

# Hypertension

JOURNAL OF THE AMERICAN HEART ASSOCIATION



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*Hypertension* 2006;47;145-146; originally published online Dec 27, 2005;

DOI: 10.1161/01.HYP.0000200278.64801.4e

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

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## Targeting Brain AT<sub>1</sub> Receptors By RNA Interference

Curt D. Sigmund, Robin L. Davisson

The dipsogenic and pressor actions of angiotensin II (Ang II) in the central nervous system (CNS) have been well documented in many species and are now accepted as dogma. The major central cardiovascular effects of Ang II are elicited by a complex receptor-dependent signaling cascade initiated by the detection of circulating Ang II in regions of the brain lacking a blood brain barrier (BBB) through local production of Ang II in regions outside and inside the BBB or through the neurotransmitter actions of Ang II. It is generally accepted that most of the central neurocardiovascular actions of Ang II occur through the activation of intracellular signaling cascades that originate at the Ang II type 1 (AT<sub>1</sub>) receptor. A second Ang II receptor, termed AT<sub>2</sub>, has been implicated in some cardiovascular and behavioral responses. However, the AT<sub>2</sub> receptor is distantly related to AT<sub>1</sub> and can be effectively distinguished from AT<sub>1</sub> pharmacologically.

In humans, a single gene encodes the AT<sub>1</sub> Ang II receptor, whereas in rodents a duplication resulted in 2 AT<sub>1</sub> receptor genes, located on different chromosomes, each known to encode 1 of 2 distinct subtypes of the receptor, AT<sub>1a</sub> and AT<sub>1b</sub>. Both receptors are highly homologous, use the same signaling mechanisms, and cannot be distinguished using currently available pharmacological reagents or antisera. Despite their high similarity at the protein level, sufficient divergence is present in the sequence of the mRNA that allows them to be distinguished by RT-PCR and in situ hybridization. Through this analysis, it has become clear that these receptor subtypes exhibit quite different expression patterns, especially outside of the CNS. The rodent AT<sub>1a</sub> subtype is thought to most resemble human AT<sub>1</sub>, because it exhibits a similar cellular and tissue distribution, whereas AT<sub>1b</sub> exhibits a much more restricted expression profile. In the brain, there is likely both overlapping and divergent localization of the receptor subtypes.<sup>1</sup> In regions where they are colocalized, including some hypothalamic, pituitary, and brain stem nuclei, AT<sub>1a</sub> and AT<sub>1b</sub> mRNA expression may be differentially regulated in response to osmotic challenges.<sup>2</sup>

Whereas these subtle differences in AT<sub>1a</sub> and AT<sub>1b</sub> mRNA sequence provided tools to assess their differential expression, studies aimed at understanding their potential functional divergence have been more challenging. Early studies in AT<sub>1a</sub>-null mutant mice provided indisputable evidence for the importance of AT<sub>1a</sub> receptors in basal blood pressure regulation and, for the first time, implicated a physiological role for AT<sub>1b</sub> in this physiological parameter.<sup>3</sup> Our studies were the first to identify divergent functional roles for AT<sub>1a</sub> and AT<sub>1b</sub> in the actions of Ang II in the brain.<sup>4</sup> Using a combination of AT<sub>1a</sub>- and AT<sub>1b</sub>-deficient mice and monitoring blood pressure and drinking activity in response to intracerebroventricular (ICV) Ang II, we showed that the pressor effects of central Ang II could be ascribed selectively to AT<sub>1a</sub> receptors, whereas the central dipsogenic actions of Ang II required the presence of AT<sub>1b</sub>. However, because the receptors were ablated throughout the brain (and periphery), our studies did not address the relative functional roles of AT<sub>1a</sub> and AT<sub>1b</sub> in specific neurocardiovascular nuclei. In addition, as with any knockout model, the effects of life-long deletion of a gene and its product must be considered. This is underscored by a recent report by Rocha et al<sup>5</sup> demonstrating an increase in AT<sub>1b</sub> mRNA in the brain stem but not the hypothalamus of AT<sub>1a</sub>-deficient mice, suggesting the possibility of region-specific compensation.

The development of reagents that can effectively discriminate highly homologous mRNAs from each other and that can result in the selective local ablation (or knockdown) of 1 receptor subtype while preserving expression of others, all at the same time avoiding chronic compensatory changes, could provide important new molecular tools to probe the function of closely related receptors. So enters the era of RNA interference, perhaps the promise of antisense methodology in vivo finally revealed.<sup>6</sup> The discovery of RNA interference has revolutionized genetic analyses in lower organisms and in mammalian cell culture, and the recent combination of this technology with viral-mediated delivery of these reagents offers a powerful new means for inducing spatiotemporally localized gene silencing in the mammalian CNS in vivo.<sup>7</sup>

In an accompanying article in this issue of *Hypertension*, Chen et al<sup>8</sup> apply this methodology and take advantage of subtle sequence differences between AT<sub>1a</sub> and AT<sub>1b</sub> mRNA to develop small hairpin RNAs (shRNA) for selective silencing of one subtype while preserving expression of the other. In initial screening experiments, they demonstrate subtype-specific downregulation ( $\approx 60\%$ ) using cultured Neuro-2a cells, which express both forms of the AT<sub>1</sub> receptor. To address whether selective downregulation of AT<sub>1a</sub> receptors in specific brain regions causes alterations in basal regulation of blood pressure and drinking activity, they administer adenoviral vectors encoding both an shRNA directed against AT<sub>1a</sub> and the reporter gene  $\beta$ -Gal, either ICV (to target the

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(*Hypertension*. 2006;47:145-146.)

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*Hypertension* is available at <http://www.hypertensionaha.org>  
DOI: 10.1161/01.HYP.0000200278.64801.4e

subfornical organ; SFO) or directly into a brain stem region (nucleus tractus solitarius/dorsal vagal nucleus).<sup>9</sup>  $\beta$ -Gal provides an indicator for those cells infected by the virus (but not necessarily expressing the shRNA, because it is driven from a different promoter), and Ang II-binding assays are used as an index of AT<sub>1</sub> receptor levels. Interestingly, although  $\beta$ -Gal activity was detected as early as 24 hours after viral delivery, 10 days was the earliest time point when Ang II binding was significantly reduced in brain regions accessed by either injection route. ICV administration of the virus caused a  $\approx$ 60% decrease in Ang II binding in the SFO at this time, returning to baseline by 20 days. A greater and more persistent reduction in Ang II binding was evident in the brain stem. Given that these shRNAs are not 100% effective in gene silencing, coupled with the use of Ang II binding assays, which do not distinguish between the 2 receptor subtypes, it remains to be seen whether the residual AT<sub>1</sub> receptor activity detected in these regions is because of inefficient silencing of AT<sub>1a</sub> or is because of binding of Ang II to AT<sub>1b</sub> receptors, which were not targeted by the AT<sub>1a</sub>-specific shRNA.

There were 2 unexpected physiological findings from this study. First, ICV administration of the shRNA adenovirus resulted in a transient increase in basal drinking activity and water intake, which correlated with the time course of AT<sub>1a</sub> receptor ablation. This effect was only observed during the period of nocturnal activity and did not involve changes in arterial pressure. At first, the increase in drinking seems counterintuitive given the decrease in Ang II binding in the SFO, a region known to mediate the dipsogenic effects of Ang II,<sup>10</sup> until one considers that there was a concomitant increase (although not statistically significant) in plasma Ang II (129.1 versus 83.6 pg/mL) at the same time point. Although the cause for this increase in circulating Ang II in shRNA-treated mice is unclear, the increased drinking could potentially be explained by augmented activity of the AT<sub>1b</sub> receptor in the SFO of these mice, a region devoid of a BBB. This would certainly be consistent with our previous findings on the importance of AT<sub>1b</sub> in mediating the drinking response to Ang II<sup>4</sup> and the localization of AT<sub>1b</sub> receptors in SFO.<sup>2</sup> That the converse experiment using an shRNA directed against AT<sub>1b</sub> was not performed makes this a viable, yet unproven, hypothesis. Naturally, this hypothesis is based on a retention of the subtype specificity of the shRNA identified in Neuro-2a cells in vitro but not proven in vivo. This point is particularly important when one considers the extraordinarily high titers of the adenoviruses used in these experiments.

The second interesting finding was a reduction in basal arterial pressure, without a change in drinking or heart rate, in mice where the shRNA-containing adenovirus was directly injected into the brain stem. Like the drinking behavior, this effect was confined to the active nocturnal period. It is notable that the decrease in arterial pressure was only observed at 10 days after injection and returned to baseline by 20 days, despite the persistent loss of Ang II binding at the later time point. To explain that result, one has to consider a

possible threshold effect or alterations in other cardiovascular parameters, such as the baroreflex, that could provide a mechanism for arterial pressure to return to baseline before Ang II binding. Compensatory effects of AT<sub>1a</sub> and AT<sub>1b</sub> receptor activation in other nuclei that were not targeted by the adenovirus also need to be considered.

The results presented in the article by Chen et al<sup>8</sup> provide additional confirmation for the differential cardiovascular and dipsogenic effects of AT<sub>1</sub> receptor activation in the brain. Importantly, the data suggest the potential for viral-mediated delivery of shRNAs as a strategy for mapping the differential functions of AT<sub>1</sub> receptor subtypes in specific regions of the brain. However, as with any study using novel technology, this one underscores the need for additional experimentation. Do the shRNAs retain their subtype selectivity in vivo, and, if so, how efficient and stable is the AT<sub>1</sub> subtype knockdown? Are the cardiovascular effects of AT<sub>1a</sub> ablation caused only by a loss of AT<sub>1a</sub> receptors or a compensating effect of AT<sub>1b</sub>-receptor activation? What are the effects of AT<sub>1a</sub> and AT<sub>1b</sub> knockdown in the context of increased central and/or systemic Ang II levels as seen in a variety of experimental models of disease? In the end, this strategy may also help to finally answer the important question of how the 2 receptor subtypes in rodents functionally relate to the single AT<sub>1</sub> receptor in humans. Finally, one has to contemplate whether AT<sub>1</sub> receptor shRNAs will ever be considered a new class of therapeutic angiotensin receptor blockers. Answering the question of whether these reagents will ever be effective clinically must await future research and novel and innovative iterations of this technology.

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