

# Flow-Dependent Dilation Mediated by Endogenous Kinins Requires Angiotensin AT<sub>2</sub> 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 mice carotid arteries, by activating bradykinin B<sub>2</sub> receptors coupled to endothelial nitric oxide (NO) release. Because the bradykinin/NO pathway may mediate some of the effects of angiotensin II AT<sub>2</sub> receptors, we examined the possible contribution of AT<sub>2</sub> 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<sup>+/+</sup>) and in tissue kallikrein-deficient mice (TK<sup>-/-</sup>) in which the presence of AT<sub>2</sub> receptor expression was verified. Saralasin, a nonselective angiotensin II receptor antagonist, impaired significantly flow-induced dilation in TK<sup>+/+</sup>, whereas it had no effect in TK<sup>-/-</sup> mice. In both groups, blockade of AT<sub>1</sub> receptors with losartan or candesartan did not affect the response to flow. Inhibition of AT<sub>2</sub> receptors with PD123319 reduced significantly flow-induced dilation in TK<sup>+/+</sup> mice, but had no significant effect in TK<sup>-/-</sup> mice. Combining PD123319 with the bradykinin B<sub>2</sub> receptor antagonist HOE-140 had no additional effect to AT<sub>2</sub> receptor blockade alone in TK<sup>+/+</sup> arteries. Flow-dependent-dilation was also impaired in AT<sub>2</sub> receptor deficient mice (AT<sub>2</sub><sup>-/-</sup>) when compared with wild-type littermates. Furthermore, HOE-140 significantly reduced the response to flow in the AT<sub>2</sub><sup>+/+</sup>, but not in AT<sub>2</sub><sup>-/-</sup> mice. In conclusion, this study demonstrates that the presence of functional AT<sub>2</sub> receptors is necessary to observe the contribution of the vascular kinin-kallikrein system to flow-dependent dilation. (*Circ Res.* 2004;94:1623-1629.)

**Key Words:** kinins ■ angiotensin II ■ flow-dependent vasodilation ■ angiotensin AT<sub>2</sub> receptor ■ bradykinin B<sub>2</sub> receptor

Kallikreins are a distinct group of serine proteases, which are capable of generating vasoactive kinins from kininogen *in vitro*. Vascular tissue contains kininogen and kallikrein mRNA, suggesting the existence of an endogenous kallikrein-kinin system (KKS). Tissue KKS is present in both endothelial and smooth muscle cells of various vascular territories and is different from the circulating plasma kallikrein-kinin system.<sup>1-4</sup> Indeed, tissue kallikrein (TK) is encoded by the kallikrein *klk1* gene, and experimental evidence suggests that endogenously formed kinins could participate in the regulation of vascular tone.<sup>5-9</sup> We recently showed that tissue kallikrein plays an essential role in kinins synthesis within the vascular wall of mice carotid arteries.<sup>10</sup> In addition, this tissue KKS contributes to about one third of flow-induced dilation through activation of bradykinin B<sub>2</sub> receptors leading to NO release in mouse carotid arteries.<sup>10</sup> Flow-dependent dilation enables arteries to increase their diameter in response to changes in blood flow after the release of endothelial factors such as nitric oxide (NO) or prostacyclin to ensure appropriate

organ perfusion.<sup>11-14</sup> Flow-dependent dilation is a complex response involving not only local mediators (such as kinins) but also mechanical activation of endothelial cells, integrins, and cytoskeleton.<sup>14</sup> Other peptide systems such as angiotensin II can also contribute to flow-dependent dilation by activating AT<sub>2</sub> receptors, as demonstrated in resistance arteries.<sup>15</sup>

Interestingly, several studies suggested a possible link between angiotensin II receptors and bradykinin B<sub>2</sub> receptors regarding nitric oxide production.<sup>16-24</sup> In vascular endothelial cells, the production of NO by angiotensin II is caused by activation of the angiotensin II type 2 (AT<sub>2</sub>) receptor.<sup>16</sup> In addition, AT<sub>2</sub> receptors stimulation by exogenous angiotensin II leads to an increase in cyclic GMP level, through a mechanism involving bradykinin B<sub>2</sub> receptors and NO release.<sup>17-23</sup> Furthermore, overexpression of AT<sub>2</sub> receptors increases bradykinin production presumably by activating kininogenase(s).<sup>25</sup> However, there is no information regarding a possible relation between AT<sub>2</sub> and bradykinin B<sub>2</sub>

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receptor pathways during flow-induced dilation. Therefore, the purpose of the present study is to determine whether or not angiotensin AT<sub>2</sub> receptors contribute to flow-dependent dilation mediated by endogenously formed kinins using isolated carotid arteries from control mice and mice lacking the tissue kallikrein *kkl1* gene.

## Materials and Methods

### Animal Groups

Littermate 12-week-old male wild-type (TK<sup>+/+</sup>) and tissue kallikrein null (TK<sup>-/-</sup>) mice were used as previously described.<sup>10,26</sup> Twelve-week-old male AT<sub>2</sub> receptor-deficient mice (AT<sub>2</sub><sup>-/-</sup>) and their age-matched wild-type littermates (AT<sub>2</sub><sup>+/+</sup>) were produced as described.<sup>27,28</sup> They were backcrossed 10 times in the C57BL/6 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  $\mu$ L of reaction buffer, consisting of first strand buffer 5 $\times$  (GibcoBRL), RNase inhibitor (40 UI/ $\mu$ L), dinucleotide triphosphate (25 mmol/L), DTT (100 mmol/L) (Amersham), and Reverse MMLV (200 UI/ $\mu$ L). 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'-GGTTTTCCAAGGAAGGGG-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'-CATGCTTTGTCTGGGCTTCGTC). These primers were taken from the unique exon from position 2176 to 2749 of the angiotensin II AT<sub>2</sub> receptor gene, localized in chromosome X, thus, producing a 0.573-kb fragment of the AT<sub>2</sub> receptor cDNA.<sup>29</sup> The cDNA was amplified using 5 IU/ $\mu$ L of *Taq*DNA polymerase (Life Technologies) and 20  $\mu$ mol/L of each set of primers in 50  $\mu$ L of buffer 10 $\times$  (22 mmol/L Tris-HCl; pH 8.4; 55 mmol/L KCl), MgCl<sub>2</sub> (1.65 mmol/L), dinucleotide triphosphate (25 mmol/L) and 10  $\mu$ L of loading dye (0.02% of red cresol and 60% of sucrose). The PCR products were sequenced and corresponded to the mouse AT<sub>2</sub> receptor sequence.

In addition, expression of GAPDH was evaluated in parallel to that of AT<sub>2</sub> receptor. Briefly, total RNA was reverse transcribed as mentioned above using the 3' primer (5'-CATGTAGGCCAT-GAGGTCCACCAC-3'). Then, the RT-product was amplified by 35 cycles as described for AT<sub>2</sub> receptor, using the 3'-primer and the 5'-oligonucleotide (5'-TGAAGGTCGGTGTGAACGGATTGGC-3'). The migration of both PCR products (AT<sub>2</sub> 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° 07430). They were cannulated 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.<sup>10</sup> Briefly, arteries were bathed in a physiological salt solution warmed (37°C) and gassed (95% O<sub>2</sub>/5% CO<sub>2</sub>) throughout the experiments. The pressure was monitored by a servoperfusion 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  $\mu$ mol/L)

during phenylephrine-induced (1  $\mu$ mol/L) contraction. Experiments were discarded when the relaxation by acetylcholine was smaller than 60% of that induced by sodium nitroprusside (0.1 mmol/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 Ca<sup>2+</sup>-free control solution containing EGTA (2 mmol/L) and sodium nitroprusside (0.1 mmol/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  $\mu$ mol/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  $\mu$ L/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 AT<sub>1</sub> and AT<sub>2</sub> blocker; 1  $\mu$ mol/L), losartan (an angiotensin AT<sub>1</sub> antagonist; 0.1  $\mu$ mol/L), candesartan (an angiotensin AT<sub>1</sub> antagonist; 10 nmol/L), PD123319 (an angiotensin AT<sub>2</sub> antagonist; 1  $\mu$ mol/L), or HOE-140 (a bradykinin B<sub>2</sub> receptor antagonist; 1  $\mu$ mol/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 arteries basal diameters in AT<sub>2</sub><sup>+/+</sup> and AT<sub>2</sub><sup>-/-</sup> mice were 177 $\pm$ 12 (n=7) and 181 $\pm$ 20  $\mu$ m (n=6), respectively. The arteries were perfused (40  $\mu$ L/min), pressurized (80 mm Hg), and dilatation to increasing concentrations of exogenous bradykinin (0.1 nmol/L to 10  $\mu$ mol/L; given extraluminally) was recorded during contractions induced by the thromboxane analog U46619 (1 to 10 nmol/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. Schölkens (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 $\pm$ SEM of n experiments; n represents the number of animals used for each experimental protocol. pD<sub>2</sub> 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.<sup>30</sup> Values of *P* $\leq$ 0.05 were considered to be statistically significant.

## Results

### Vasoactive Responses in Perfused TK<sup>+/+</sup> and TK<sup>-/-</sup> Mouse Carotid Arteries

Exposure of perfused TK<sup>+/+</sup> and TK<sup>-/-</sup> 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<sup>+/+</sup> and TK<sup>-/-</sup> animals. At high flow rates, this response to flow was significantly smaller in preparations from TK<sup>-/-</sup> mice when compared with TK<sup>+/+</sup> mice, as previously observed<sup>10</sup> (*P*=0.0003) (Figure 1).

**Changes in Outer Diameter of Carotid Arteries From TK<sup>+/+</sup> and TK<sup>-/-</sup> Mice, in Response to Phenylephrine, Acetylcholine, and Sodium Nitroprusside**

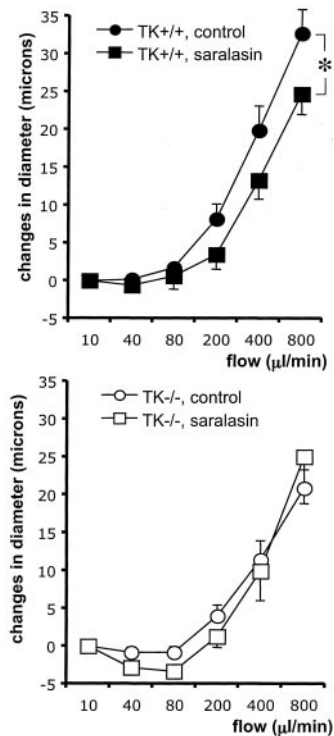
	Control	Losartan	Losartan+PD123319	Saralasin	PD123319	PD123319+HOE140	P
<b>TK<sup>+/+</sup></b>							
n	18	5	6	7	6	6	
PHE	-58±5	-56±11	-74±8	-65±14	-67±14	-70±14	0.87
ACH	94±5	91±4	93±6	100±2	99±3	99±3	0.05
SNP	83±12	80±16	93±11	91±2	77±15	83±15	0.96
<b>TK<sup>-/-</sup></b>							
n	17	5	6	6	6	...	
PHE	-58±8	-71±7	-72±9	-63±12	-68±9	...	0.81
ACH	94±4	85±5	84±4	101±1	95±4	...	0.12
SNP	72±10	79±8	78±9	74±14	92±9	...	0.89

PHE indicates phenylephrine; ACH, acetylcholine; SNP, sodium nitroprusside.

The contractions to PHE and the relaxations to SNP are given as changes in μm 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.

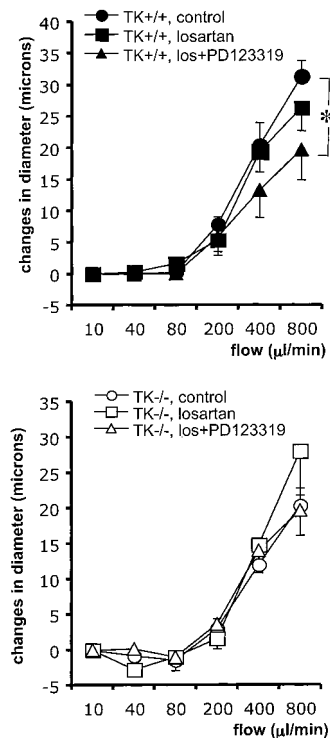
**Effect of Angiotensin II Receptor Antagonists on Flow-Dependent Response**

In TK<sup>+/+</sup> mice, flow-dependent dilation was significantly impaired by the nonspecific angiotensin II receptors antagonist saralasin (*P*=0.02 between 200 and 800 μL/min) (Figure 1). Conversely, saralasin did not significantly modify the response to flow of TK<sup>-/-</sup> carotid arteries (*P*=0.84) (Figure 1). We then investigated the effect of preferential angiotensin AT<sub>1</sub> and AT<sub>2</sub> receptor antagonists on the response to flow.

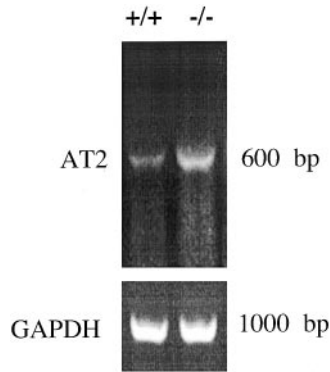


**Figure 1.** Changes in diameter (μm) after step-increases in flow rate in carotid arteries from TK<sup>+/+</sup> (top, closed symbols) (n=7) and TK<sup>-/-</sup> mice (bottom, open symbols) (n=6). Experiments were performed under control condition (●; ○) or in the presence of saralasin (■; □). \*Significant difference when compared with control conditions (*P*≤0.05).

The response to flow was not affected by the AT<sub>1</sub> receptor antagonist losartan, in both TK<sup>+/+</sup> and TK<sup>-/-</sup> 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).



**Figure 2.** Changes in diameter (μm) after step-increases in flow rate in carotid arteries from TK<sup>+/+</sup> (top, closed symbols) and TK<sup>-/-</sup> mice (bottom, open symbols). Experiments were performed under control condition (●, n=6; ○, n=6), in the presence of losartan (los; ■, n=5; □, n=5), or in the presence of both losartan plus PD123319 (▲, n=6; △, n=6). Control responses and experiments with losartan were performed on carotid arteries from the same mice, whereas experiments with losartan plus PD123319 were performed were obtained in a different set of animals. \*Significant effect (*P*≤0.05).



**Figure 3.** Expression of the angiotensin II AT<sub>2</sub> receptor gene in carotid arteries of TK<sup>+/+</sup> and TK<sup>-/-</sup> mice. This typical RT-PCR experiment is representative of a total of 2 different experiments obtained on different pools of 10 carotid arteries. Expression of GAPDH was observed in the same samples in parallel experiments.

Interestingly, the AT<sub>2</sub> receptor antagonist PD123319 significantly impaired the response to high flow rates in TK<sup>+/+</sup> 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<sup>-/-</sup> 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 AT<sub>2</sub> receptors was examined in carotid arteries of both TK<sup>+/+</sup> and TK<sup>-/-</sup> mice by RT-PCR experiments. The AT<sub>2</sub> receptor mRNA was present in carotid arteries of both groups, as demonstrated by the band at 0.6 kb (Figure 3).

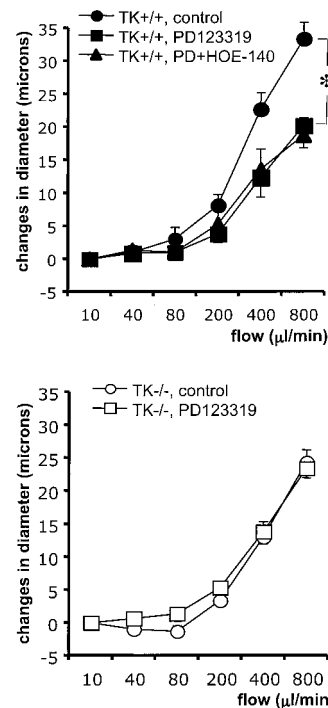
### Effect of AT<sub>2</sub> and B<sub>2</sub> Receptors Antagonists on Flow-Dependent Response

In TK<sup>+/+</sup> arteries, blockade of AT<sub>2</sub> receptors with PD123319 alone reduced significantly flow-dependent dilation ( $P=0.005$  from 200 to 800  $\mu\text{L}/\text{min}$ ). Interestingly, the response to flow of TK<sup>+/+</sup> arteries exposed to PD123319 was not different from that of TK<sup>-/-</sup> arteries under control conditions ( $P=0.59$ ) (Figure 4). Furthermore, PD123319 alone had no significant effect on the response to flow of TK<sup>-/-</sup> arteries ( $P=0.19$ ) (Figure 4).

The bradykinin B<sub>2</sub> receptor antagonist HOE-140 also decreased the response to flow in TK<sup>+/+</sup> arteries, as previously shown.<sup>10</sup> Combining PD123319 plus HOE-140 significantly altered the response to flow when compared with control conditions ( $P=0.002$  from 400 to 800  $\mu\text{L}/\text{min}$ ) (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 AT<sub>2</sub>-Deficient Mice

Flow-dependent dilation was also significantly reduced in carotid arteries of AT<sub>2</sub><sup>-/-</sup> mice when compared with their wild-type littermates AT<sub>2</sub><sup>+/+</sup> ( $P=0.04$ ) (Figure 5). In addition, HOE-140 significantly decreased the response to flow in wild-type mice, but had no significant effect in the AT<sub>2</sub><sup>-/-</sup>



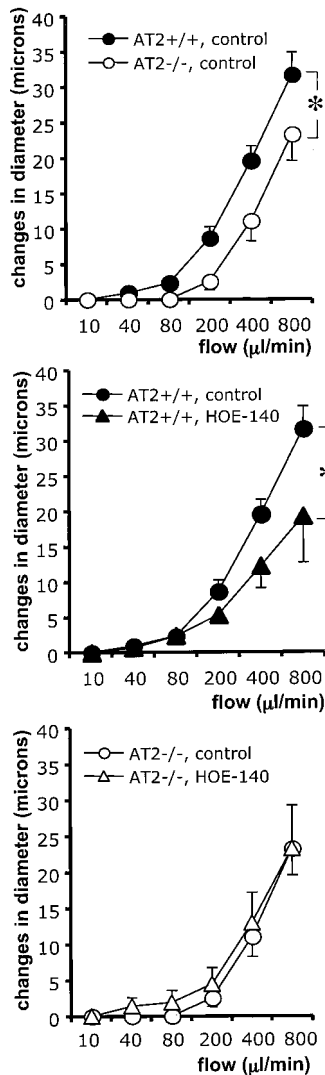
**Figure 4.** Changes in diameter ( $\mu\text{m}$ ) after step-increases in flow rate in carotid arteries from TK<sup>+/+</sup> (top, closed symbols) and TK<sup>-/-</sup> mice (bottom, open symbols). Experiments were performed under control condition (●,  $n=6$ ; ○,  $n=6$ ), in the presence of PD123319 (PD; ■,  $n=6$ ; □,  $n=6$ ), or in the presence of both PD123319 plus HOE140 (▲,  $n=6$ ). Control experiments and experiments with PD123319 were obtained on arteries from the same animals. Experiments with PD123319 plus HOE-140 were performed on carotid arteries from different mice. \*Significant difference ( $P\leq 0.05$ ).

animals ( $P=0.01$  and  $P=0.80$ , respectively) (Figure 5). Dilation to exogenous bradykinin was investigated in perfused AT<sub>2</sub><sup>+/+</sup> and AT<sub>2</sub><sup>-/-</sup> mesenteric arteries ( $n=6$ ), where full dose-response curves to the peptide could be obtained. There was no significant difference in pD<sub>2</sub> values ( $7.89\pm 0.31$  versus  $8.18\pm 0.33$ ;  $P=0.54$ ) and maximal responses ( $52\pm 12$  and  $47\pm 7\%$ ;  $P=0.63$ ) to bradykinin between AT<sub>2</sub><sup>+/+</sup> and AT<sub>2</sub><sup>-/-</sup> mesenteric arteries, respectively.

### Discussion

Tissue kallikrein activation contributes to one third of flow-mediated dilation by activating bradykinin B<sub>2</sub> receptors coupled to endothelial NO synthesis.<sup>6–10,31</sup> In this study, we demonstrate that the angiotensin AT<sub>2</sub> receptor mediates the tissue-kallikrein-dependent dilation induced by flow in perfused murine carotid arteries.

Before investigating the possible contribution of AT<sub>2</sub> receptors in flow-induced dilation mediated by the endogenous kinin-kallikrein system, we verified the presence of AT<sub>2</sub> receptors in this preparation because these receptors are either absent or expressed at a low level in blood vessels from adult animals.<sup>32</sup> RT-PCR experiments demonstrated the presence of angiotensin II AT<sub>2</sub> receptor mRNA in arteries from control mice and from mice lacking tissue kallikrein (TK<sup>-/-</sup>). In perfused carotid arteries, we observed that the response to an increase in flow rate was significantly smaller in TK<sup>-/-</sup> when



**Figure 5.** Changes in diameter ( $\mu\text{m}$ ) after step-increases in flow rate in carotid arteries from AT<sub>2</sub><sup>+/+</sup> (closed symbols;  $n=7$ ) and AT<sub>2</sub><sup>-/-</sup> (open symbols;  $n=6$ ) mice. Experiments were performed under control conditions (●; ○) or in the presence of HOE-140 (▲; △) in carotid arteries from the same animal. \*Significant difference ( $P \leq 0.05$ ).

compared with TK<sup>+/+</sup>, thus confirming our previous study.<sup>10</sup> 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<sub>1</sub>-AT<sub>2</sub> receptor antagonist. Saralasin impaired the response to flow in TK<sup>+/+</sup> arteries, but not in TK<sup>-/-</sup> arteries, which lack the formation of endogenous kinins.<sup>10,26</sup>

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<sub>1</sub> or AT<sub>2</sub> receptors. Neither losartan nor candesartan modified flow-induced dilation in both TK<sup>+/+</sup> and TK<sup>-/-</sup> animals, demonstrating that AT<sub>1</sub> 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<sub>1</sub> receptor antagonists in flow-mediated

dilation,<sup>15</sup> but may contrast with previous studies showing that angiotensin II increases cyclic GMP levels by activating an endothelial AT<sub>1</sub> receptor.<sup>33,34</sup> 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<sub>1</sub> receptor alone, exposing arteries to the combination of an AT<sub>1</sub> and AT<sub>2</sub> receptor antagonist decreased the response to flow in TK<sup>+/+</sup> arteries, thus implying that angiotensin AT<sub>2</sub> but not AT<sub>1</sub> receptors contribute to flow-induced dilation in wild-type arteries. The inhibitory effects of PD123319 on flow-induced dilation in TK<sup>+/+</sup> arteries further reinforce this interpretation. Interestingly, PD123319 reduced flow-induced dilation in wild-type mice to reach comparable levels to those observed in TK<sup>-/-</sup> mice under control conditions. The effect of AT<sub>1</sub> and AT<sub>2</sub> receptor antagonists was also examined in TK<sup>-/-</sup> arteries. Unlike TK<sup>+/+</sup> arteries, blockade of AT<sub>1</sub> and/or AT<sub>2</sub> receptors did not modify the flow response in TK<sup>-/-</sup> arteries. As TK<sup>-/-</sup> arteries express both AT<sub>2</sub> and B<sub>2</sub> receptors,<sup>10</sup> we can conclude from the present data that the participation of AT<sub>2</sub> 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<sub>2</sub> receptor antagonist PD123319 in wild-type mice, by investigating the response to flow of carotid arteries obtained from AT<sub>2</sub> receptor-deficient mice (AT<sub>2</sub><sup>-/-</sup>).<sup>27</sup> Flow-induced dilation was impaired in AT<sub>2</sub><sup>-/-</sup> arteries, as compared with their wild-type littermates. In addition, the B<sub>2</sub> receptor antagonist HOE-140 reduced the response to flow in AT<sub>2</sub><sup>+/+</sup> mice but not in AT<sub>2</sub><sup>-/-</sup> mice, although deletion of AT<sub>2</sub> receptor gene expression did not affect the functional response to B<sub>2</sub> receptor activation with exogenous bradykinin. Taken together, these findings indicate that the participation of bradykinin B<sub>2</sub> receptors to flow-induced dilation requires the presence of functional AT<sub>2</sub> receptors.

Then, we investigated in wild-type mice the possible contribution of bradykinin B<sub>2</sub> receptors to the AT<sub>2</sub>-dependent dilation in response to flow. The B<sub>2</sub> receptor antagonist HOE-140 did not further decrease the response to flow in TK<sup>+/+</sup> 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.<sup>10</sup> Taken together, these results show that if bradykinin B<sub>2</sub> receptors are blocked or if the vascular kinin-kallikrein system is inactivated, the AT<sub>2</sub> receptor antagonist PD123319 no longer decreases the response to flow. Similarly, if AT<sub>2</sub> receptors are blocked or not expressed, the bradykinin B<sub>2</sub> receptor antagonist HOE-140 no longer inhibits flow-induced dilation. Thus, the present data demonstrate that the involvement of AT<sub>2</sub> receptors in flow-dependent dilation requires the presence of both functional bradykinin B<sub>2</sub> 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<sub>2</sub> receptors. This interpretation is in agreement with previous studies indicating that AT<sub>2</sub>-dependent vasodilatation after

exposure to exogenous angiotensin II involves bradykinin B<sub>2</sub> receptor activation.<sup>17,20,23</sup>

Although the exact mechanism linking AT<sub>2</sub> receptors and B<sub>2</sub> 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.<sup>35,36</sup> 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.<sup>37</sup> However, an increase in ACE activity would also contribute to a greater degradation of locally formed kinins,<sup>38</sup> thus counterbalancing the effect of an augmented synthesis of angiotensin II. Interestingly, the lack of additional effect of the AT<sub>2</sub> and the B<sub>2</sub> 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 AT<sub>2</sub> pathway might precede the stimulation of endogenous kinins synthesis and B<sub>2</sub> receptors, as indicated from a recent study by Katada and Majima.<sup>23</sup> This interpretation is also supported by data from this study and previous work<sup>10</sup> as bradykinin response was unchanged after inactivation of either vascular kallikrein or AT<sub>2</sub> receptors. Furthermore, overexpression of AT<sub>2</sub> receptors in vascular smooth muscle cells increases intracellular acidosis, resulting in an increase in kininogenase activity and in turn the generation of kinins.<sup>25</sup> However, activation of kininogenase(s) by lowering intracellular pH after AT<sub>2</sub> receptor activation is not fully compatible with the known in vitro characteristics of tissue kallikrein activation.<sup>39</sup> Alternatively, the present results may suggest a direct molecular interaction between B<sub>2</sub> and AT<sub>2</sub> receptors, comparable to the one recently described for AT<sub>1</sub> and AT<sub>2</sub>,<sup>40</sup> but these different hypotheses would require further investigations.

In conclusion, the present study demonstrates that in mice carotid arteries, the vascular kinin-kallikrein system contributes to one third of flow-induced dilation and requires the presence of both functional angiotensin AT<sub>2</sub> and bradykinin B<sub>2</sub> receptors.

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