unlike TK\(^{+/+}\) arteries, blockade of AT\(_1\) and/or AT\(_2\) receptors did not modify the flow response in TK\(^{-/-}\) arteries. As TK\(^{-/-}\) arteries express both AT\(_1\) and B\(_2\) receptors,\(^{19}\) we can conclude from the present data that the participation of AT\(_1\) receptors to flow-induced dilation requires the presence of a functional vascular kallikrein-kinin system.

We further confirmed the results obtained with saralasin and the angiotensin AT\(_2\) receptor antagonist PD123319 in wild-type mice, by investigating the response to flow of carotid arteries obtained from AT\(_2\)-deficient mice (AT\(_2\)^{-/-}).\(^{27}\) Flow-induced dilation was impaired in AT\(_2\)^{-/-} arteries, as compared with their wild-type littermates. In addition, the B\(_2\) receptor antagonist HOE-140 reduced the response to flow in AT\(_2\)^{-/-} mice but not in AT\(_2\)^{-/-} mice, although deletion of AT\(_2\) receptor gene expression did not affect the functional response to B\(_2\) receptor activation with exogenous bradykinin. Taken together, these findings indicate that the participation of bradykinin B\(_2\) receptors to flow-induced dilation requires the presence of functional AT\(_2\) receptors.

Then, we investigated in wild-type mice the possible contribution of bradykinin B\(_2\) receptors to the AT\(_2\)-dependent dilation in response to flow. The B\(_2\) receptor antagonist HOE-140 did not further decrease the response to flow in TK\(^{+/+}\) arteries already exposed to PD123319, although previous results demonstrated that in this strain, HOE-140 alone significantly impairs the response to flow under control conditions.\(^{10}\) Taken together, these results show that if bradykinin B\(_2\) receptors are blocked or if the vascular kinin-kallikrein system is inactivated, the AT\(_3\) receptor antagonist PD123319 no longer decreases the response to flow. Similarly, if AT\(_3\) receptors are blocked or not expressed, the bradykinin B\(_2\) receptor antagonist HOE-140 no longer inhibits flow-induced dilation. Thus, the present data demonstrate that the involvement of AT\(_1\) receptors in flow-dependent dilation requires the presence of both functional bradykinin B\(_2\) receptors and an active vascular kinin-kallikrein system. The present study also supports the conclusion that the part of flow-dependent dilation that is mediated by the local kinin-kallikrein system requires the presence of functional AT\(_2\) receptors. This interpretation is in agreement with previous studies indicating that AT\(_2\)-dependent vasodilatation after
exposure to exogenous angiotensin II involves bradykinin B₂ receptor activation.17,20,23 Although the exact mechanism linking AT₁ receptors and B₂ receptors in flow-induced dilation remains to be elucidated, several hypotheses could be brought forward. The increase in flow rate may favor and augment the boundary layer mass transport of kinins and angiotensin II to their receptors, therefore decreasing their degradation rate.35,36 However, we cannot exclude the possibility that shear stress might also modulate endogenous peptides synthesis. Indeed, as a short-term increase in shear stress enhanced ACE activity, flow stimulation may augment the local production of angiotensin II.37 However, an increase in ACE activity would also contribute to a greater degradation of locally formed kinins,38 thus counterbalancing the effect of an augmented synthesis of angiotensin II. Interestingly, the lack of additional effect of the AT₁ and the B₂ receptor antagonists supports the conclusion that these two receptors do not act synergistically. This observation also favors the interpretation that these two receptor pathways lie upstream/downstream of each other. Activation of the angiotensin AT₁ pathway might precede the stimulation of endogenous kinins synthesis and B₂ receptors, as indicated from a recent study by Katada and Majima.23 This interpretation is also supported by data from this study and previous work10 as bradykinin response was unchanged after inactivation of either vascular kallikrein or AT₁ receptors. Furthermore, overexpression of AT₂ receptors in vascular smooth muscle cells increases intracellular acidosis, resulting in an increase in kininogenase activity and in turn the generation of kinins.25 However, activation of kininogenase(s) by lowering intracellular pH after AT₁ receptor activation is not fully compatible with the known in vitro characteristics of tissue kallikrein activation.39 Alternatively, the present results may suggest a direct molecular interaction between B₂ and AT₂ receptors, comparable to the one recently described for AT₁ and AT₂,40 but these different hypotheses would require further investigations.

In conclusion, the present study demonstrates that in mouse carotid arteries, the vascular kinin-kallikrein system contributes to one third of flow-induced dilation and requires the presence of both functional angiotensin AT₁ and bradykinin B₂ receptors.

Acknowledgments

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References


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