Angiotensin II Type 2 Receptor Expression After Vascular Injury
Differing Effects of Angiotensin-Converting Enzyme Inhibition and Angiotensin Receptor Blockade

Thomas A. Barker, Michael P. Massett, Vyacheslav A. Korshunov, Amy M. Mohan, Amy J. Kennedy, Bradford C. Berk

Abstract—It has been suggested that the effects of angiotensin II type 1 receptor (AT₁R) blockers are in part because of angiotensin II type 2 receptor (AT₂R) signaling. Interactions between the AT₂R and kinins modulate cardiovascular function. Because AT₂R expression increases after vascular remodeling of the AT₁R blocker valsartan and the ACE inhibitor benazepril require AT₂R signaling through the bradykinin 1 and 2 receptors (B₁R and B₂R). To test this hypothesis, Brown Norway rats were assigned to 8 treatments (n=16): valsartan, valsartan+PD123319 (AT₂R inhibitor), valsartan+des-arg⁹-[Leu⁹]-bradykinin (B₂R inhibitor), valsartan+HOE140 (B₂R inhibitor), benazepril, benazepril+HOE140, amlodipine, and vehicle. After 1 week of treatment, carotid balloon injury was performed. Two weeks later, carotids were harvested for morphometry and analysis of receptor expression by immunohistochemistry and Western blotting. Valsartan and benazepril significantly reduced the intima/media ratio compared with vehicle. Blockade of AT₂R, B₁R, or B₂R in the presence of valsartan prevented the reduction seen with valsartan alone. B₂R blockade inhibited the effect of benazepril. Injury increased AT₁R, AT₂R, B₁R, and B₂R expression. Treatment with valsartan but not benazepril significantly increased intima AT₂R expression 2-fold compared with vehicle, which was not reversed by inhibition of AT₁R, B₁R, and B₂R. Functionally, valsartan increased intimal cGMP levels compared with vehicle, and this increase was inhibited by blocking the AT₁R, B₁R, and B₂R. Results suggest that AT₂R expression and increased cGMP represent a molecular mechanism that differentiates AT₁R blockers, such as valsartan, from angiotensin-converting enzyme inhibitors like benazepril. (Hypertension. 2006; 48:1-8.)

Key Words: valsartan ■ angiotensin ■ AT₂R ■ restenosis ■ rat

Angiotensin II (Ang II) exerts its effects primarily through 2 functionally distinct receptors, the angiotensin type 1 receptor (AT₁R) and angiotensin type 2 receptor (AT₂R). The AT₁R is widely distributed in adults, whereas the AT₂R is only highly expressed in the fetus. However, AT₂R expression increases in pathological conditions, such as vascular injury. These 2 receptors exert effects that are generally considered antagonistic. Blocking Ang II activity with angiotensin-converting enzyme (ACE) inhibitors reduces the risk of mortality in patients at high risk for cardiovascular events. Recently, AT₂R blockers (ARBs) have been shown to be effective in reducing stroke in patients with left ventricular hypertrophy and mortality in heart failure patients.

Kinins exert diverse physiological actions, including vasodilation, increased capillary permeability, and inflammation. The major kinins, which are agonists at the bradykinin type 2 receptor (B₂R), are metabolized to produce the des-Arg⁹-kinins, which are agonists at bradykinin type 1 receptors (B₁Rs). Although the B₁R is widely and constitutively expressed, the B₂R is normally expressed at low levels, although it can be induced by growth factors and cytokines.

The renin–angiotensin and kinin systems are intimately linked, because ACE metabolizes both angiotensin I and bradykinin. Recently, an AT₂R pathway has been shown to signal via the B₂R to produce vasodilation in resistance vessels and coronary microvessels, to regulate blood pressure, to mediate renal production of NO, and to promote cardiac fibrosis.

Treatment of hypertensive patients with ARBs and ACE inhibitors decreases cardiovascular events and renal failure to an extent greater than predicted by the reduction in blood pressure. Because there is increased Ang II during blockade of the AT₁R with ARBs, it has been suggested that some beneficial effects of ARBs may be because of actions of Ang II at the AT₂R. As discussed above, there may be an

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important link between AT₉R signaling and B₁R pathways. This interaction might be particularly important in vascular injury where both the AT₁R and B₁R are known to modulate the tissue response as shown by pharmacological and genetic inhibition of these receptors. Previous work showed that ACE inhibition and the ARB valsartan decreased neointima formation after rabbit carotid injury. Specifically, both treatments reduced circulating endothelin-1, thromboxane B₂, and 6-keto-prostaglandin F₁α, but changes in the vessel wall itself were not studied. Here we hypothesize that the beneficial effects in vascular injury of AT₁R blockade and ACE inhibition are mediated, in part, by a pathway involving AT₁R signaling via the B₁R and B₂R, which increases vascular cGMP levels. We measured expression of these receptors quantitatively in the injured vessel, as well as the effects of receptor blockade on intima formation. Our results show that the valsartan reduction in intima formation correlates with expression of the AT₁R and cGMP in the intima and media. In contrast, the ACE inhibitor benazepril also decreased intima formation but did not increase AT₁R expression or intima and media cGMP levels, suggesting a different mechanism of action.

Methods

Animal Surgery, Drug Treatment, and Analyses

Fourteen-week-old male Brown Norway rats purchased from Charles River (Wilmington, MA) were used in accordance with the guidelines of the National Institutes of Health and American Heart Association for the care and use of laboratory animals (approved by the University of Rochester Committee on Animal Resources). Rats (250 to 300 g) were randomly assigned to 8 treatments (n = 16 per group): (1) valsartan at 30 mg/kg per day (AT₁R inhibitor, Novartis Pharmaceuticals), (2) valsartan at 30 mg/kg per day plus PD123319 at 30 mg/kg per day (AT₁R inhibitor); (3) valsartan at 30 mg/kg/d plus des-arg-[Leu]⁻bradykinin at 10 mg/kg per day (B₁R inhibitor, Bachem USA); (4) valsartan at 30 mg/kg per day plus HOE140 at 500 µg/kg per day (B₁R inhibitor, Bachem USA); (5) benazepril at 20 mg/kg per day (Novartis Pharmaceuticals); (6) benazepril at 30 mg/kg per day plus HOE140 at 500 µg/kg per day; (7) amlodipine at 20 mg/kg per day (Novartis Pharmaceuticals); and (8) vehicle (0.15 mol/L NaOH). In preliminary experiments, we compared the blood pressure reduction obtained with several doses of valsartan and benazepril and chose doses that gave an equivalent reduction after 2 weeks. We did not have groups with individual AT₁R, B₁R, and B₂R blockers, because previous work already showed the effects of these compounds alone on neointima formation. Drugs were administered daily by intraperitoneal injection at 9:00 AM. We did not include HOE140 or des-arg-[Leu]⁻bradykinin alone, because previous studies showed no effect on intima formation. Drugs were administered immediately after the procedure and 6 hours later. Fourteen days after injury, half of the rats (n = 8) were perfused with 10% formaldehyde (100 mm Hg), and carotids were paraffin embedded. Sections (4 µm thick) at the midpoint of the common carotid and 0.5 mm proximal and distal to the midpoint were obtained. Carotids from the remaining animals were frozen in liquid nitrogen for biochemical analysis.

Morphometry and Immunohistochemistry

After staining with hematoxylin/eosin, sections were analyzed by MicroComputer Imaging Device (MCID) and software (Imaging Research Incorporated). Measurements were made for each of the 3 sites sampled and averaged. Antibodies included: AT₁R (1:200 polyclonal rabbit, Santa Cruz Biotechnology), AT₁R (1:2500 polyclonal goat, Santa Cruz Biotechnology), B₁R (1:1000 polyclonal goat Santa Cruz Biotechnology), B₂R (1:100 polyclonal mouse BD Biosciences), and cGMP (1:2000 polyclonal sheep, kindly donated by Dr J de Vente, Maastricht University, Maastricht, the Netherlands). With the exception of the cGMP, antigen retrieval with 10 mmol/L of citrate buffer at pH 6.0 heated to 120°C and pressure to 10 PSI for 20 minutes was used. For quantitation, thresholds were set based on no primary antibody and converted to grayscale for MCID software. No counterstain was used. Grayscale quantitation had an interobserver correlation of 0.97. After analysis, coverslips were removed and slides counterstained with hematoxylin.

Immunoblot Analysis

Carotids were lysed and sonicated, nonsolubilized proteins removed by centrifugation, and the supernatant subjected to electrophoresis on a 7% sodium dodecyl sulfate polyacrylamide gel. Immunoblots were performed at dilutions of 1:100 (AT₁R, AT₁R, and B₁R) or 1:500 (B₂R). The dominant band on Western blot chosen for quantitative analysis was of the following molecular mass (based on expression of receptor cDNAs in Chinese hamster ovary cells, vessel wall, and the manufacturer’s information): AT₁R 60 kDa, AT₁R 43 kDa, B₂R 45 kDa, and B₁R 70 kDa. It is likely that the 60-kDa form of the AT₁R is the fully glycosylated form. A limitation of the immunoblot analysis is the unavailability of the immunizing peptides for preadsorption control studies to demonstrate specificity. Odyssey infrared imaging system (LiCOR) was used to visualize proteins and Odyssey software used for quantification.

Statistical Analysis

Analyses were performed using SAS Version 9.1 (SAS Institute). Because of sample sizes and number of comparisons, only comparisons with P < 0.01 were classified as significant (0.01 set a priori). Baseline mean arterial pressure (MAP), heart rate (HR), heart weight/body weight ratio, and Western blot group comparisons were computed using PROC GLM. If group was found to be a significant predictor, then the LSMEANS statement was added to compute the multiple comparisons. The last 3 MAP and HR measurements were averaged for each subject and then modeled in the same fashion as the baseline MAP measurement. The morphometry measurements were analyzed using repeated measures ANOVA. If the group was found to be significant, then the LSMEANS statement was used again for the multiple comparisons, and P < 0.01 was classified as significant. When comparing the morphometry measurements for right carotid artery (RCA) versus left carotid artery (LCA), the difference between the 2 measurements was analyzed to remove the correlation of the measurements using PROC GENMOD with the same predictors as for RCA and LCA alone.

The immunohistochemistry receptor data were all paired; therefore, all of the analyses were completed using a paired t test, if the data were normal. For Western blot RCA–LCA data, the difference between the measurements was calculated, and a nonparametric signed rank test was performed.

Results

Physiological Parameters

Compared with vehicle, amlodipine-treated rats had a 10-mm Hg reduction in MAP after 1 week of treatment (Figure IA, available online at http://hyper.ahajournals.org). Valsartan and benazepril significantly reduced MAP to an extent similar to amlodipine. There was no significant difference in the MAP between amlodipine and valsartan or benazepril groups. The effect of valsartan on MAP was unaltered by the
Specifically, there was a significant 50% reduction in lumen area expected changes in the vehicle group (Figure 2A and 2B).

Vessel Morphometry of the uninjured RCA was not altered significantly by drug treatment. The lumen, media, and external elastic lamina (EEL) areas for the RCA did not differ across groups (data not shown). There was no significant difference in lumen areas among the vascular remodeling showed no significant changes among groups.

To correct for variation in vessel size, the intima:media (I:M) ratio was calculated for each group (Figure 1E). Compared with vehicle, amlodipine showed no reduction in I:M ratio, whereas valsartan significantly reduced the I:M ratio. When PD123319 was given with valsartan, the I:M ratio returned to vehicle levels. HOE140. There was no significant difference in HR at baseline:body weight ratio also was not significantly different between groups. (Table).

Effect of Injury and Treatment on Vessel Morphometry

Morphometry of the uninjured RCA was not altered significantly by drug treatment. The lumen, media, and external elastic lamina (EEL) areas for the RCA did not differ across groups (Table IS). An intima developed 2 weeks after balloon injury of the LCA (Figure 1B). Comparison of vessel morphology between injured LCA and uninjured RCA (Table and Table IS, available online at http://hyper.ahajournals.org) showed the expected changes in the vehicle group (Figure 2A and 2B). Specifically, there was a significant 50% reduction in lumen area of the LCA (LCA: 137±10 versus RCA: 256±13×10³ μm²; P=0.0001) that was primarily because of intima formation in the LCA (134±13×10³ μm²). There were small increases in media and EEL area in the LCA that did not differ significantly from the RCA.

Treatment with valsartan had no effect on the RCA but dramatically altered morphology in the injured LCA (Figure 1C). Valsartan significantly decreased intima area by 51% compared with the vehicle (Figure 1C and Table; P<0.0001). There was no significant difference in lumen areas among the valsartan treatment groups (Table), although there was a trend toward valsartan alone having a larger lumen than vehicle (32% increase). Blocking AT₂R with PD123319 in the presence of valsartan also increased intima area compared with valsartan alone. Similarly, valsartan plus des-arg⁹-[Leu¹]-bradykinin had significantly greater intima than valsartan alone. Blocking B₁R with HOE140 in the presence of valsartan also increased intima area compared with valsartan alone, but this was of borderline significance (P=0.03). Amlodipine did not inhibit intima formation, suggesting that the effect of valsartan was not primarily because of lowering blood pressure. Although AT₂R, B₁R, and B₂R blockade in association with valsartan reversed the effect of valsartan alone on intima, these groups showed a trend for smaller intima than the vehicle-treated group. This result is not expected because it is unlikely that any individual receptor completely mediates the valsartan effect (Table).

Valsartan alone also increased the media area compared with vehicle (99±6 versus 75±6×10³ μm²; P=0.003). Inhibiting the AT₂R, B₁R, or B₂R completely blocked the effect of valsartan on media area. Other measurements of vascular remodeling showed no significant changes among groups.

All data shown as mean±SEM. Val indicates valsartan; PD, PD123319; des-BK, des-arg⁹-[Leu¹]-bradykinin.

†Significantly different from other groups (P<0.01).

*Significantly different from other groups except valsartan plus HOE140, P<0.01.

†Significantly different from other groups (P<0.01).

Morphometry Measurements of Injured LCA Treatment Groups

<table>
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<tr>
<th>Group</th>
<th>Lumen (×10³ μm²)</th>
<th>Intima (×10³ μm²)</th>
<th>Media (×10³ μm²)</th>
<th>EEL (×10³ μm²)</th>
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<tr>
<td>Ben+HOE</td>
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<td>128±18</td>
<td>82±6</td>
<td>366±22</td>
</tr>
</tbody>
</table>

All data shown as mean±SEM. Val indicates valsartan; PD, PD123319; des-BK, des-arg⁹-[Leu¹]-bradykinin.

†Significantly different from other groups (P<0.01).

*Significantly different from other groups except valsartan plus HOE140, P<0.01.

†Significantly different from other groups (P<0.01).
I:M ratio. These data strongly suggest that the AT,R, B,R, and B,R participate in the valsartan effect to decrease intima formation.

Benazepril significantly decreased the intima area by 69% and the I:M ratio compared with vehicle (Figure 1D and 1E and Table; P<0.0001). Benazepril significantly increased the lumen area by 59% compared with vehicle (Table). The changes in intima, I:M ratio, and lumen were inhibited by the addition of HOE140. Media and EEL area were unchanged by treatment with benazepril (Table).

**Immunohistochemistry of Ang II and Bradykinin Receptors in Vehicle-Treated Rats**

To quantitate changes in receptor expression, we performed immunohistochemistry and used the MCID system. Controls without primary antibody were optimized to minimize background staining. In the uninjured RCA of the vehicle group, there was substantial expression of both AT,R and B,R in media but not in adventitia (Figure II A and III D). In contrast, there was little AT,R expression or B,R expression anywhere (Figure III B and IIC). A previous study by Hutchinson et al² showed that after injury, AT,R expression peaked at 48 to 72 hours, whereas AT,R expression was slower, peaking at 96 to 144 hours.

After injury, AT,R was highly expressed in intima, with no expression in media or adventitia (Figure 2A). The AT,R and B,R were both highly expressed in intima and media (Figure III A and III C) but not adventitia. The B,R was expressed primarily in the intima after injury but was also present to a lesser extent in media (Figure III B).

**Immunohistochemistry of Ang II and Bradykinin Receptors in Drug-Treated Rats**

In the uninjured RCA, there was no change in receptor expression by any of the drugs compared with vehicle (data not shown). In the LCA, amlodipine did not alter expression of the 4 receptors, including the AT,R (Figure 2B and Figures IV to VI B). This finding suggests that altered Ang II and kinin receptor expression are primarily regulated by tissue injury, rather than by blood pressure.

In rats treated with valsartan, the most impressive change in the LCA was increased AT,R expression in the intima that was significantly greater compared with vehicle or amlodipine (>2 fold increase; P=0.002; Figures 2C and 3). There was also a small increase in AT,R expression in the media. There were no differences in AT,R, B,R, and B,R expression with valsartan compared with vehicle or amlodipine in the LCA (Figures IV to VI).

Surprisingly, the addition of AT,R, B,R, or B,R blockers to valsartan did not affect the increase in AT,R, which was ≈2-fold increased for all of the groups compared with vehicle (Figure 2D through 2F and 2I). Together these results suggest that the effect of valsartan on AT,R expression after injury is related to elevated Ang II levels, because blockade of the AT,R did not reverse this effect. Finally, there was no significant difference in expression of AT,R, B,R, and B,R in these groups compared with vehicle, amlodipine, or valsartan (Figure IV through VI). There was no nonspecific immunoreactivity for the receptor antibodies (Figure VII). Benazepril did not alter AT,R, AT,R, B,R, or B,R expression compared with vehicle (Figures 2G and 2H, 3, and IV through VI), suggesting that the mechanism by which ACE inhibitors reduce intima formation differs from ARBs, especially in relation to AT,R expression.

**Immunoblot Analysis**

The increase in expression of AT,R, AT,R, B,R, and B,R after balloon injury was confirmed by Western blotting (Figure 4A). Ponceau staining showed equal protein loading (data not shown). Increased AT,R expression in the injured LCA of valsartan-treated groups was apparent by immunoblot (Figure 4B). Consistent with the immunohistochemistry, the increase in AT,R with valsartan differed significantly from vehicle and amlodipine (Figure 4C), whereas there was no difference in AT,R protein levels in the benazepril group (Figure 4B and 4C). Valsartan in the absence of injury caused no change in expression of AT,R, AT,R, B,R, and B,R (data not shown).

**Correlation Analysis of I:M Ratio and Receptor Expression**

To evaluate the relationship between receptor expression and intima formation, correlation analyses were performed using quantitative measurements of immunohistochemical receptor abundance. Among multiple comparisons, the only significant correlation between I:M ratio and receptor expression was a negative correlation between AT,R and I:M ratio (details in Figure 5). Equally important is the obvious difference between benazepril and valsartan; both drugs significantly reduced I:M ratio, but only valsartan increased AT,R expression, suggesting that their mechanism of action is different.

**Immunohistochemistry of cGMP in Drug-Treated Rats**

The valsartan group had a 2.6±1.1-fold increase in cGMP in the intima (Figure 6C and 6E, normalized to media) compared with vehicle-treated rats (Figure 6A and 6E). The increase in intima cGMP was completely blocked by both PD123319 and HOE140 (Figure 6E). Benazepril increased cGMP in the endothelium (Figure 6D, arrows), but intima and media cGMP did not differ from vehicle. These findings further support the concept that valsartan and benazepril inhibit intima formation by different mechanisms.

**Discussion**

The 4 major findings of this study are that the valsartan-mediated reduction in intima formation after rat carotid balloon injury: (1) differs mechanistically from benazepril; (2) correlates with AT,R and cGMP expression in the intima; (3) depends on receptor-mediated events that require the AT,R, B,R, and B,R; and (4) is not mediated by lowering blood pressure. We confirmed previous findings that expression levels of the AT,R, AT,R, B,R, and B,R were increased by balloon injury. The present study is the first to measure receptor expression in response to both injury and drug treatment. Our results are supported by previous work showing that both captopril and valsartan decreased intima formation after rabbit carotid injury, although this article...
did not measure receptor expression. Importantly, we show for the first time that treatment with valsartan further increased AT$_2$R protein expression, primarily in the intima. Increased expression of the AT$_1$R was not affected by AT$_2$R blockade or by blockade of B$_1$R or B$_2$R. Because AT$_1$R, B$_1$R, and B$_2$R expression were not affected by treatment with valsartan, our results suggest a critical role for an AT$_2$R-cGMP pathway in the ability of valsartan to decrease intima. This concept is supported by the inverse relationship between expression of AT$_2$R and cGMP with the I:M ratio for valsartan (Figure 5). Another novel finding is the difference between valsartan and benazepril with respect to changes in AT$_2$R expression and cGMP. Valsartan increased both AT$_2$R expression and cGMP in the neointima, whereas benazepril had no effect on the neointima and increased cGMP only in the endothelium. This association suggests that increased AT$_2$R expression by valsartan may explain increased cGMP, as proposed by Carey and colleagues, for the kidney.

Based on these results, we propose the following mechanism for valsartan. Valsartan blocks the AT$_1$R and increases Ang II levels. Valsartan increases AT$_2$R expression that now increases kininogenase activity and generates bradykinin. Bradykinin-mediated activation of B$_1$R and B$_2$R inhibits intima formation, in part via endothelial cell generation of NO and increased cGMP in vascular smooth muscle cell (VSMC). Although it is possible that the effects on AT$_2$R are parallel to the B$_1$R and B$_2$R, this seems unlikely, because individual blockade would result in only a partial reversal of the effects of valsartan. Finally, there may be direct effects mediated by the AT$_2$R, especially via heterodimerization with the B$_1$R and B$_2$R, to inhibit intima formation. Data to support the model include reports that valsartan increases Ang II levels, thus increasing AT$_2$R activation. The importance of increased Ang II has been demonstrated in the angiotensinogen transgenic mouse in which AT$_1$R signaling induces angiogenesis. We propose that a similar mechanism exists.
in vascular injury. AT2R mRNA is induced by vascular injury, and we confirm increased AT2R protein using immunohistochemistry and Western blotting. Overexpression of the AT2R has been shown to reduce intima formation in the balloon-injured rat. Here we show by pharmacological inhibition that the AT2R is required for valsartan inhibition of intima formation.

Because AT2R expression is normally low in adults, the increased expression in pathological situations is likely an important mechanism by which this receptor contributes to the tissue response to injury. Crosstalk between the AT1R and AT2R potentially occurs by several mechanisms, the best characterized being generation of regulatory peptides, such as bradykinin and antagonism of intracellular signaling, whereas heterodimerization remains more speculative. Thus, the effects of ARBs in this situation are likely because of increased AT2R and bradykinin signaling, as well as AT1R inhibition.

Our study confirms that AT2R expression is important after vascular injury and shows that AT2R expression occurs primarily in the intima.

We show for the first time that AT2R expression is induced by valsartan in blood vessels. Because AT1R, B1R, and B2R inhibition in association with AT1R blockade did not change this effect, the most likely explanation is that increased Ang II levels upregulate AT2R expression. Increased Ang II levels were shown previously to induce AT2R and bradykinin signaling, as well as AT1R inhibition. Our study confirms that AT2R expression is important after vascular injury and shows that AT2R expression occurs primarily in the intima.

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Figure 3. Intimal AT2R immunoreactivity. The relative intimal area stained by AT2R antibody was quantitated relative to the vehicle group, which was arbitrarily set to 1.0. *Significantly different from vehicle.

Figure 4. Immunoblot analysis of receptor expression. (A) Comparison of expression for AT1R, AT2R, B1R, and B2R proteins in the vehicle group. (B) Comparison of LCA AT1R protein expression between the treatment groups. (C) The relative AT2R immunoreactivity was quantitated relative to the vehicle group, which was arbitrarily set to 1.0. *Significantly different from vehicle and amlodipine.

Figure 5. Correlation analysis of AT2R expression and I:M ratio. Shown is a plot of means for I:M ratio and AT2R for each treatment group. Correlation analysis was performed using linear regression. Significant correlations between I:M ratio and AT2R were observed for combined control, valsartan, and amlodipine groups ($R^2=0.47; P=0.013$). No significant correlation was observed for combined control, benazepril, benazepril+HOE, and amlodipine groups ($R^2=0.05$) or for combined control, valsartan+PD, valsartan+HOE, valsartan+des-bradykinin, and amlodipine groups ($R^2=0.00$).
AT2R, which signals downstream through both the B1R and the B2R, most likely by altering cGMP levels in the injured vessels. A more speculative mechanism may be heterodimerization of the AT2R with the B1R and B2R, which may augment signaling by AT2R. This mechanism would explain why increased AT2R in the intima would inhibit VSMC proliferation and why blockade of either the B1R or B2R blunted the effect of valsartan on intima formation.

Previous studies of intima formation in the mouse carotid after ligation showed no effect of B1R or B2R antagonists on intima.35 In cultured VSMCs, bradykinin was shown to both stimulate36 and inhibit growth.23 The fact that blocking either B1R or B2R abrogated the ability of valsartan to limit intima formation is somewhat surprising, especially because blockade did not decrease AT2R expression. However, it is reasonable to speculate that bradykinin receptor–specific signals from both receptors (either in the same cell or different cells) are required to limit VSMC proliferation.

ARBs clearly exert effects through mechanisms that differ from ACE inhibitors. Benazepril did not alter AT2R receptor expression level (Figure 5), suggesting that ACE inhibitors primarily reduce intima formation by affecting bioavailability of receptor ligands (ie, decreased Ang II and increased bradykinin). In addition, ACE inhibitors may also directly affect intracellular signaling as shown by Kohlstedt et al.37 Previous studies found that HOE140 reversed ACE inhibitor-mediated reduction of intima, suggesting a key role for B2R. Our results (Figure 1) support this concept.

**Perspectives**

The present study provides insights into the similarities and differences in the vascular protective mechanisms of ACE inhibitors and ARBs. Our data suggest that the major similarity between ACE inhibition and AT1R blockade is via bradykinin actions at the B2R. An important mechanistic difference is the role of increased AT2R expression in the actions of ARBs. Despite the fact that both ACE inhibition and AT1R blockade require B2R function, the increase in vascular cGMP was much greater in the presence of AT1R blockade and increased AT2R expression. These results suggest that heterodimerization of the AT1R with the B1R and B2R or a unique feature of AT2R signaling increases cGMP. An interesting possibility for future study is the effect of AT2R on vascular cGMP phosphodiesterases, which, if downregulated, would increase cGMP levels.

Another concept that could be explored is the effect of agonists that stimulate increase AT2R expression or increase its activity on the vascular response to injury. Finally, our results provide further mechanistic insight into why combined ARB–ACE inhibitor therapy may be clinically beneficial in some situations.

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**Disclosures**

None.

**References**


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AT_{2}R expression following vascular injury:

Differing effects of ACE inhibition and angiotensin receptor blockade


Online Data Supplement
# Table I. Morphometry measurements of uninjured RCA treatment groups

<table>
<thead>
<tr>
<th></th>
<th>Lumen x10³ [m²]</th>
<th>Media x10³ [m²]</th>
<th>EEL x10³ [m²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>256 ± 13</td>
<td>63 ± 2</td>
<td>318 ± 13</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>249 ± 8</td>
<td>59 ± 2</td>
<td>309 ± 8</td>
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<tr>
<td>Valsartan</td>
<td>240 ± 18</td>
<td>65 ± 3</td>
<td>305 ± 19</td>
</tr>
<tr>
<td>Val + PD</td>
<td>220 ± 15</td>
<td>64 ± 3</td>
<td>284 ± 12</td>
</tr>
<tr>
<td>Val + des-BK</td>
<td>264 ± 16</td>
<td>63 ± 2</td>
<td>327 ± 15</td>
</tr>
<tr>
<td>Val + HOE</td>
<td>244 ± 16</td>
<td>60 ± 3</td>
<td>303 ± 16</td>
</tr>
<tr>
<td>Benazepril</td>
<td>281 ± 10</td>
<td>61 ± 2</td>
<td>342 ± 11</td>
</tr>
<tr>
<td>Ben + HOE</td>
<td>259 ± 17</td>
<td>63 ± 3</td>
<td>322 ± 20</td>
</tr>
</tbody>
</table>

All data shown as mean ± SEM. Val = valsartan; PD = PD123319; des-BK = des-arg⁸-[Leu⁴]-bradykinin; Ben = benazepril; EEL = external elastic lamina area.
Supplement Figure Legends

**Figure I.** Comparison of (A) mean arterial blood pressure (n = 5 per group) and (B) heart rate (n = 5 per group) between groups. * Significantly different from vehicle (p<0.01).

**Figure II.** Representative vehicle treated uninjured RCA cross-sections stained (brown) with antibodies for (A) AT\(_1\)R, (B) AT\(_2\)R, (C) B\(_1\)R and (D) B\(_2\)R.

**Figure III.** Representative injured LCA cross-sections stained (brown) with antibody for (A) AT\(_1\)R, (B) B\(_1\)R and (C) B\(_2\)R.

**Figure IV.** Representative injured LCA cross-sections stained (brown) with AT\(_1\)R antibody for (A) vehicle, (B) amlodipine, (C) valsartan, (D) valsartan plus PD123319, (E) valsartan plus des-arg\(^9\)-[Leu\(^8\)]-bradykinin, (F) valsartan plus HOE140, (G) benazepril, and (H) Benazepril plus HOE140.

**Figure V.** Representative injured LCA cross-sections stained (brown) with B\(_1\)R antibody for (A) vehicle, (B) amlodipine, (C) valsartan, (D) valsartan plus PD123319, (E) valsartan plus des-arg\(^9\)-[Leu\(^8\)]-bradykinin, (F) valsartan plus HOE140, (G) benazepril, and (H) Benazepril plus HOE140.
**Figure VI.** Representative injured LCA cross-sections stained (brown) with B,\(_2\)R antibody for (A) vehicle, (B) amlodipine, (C) valsartan, (D) valsartan plus PD123319, (E) valsartan plus des-arg\(^9\)-[Leu\(^8\)]-bradykinin, (F) valsartan plus HOE140, (G) benazepril, and (H) Benazepril plus HOE140.

**Figure VII.** Immunohistochemistry negative controls for (A) AT\(_1\)R, (B) AT\(_2\)R, (C) B,\(_1\)R and (D) B,\(_2\)R.
Figure I

A

<table>
<thead>
<tr>
<th>Baseline</th>
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<tr>
<td>Veh</td>
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<tr>
<td>Val</td>
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<td>Val + PD</td>
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<td>Val + des-BK</td>
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<td>Val + HOE</td>
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<tr>
<td>Ben</td>
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<td>Ben + HOE</td>
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B

<table>
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<tr>
<th>Veh</th>
<th>Aml</th>
<th>Val</th>
<th>Val + PD</th>
<th>Val + des-BK</th>
<th>Val + HOE</th>
<th>Ben</th>
<th>Ben + HOE</th>
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Barker et al.

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Figure II

A

AT\textsubscript{1}R RCA Vehicle

B

AT\textsubscript{2}R RCA Vehicle

C

B\textsubscript{1}R RCA Vehicle

D

B\textsubscript{2}R RCA Vehicle
Figure III

A
AT\textsubscript{1}R LCA
Vehicle

B
B\textsubscript{1}R LCA
Vehicle

C
B\textsubscript{2}R LCA
Vehicle
Figure IV

A. LCA Veh
B. LCA Aml
C. LCA Val
D. LCA Val+PD
E. LCA Val+des-BK
F. LCA Val+HOE
G. LCA Ben
H. LCA Ben+HOE
Figure V

A
LCA Veh

B
LCA Aml

C
LCA Val

D
LCA Val+PD

E
LCA Val+des-BK

F
LCA Val+HOE

G
LCA Ben

H
LCA Ben+HOE
Figure VI

A: LCA Veh

B: LCA Ami

C: LCA Val

D: LCA Val+PD

E: LCA Val+des-BK

F: LCA Val+HOE

G: LCA Ben

H: LCA Ben+HOE
Figure VII

A

AT$_1$R

B

AT$_2$R

C

B$_1$R

D

B$_2$R