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 AT1R and kinins modulate cardiovascular function. Because AT2R expression increases after vascular injury, we hypothesized that the effects on vascular remodeling of the AT1R blocker valsartan and the ACE inhibitor benazepril require AT1R 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 (AT1R inhibitor), valsartan+des-Arg^9-[Leu^9]-bradykinin (B1R 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 AT1R, 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 AT1R 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 AT1R expression and increased cGMP represent a molecular mechanism that differentiates AT1R blockers, such as valsartan, from angiotensin-converting enzyme inhibitors like benazepril. (Hypertension. 2006; 48:942-949.)

Key Words: valsartan ▪ angiotensin ▪ AT1R ▪ restenosis ▪ rat
important link between AT\(_R\) signaling and B\(_R\) pathways.\(^{11}\) This interaction might be particularly important in vascular injury where both the AT\(_R\) and B\(_R\) are known to modulate the tissue response as shown by pharmacological\(^{2,19}\) and genetic\(^{20}\) inhibition of these receptors. Previous work showed that ACE inhibition and the ARB valsartan decreased neointima formation after rabbit carotid injury.\(^{21}\) Specifically, both treatments reduced circulating endothelin-1, thromboxane B\(_2\), and 6-keto-prostaglandin F\(_1\alpha\), but changes in the vessel wall itself were not studied. Here we hypothesize that the beneficial effects in vascular injury of AT\(_R\) blockade and ACE inhibition are mediated, in part, by a pathway involving AT\(_R\) signaling via the B\(_R\) and B\(_2\)R, 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 AT\(_R\) and cGMP in the intima and media. In contrast, the ACE inhibitor benazepril also decreased intima formation but did not increase AT\(_R\) 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 (AT\(_R\) inhibitor, Novartis Pharmaceuticals), (2) valsartan at 30 mg/kg per day plus PD123319 at 30 mg/kg per day (AT\(_R\) inhibitor); (3) valsartan at 30 mg/kg/d plus des-arg\(_9\)-[Leu\(_8\)]-bradykinin at 10 mg/kg per day (B\(_1\)R inhibitor, Bachem USA); (4) valsartan at 30 mg/kg per day plus HOE140 at 500 μg/kg per day (Novartis Pharmaceuticals); (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 molar/L of citrate buffer at pH 6.0 heated to 120°C and pressured 10 mmol/L of citrate buffer at pH 6.0 heated to 120°C and pressured for 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 lyzed and sonicated, nonsolubilized proteins removed by centrifugation, and the supernatant subjected to electrophoresis on a 7.5% sodium dodecyl sulfate polyacrylamide gel. Immunoblots were performed at dilutions of 1:100 (AT\(_R\), AT\(_R\), and B\(_R\)) or 1:500 (B\(_R\)). 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): AT\(_R\), 60 kDa, AT\(_R\), 43 kDa, B\(_R\), 45 kDa, and B\(_R\), 70 kDa. It is likely that the 60-kDa form of the AT\(_R\) 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 measurements.

The morphometry measures 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 left carotid artery (LCA) versus right carotid artery (RCA), 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 of the LCA (LCA: 137±10 versus RCA: 256±13×10^3 μm^2; P=0.0001) that was primarily because of intima formation in the LCA (134±13×10^3 μm^2). 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 AT\(_2\)R with PD123319 in the presence of valsartan significantly increased the intima area compared with valsartan alone. Similarly, valsartan plus des-arg^9-[Leu^8]-bradykinin had significantly greater intima than valsartan alone. Blocking B\(_1\)R 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 AT\(_2\)R, B\(_1\)R, and B\(_2\)R 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±6×10^3 μm^2; P=0.003). Inhibiting the AT\(_2\)R, B\(_1\)R, or B\(_2\)R completely blocked the effect of valsartan on media area. Other measurements of 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. In addition, the B\(_2\)R blocker (des-arg^9-[Leu^8]-bradykinin) and the B\(_2\)R blocker (HOE140) also reversed the valsartan decrease in

**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 addition of PD123319, des-arg^9-[Leu^8]-bradykinin, or HOE140.

The effect of benazepril on MAP was partially inhibited by HOE140. There was no significant difference in HR at baseline or after treatment between groups (Figure 1B). Heart weight:body weight ratio also was not significantly different between groups (data not shown).

**Morphometry Measurements of Injured LCA Treatment Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Lumen (\times10^3 \mu m^2)</th>
<th>Intima (\times10^3 \mu m^2)</th>
<th>Media (\times10^3 \mu m^2)</th>
<th>EEL (\times10^3 \mu m^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>137±10</td>
<td>134±13</td>
<td>75±6</td>
<td>347±12</td>
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<tr>
<td>Amlodipine</td>
<td>129±16</td>
<td>146±14</td>
<td>81±7</td>
<td>356±18</td>
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<td>Valsartan</td>
<td>182±18</td>
<td>68±10</td>
<td>99±6†</td>
<td>351±27</td>
</tr>
<tr>
<td>Val+PD</td>
<td>150±10</td>
<td>113±13</td>
<td>73±4</td>
<td>338±9</td>
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<tr>
<td>Val+des-BK</td>
<td>162±23</td>
<td>121±11</td>
<td>76±6</td>
<td>359±22</td>
</tr>
<tr>
<td>Val+HOE</td>
<td>155±22</td>
<td>118±20</td>
<td>67±8</td>
<td>341±24</td>
</tr>
<tr>
<td>Benazepril</td>
<td>219±26†</td>
<td>55±15†</td>
<td>92±7</td>
<td>366±17</td>
</tr>
<tr>
<td>Ben+HOE</td>
<td>156±18</td>
<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-arg^9-[Leu^8]-bradykinin. *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 IIA and IIB). In contrast, there was little AT,R expression or B,R expression anywhere (Figure IIB and IIC). A previous study by Hutchinson et al \(^2\) 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 IIIA and IIIC) 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 IIIB).

**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 and B,R in media but not in adventitia (Figure II A and III A). In contrast, there was little AT,R expression or B,R expression anywhere (Figure IIIB and IIIC). A previous study by Hutchinson et al \(^2\) 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.

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**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 and B,R in media but not in adventitia (Figure II A and III A). In contrast, there was little AT,R expression or B,R expression anywhere (Figure IIIB and IIIC). A previous study by Hutchinson et al \(^2\) 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.

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 \( \approx 2 \)-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 \( 2.6 \pm 1.1 \)-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,\(^{21}\) although this article.
did not measure receptor expression. Importantly, we show for the first time that treatment with valsartan further increased AT2R protein expression, primarily in the intima. Increased expression of the AT2R was not affected by AT1R blockade or by blockade of B1R or B2R. Because AT1R, B1R, and B2R expression were not affected by treatment with valsartan, our results suggest a critical role for an AT2R-cGMP pathway in the ability of valsartan to decrease intima. This concept is supported by the inverse relationship between expression of AT2R 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 AT2R expression and cGMP. Valsartan increased both AT2R 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 AT2R expression by valsartan may explain increased cGMP, as proposed by Carey and colleagues,14,28 for the kidney.

Based on these results, we propose the following mechanism for valsartan. Valsartan blocks the AT1R and increases Ang II levels. Valsartan increases AT2R expression that now increases kininogenase activity and generates bradykinin. Bradykinin-mediated activation of B1R and B2R 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 AT2R are parallel to the B1R and B2R, 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 AT2R, especially via heterodimerization with the B1R and B2R, to inhibit intima formation. Data to support the model include reports that valsartan increases Ang II levels,29 thus increasing AT2R activation. The importance of increased Ang II has been demonstrated in the angiotensinogen transgenic mouse in which AT1R signaling induces angiogenesis.30 We propose that a similar mechanism exists

Figure 2. Representative carotid artery cross-sections stained with AT2R antibody (brown) for (A) vehicle RCA, (B) amlodipine, (C) valsartan, (D) valsartan plus PD123319, (E) valsartan plus des-arg²-[Leu⁹]-bradykinin, (F) valsartan plus HOE140, (G) benazepril, and (H) benazepril plus HOE140.
in vascular injury. AT\textsubscript{2}R mRNA is induced by vascular injury,\textsuperscript{2} and we confirm increased AT\textsubscript{2}R protein using immunohistochemistry and Western blotting. Overexpression of the AT\textsubscript{2}R has been shown to reduce intima formation in the balloon-injured rat.\textsuperscript{31} Here we show by pharmacological inhibition that the AT\textsubscript{2}R is required for valsartan inhibition of intima formation.

Because AT\textsubscript{2}R 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.\textsuperscript{2} Crosstalk between the AT\textsubscript{1}R and AT\textsubscript{2}R 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 AT\textsubscript{2}R and bradykinin signaling, as well as AT\textsubscript{1}R inhibition.\textsuperscript{11}

Our study confirms that AT\textsubscript{2}R expression is important after vascular injury and shows that AT\textsubscript{2}R expression occurs primarily in the intima.

We show for the first time that AT\textsubscript{2}R expression is induced by valsartan in blood vessels. Because AT\textsubscript{1}R, B\textsubscript{1}R, and B\textsubscript{2}R inhibition in association with AT\textsubscript{1}R blockade did not change this effect, the most likely explanation is that increased Ang II levels upregulate AT\textsubscript{2}R expression. Increased Ang II levels were shown previously to induce AT\textsubscript{2}R and bradykinin signaling, as well as AT\textsubscript{1}R inhibition.\textsuperscript{11}

Our study confirms that AT\textsubscript{2}R expression is important after vascular injury and shows that AT\textsubscript{2}R expression occurs primarily in the intima.

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Previous reports have shown that AT\textsubscript{2}R-dependent events involve increased B\textsubscript{2}R signaling, including renal natriuresis,\textsuperscript{28} regulation of blood pressure,\textsuperscript{34} and vascular relaxation.\textsuperscript{9,11,13} An important signaling mechanism for the AT\textsubscript{2}R is crosstalk with bradykinin via increased bradykinin generation, presumed because of inhibition of the Na\textsuperscript{+}-H\textsuperscript{+} exchanger causing intracellular acidosis and activation of kininogenase activity, which generates bradykinin.\textsuperscript{11} The B\textsubscript{2}R has been shown to reduce intima formation after balloon injury in the rat.\textsuperscript{19} Our study showed that both the B\textsubscript{1}R and the B\textsubscript{2}R were increased by injury and were required for valsartan to reduce intima formation. Combining these results with previous reports of AT\textsubscript{2}R signaling through increased bradykinin and kinin receptors, we propose that the effect of valsartan on intima formation after balloon injury is initially through the

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Intimal AT\textsubscript{2}R immunoreactivity. The relative intimal area stained by AT\textsubscript{2}R antibody was quantitated relative to the vehicle group, which was arbitrarily set to 1.0. *Significantly different from vehicle.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Immunoblot analysis of receptor expression. (A) Comparison of expression for AT\textsubscript{1}R, AT\textsubscript{2}R, B\textsubscript{1}R, and B\textsubscript{2}R proteins in the vehicle group. (B) Comparison of LCA AT\textsubscript{2}R protein expression between the treatment groups. (C) The relative AT\textsubscript{2}R immunoreactivity was quantitated relative to the vehicle group, which was arbitrarily set to 1.0. *Significantly different from vehicle and amlodipine.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Correlation analysis of AT\textsubscript{2}R expression and l:M ratio. Shown is a plot of means for l:M ratio and AT\textsubscript{2}R for each treatment group. Correlation analysis was performed using linear regression. Significant correlations between l:M ratio and AT\textsubscript{2}R were obtained 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 ($R^2 = 0.00$).}
\end{figure}
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 B1Ro rB 2R blunted the effect of valsartan on intima formation.

Previous studies of intima formation in the mouse carotid after ligation showed no effect of B1Ro rB2R antagonists on intima.35 In cultured VSMCs, bradykinin was shown to both stimulate36 and inhibit growth.23 The fact that blocking either B1Ro rB2R abrogated the ability of valsartan to limit intima formation is somewhat surprising, especially because blockade did not decrease AT3R 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 AT3R 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 AT1R expression. 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 AT3R blockade is via bradykinin actions at the B3R. An important mechanistic difference is the role of increased AT3R expression in the actions of ARBs. Despite the fact that both ACE inhibition and AT3R blockade require B3R function, the increase in vascular cGMP was much greater in the presence of AT3R blockade and increased AT3R expression. These results suggest that heterodimerization of the AT3R with the B3R and B3R or a unique feature of AT3R signaling increases cGMP. An interesting possibility for future study is the effect of AT3R 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 AT3R 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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Thomas A. Barker, Michael P. Massett, Vyacheslav A. Korshunov, Amy M. Mohan, Amy J. Kennedy and Bradford C. Berk

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AT$_2$R expression following vascular injury:

Differing effects of ACE inhibition and angiotensin receptor blockade


Online Data Supplement
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lumen (x10^3 \text{m}^2)</th>
<th>Media (x10^3 \text{m}^2)</th>
<th>EEL (x10^3 \text{m}^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>256 ± 13</td>
<td>63 ± 2</td>
<td>318 ± 13</td>
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<tr>
<td>Amlodipine</td>
<td>249 ± 8</td>
<td>59 ± 2</td>
<td>309 ± 8</td>
</tr>
<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-arg⁹-[Leu⁸]-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 B₂R antibody for
(A) vehicle, (B) amlodipine, (C) valsartan, (D) valsartan plus PD123319, (E) valsartan plus des-arginine-[Leu⁸]-bradykinin, (F) valsartan plus HOE140, (G) benazepril, and (H) Benazepril plus
HOE140.

**Figure VII.** Immunohistochemistry negative controls for (A) AT₁R, (B) AT₂R, (C) B₁R and (D) B₂R.
Figure I

A

B

MAP (mmHg) vs. HR (BPM)

Baseline
Treatment

Veh, Aml, Val, Val + PD, Val + des-BK, Val + HOE, Ben, Ben + HOE

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Figure II

A. \(\text{AT}_1\text{R RCA vehicle}\)
B. \(\text{AT}_2\text{R RCA vehicle}\)
C. \(\text{B}_1\text{R RCA vehicle}\)
D. \(\text{B}_2\text{R RCA vehicle}\)
Figure III

A

AT₁R LCA
Vehicle

B

B₁R LCA
Vehicle

C

B₂R 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