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Increased Angiotensin II AT₁ receptor mRNA and binding in spleen and lung of AT₂ receptor gene disrupted mice

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Abstract

To clarify the relationship between Angiotensin II AT₁ and AT₂ receptors, we studied AT₁ receptor mRNA and binding expression in tissues from AT₂ receptor gene-disrupted (AT₂ ^{-/-}) female mice, where AT₂ receptors are not expressed *in vivo*, using *in situ* hybridization and quantitative autoradiography. Wild type mice expressed AT_{1A} receptor mRNA and AT₁ receptor binding in lung parenchyma, the spleen, predominantly in the red pulp, and in liver parenchyma. In wild type mice, lung AT₂ receptors were expressed in lung bronchial epithelium and smooth muscle, and were not present in the lung parenchyma, the spleen or the liver. This indicates that AT₁ and AT₂ receptors were not expressed in the same cells. In AT₂ ^{-/-} mice, we found higher AT_{1A} receptor mRNA and AT₁ receptor binding in lung parenchyma and in the red pulp of the spleen, but not in the liver, when compared to littermate wild-type controls. Our results suggest that impaired AT₂ receptor function upregulates AT₁ receptor transcription and expression in a tissue-specific manner and in cells not expressing AT₂ receptors. AT₁ upregulation explains the increased sensitivity to Angiotensin II characteristic of the AT₂ ^{-/-} phenotype, consistent with enhanced AT₁ receptor activation in a number of tissues.

Keywords

Renin-angiotensin system; Angiotensin receptor types; gene-disruption; autoradiography; *in situ* hybridization

INTRODUCTION

Angiotensin II (Ang II) is the principal effector peptide of the renin-angiotensin system (RAS) and there are two Ang II receptor types, the AT₁ and AT₂ receptors [1,2]. The AT₁ receptors are universally recognized as the major physiological Ang II receptors [2,3] and selective and

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safe Ang II AT₁ receptor blockers are a cornerstone in the treatment of hypertension and other cardiovascular disorders [2].

While humans express a single AT₁ receptor type, there are two AT₁ receptor subtypes in rodents, the AT_{1A} and AT_{1B} receptors [2,4]. Their high sequence homology in the translated regions makes them pharmacologically similar and indistinguishable [5,6]. However, with the use of probes directed to the low homology untranslated domains, it is possible to identify the AT_{1A} and AT_{1B} receptor subtypes by RT-PCR or *in situ* hybridization techniques [7,8,9]. In rodents, the AT_{1A} and AT_{1B} receptor subtypes are distributed and regulated differently [5]. The AT_{1A} receptor is the most abundant AT₁ receptor subtype, and most of the Ang II effects are the consequence of AT_{1A} receptor stimulation [7,10,11,12,13].

On the other hand, the role of the Ang II AT₂ receptor is still a matter of controversy [14,15,16,17,18,19]. Interest on the role of these receptors emerged from the recognition that therapeutic blockade of AT₁ receptors reduces feedback inhibition of renin formation, increasing circulating Ang II levels and possibly over stimulating AT₂ receptors [2]. Initially a matter of concern [20] the postulated AT₂ receptor activation during therapy with AT₁ receptor antagonists was later proposed as a mechanism responsible for some of the therapeutic effects of AT₁ receptor blockade [17]. This proposal was based on the cross-talk hypothesis, based on *in vitro* experiments and initially formulated as a physiological intracellular balance or feedback between AT₁ and AT₂ receptors exerting opposite effects [21,22,23,24]. A balance shift towards AT₂ receptor activation as a consequence of AT₁ receptor antagonism could significantly contribute to the beneficial effects of the AT₁ receptor blockers [17].

Gene disruption of specific receptors is one useful model to analyze receptor function, and it has been used to study the role of AT₂ receptors [25,26,27,28,29,30]. The phenotype of AT₂ ^{-/-} mice largely represents that of enhanced AT₁ receptor activation. This includes increased baseline blood pressure [29,31] enhanced pressor response as a result of peripheral or central administration of Ang II [26,25,31,29,28], enhanced aortic constrictor responses to Ang II [29], decreased urinary sodium excretion after exogenous administration of the peptide and sustained anti-natriuresis [26,31,28] and increased renal fibrosis and collagen deposition [32]. In addition, AT₂ ^{-/-} mice exhibited exaggerated HPA axis activity with increased ACTH and corticosterone release [33], enhanced adrenomedullary activity [33], increased vasopressin secretion [30], enhanced stress-induced hyperthermia [34], increased sensitivity to pain [35], and increased locomotion and anxiety [31,27]. Thus, the AT₂ ^{-/-} phenotype appears to resemble overall AT₁ receptor activation.

This phenotype was initially explained as a direct result of a deficiency in protective mechanisms, compensatory in nature, in balance with AT₁ receptor activation, and mediated by direct AT₂ receptor stimulation in the same cells [28]. A number of findings indicated, however, that the original AT₁/AT₂ receptor cross-talk hypothesis was in need of revision. First, if AT₁ and AT₂ receptors were normally expressed within the same cells, as initially assumed [13] their reciprocal influence may be the consequence of autocrine regulatory mechanisms. However, there is no *in vivo* definitive evidence of same-cell localization of AT₁ and AT₂ receptors [8,9,36,37,38,39,40,41]. Second, deletion of the AT₂ receptor gene is associated with increased expression of AT₁ receptors in aorta [29], selected kidney structures [42], adrenal gland [43] and hypothalamic paraventricular nucleus [33]. Third, we were intrigued by our finding that enhanced AT₁ receptor expression in AT₂ ^{-/-} mice occurred in structures such as the hypothalamic paraventricular nucleus and in renal cells where AT₂ receptors are may not be present *in vivo* [33,42,44,45,46].

These observations indicated that autocrine mechanisms proposed initially may not be sufficient to explain the original hypothesis of an AT₁/AT₂ interaction. However, the tissues

previously studied may contain low levels of AT₂ receptors [33,42,44,45,46] The present study was designed to test to what extent same-cell localization was necessary for the AT₂ receptor-induced regulation of AT₁ receptor expression. To this effect we chose to analyze, in AT₂ -/- mice and in search for AT₁ receptor expression, the liver, spleen and lung, organs with established AT₁ receptor function and demonstrated lack of AT₁ and AT₂ receptor cellular coexpression [9,11,12,37,38,47,48,49,50,51,52].

For our study we combined quantitative autoradiography and *in situ* hybridization, techniques that have been validated for the study of the distribution and quantification of Ang II receptor types [14].

MATERIALS AND METHODS

Animals

Mice (C57BL/6 and 129 Ola) were initially obtained from Jackson Laboratory (Bar Harbor, MA) and were subsequently housed at the Department of Biochemistry, Nashville University, where they were kept under controlled conditions with free access to water and food. The AT₂ -/- mice were produced at the Department of Biochemistry, Nashville University by the injection of AT₂ -/- disrupted embryonic stem cells (E14-1) from the 129 Ola mouse line into blastocysts derived from C57BL/6 mice, as described previously [31]. After the genotype of F₂ heterozygous females (AT₂ +/-) was clearly confirmed by Southern blot analysis of DNA isolated from tail biopsies [31], they were backcrossed with C57BL/6 wild type males for three generations. Female AT₂ +/- littermates were crossed with the backcrossed males AT₂ +/-Y and AT₂ -/Y to produce homozygous females AT₂ -/- and wild type females AT₂ +/-Y. Female littermates from the third backcross progeny were selected to minimize the effect of differences in genetic background. Adequate measures were taken to minimize pain or discomfort. The experiments were conducted in accordance with international standards on animal welfare as well as being compliant with local and national regulations, as defined by the European Communities Council Directive of 24 November 1986 (86/609/EEC). The University of Nashville and NIMH Animal Care and Use Committees approved all animal protocols. Mice were transported to NIMH when 8 weeks old, kept for one day under controlled conditions as above, and killed by decapitation between 10:00 a.m. and 11:00 a.m.

Tissue preparation

Spleen, lung and liver were immediately removed, frozen in isopentane at -30°C, and stored at -80°C. For binding studies, tissues were cut in a cryostat at -20°C to obtain 16 µm sections, which were thaw-mounted on gelatin-coated slides, dried overnight in a desiccator at 4°C, and stored at -80°C until use. For *in situ* hybridization, sections (16 µm) were thaw-mounted onto Superfrost®/Plus microslides (Daigger, Vernon Hills, IL), dried at 50°C for 5 min on a slide warmer and stored at -80°C until hybridization. We used consecutive sections for each set of experiments and for staining with hematoxylin and eosin to localize the structures expressing the binding or the receptor mRNA.

Angiotensin II receptor binding

Receptor binding was performed as described earlier [36]. Tissue sections were pre-incubated for 15 min at 22°C in 10 mM sodium phosphate buffer (pH 7.4), containing 120 mM NaCl, 5 mM MgCl₂, 5 mM EDTA, 0.005% bacitracin and 0.2% protease free bovine serum albumin (Sigma Chemical). Pre-incubation was followed by incubation for 2 hours at 22°C in fresh buffer prepared as above with addition of 50 µM Plummer's inhibitor, 100 µM phenylmethylsulfonyl fluoride and 500 µM phenanthroline. To determine receptor binding, 0.5 nM of Sarcosine¹-Angiotensin II ([¹²⁵I]-Sar¹-Ang II) obtained from Peninsula Laboratories (Belmont, CA) and iodinated by New England Nuclear (Boston, MA) to a specific activity of

2200 Ci/mmol were added to the incubation buffer. After the incubation period, sections were washed 4 times for 1 min each in ice-cold 50 mM Tris-HCl buffer (pH 7.4), followed by a 30 sec wash in ice-cold water, and dried under a stream of cold air.

Consecutive sections were incubated with 0.5 nM [125 I]Sar¹-Ang II to determine total binding. Adjacent sections were incubated as above with addition of 10 μ M losartan (DuPont-Merck, Wilmington, DE, USA), to displace binding to AT₁ receptors. Binding to AT₁ receptors was calculated as the difference between total binding and the binding remaining in adjacent sections incubated in the presence of excess concentration of losartan. Similarly, binding of [125 I]Sar¹-Ang II to AT₂ receptors was determined as the difference between total binding and binding in adjacent sections incubated in the presence of 10 μ M of PD 123319 (Parke-Davis, Ann Arbor, MI, USA) or 0.1 μ M of CGP42112 (Neosystems Laboratory, Strasbourg, France) to selectively displace binding to AT₂ receptors. The concentrations of the AT₁ and AT₂ receptor selective ligands were selected to give maximum specific displacement [36,44,53]. Non-specific or background binding was determined by incubating consecutive sections with 10⁻⁶ M Ang II (Peninsula Laboratories, Belmont, CA). The non-specific or background binding was subtracted from all determinations as described above. In the case of areas with only AT₁ receptor binding, the values obtained after displacement with excess concentrations of losartan were not significantly different from background values obtained by displacement with excess concentrations of unlabelled Ang II.

In another set of adjacent lung sections, we performed binding to [125 I]-CGP42112, a ligand for AT₂ receptors that does not recognize AT₁ binding sites, to confirm the expression of AT₂ receptors [53]. Buffers used in this assay had the same composition as those used for the binding to [125 I]-Sar¹-Ang II. Tissue sections were pre-incubated for 15 min in incubation buffer followed by incubation for 120 min in fresh buffer containing 0.2 nM [125 I]-CGP42112. At the concentrations used, [125 I]CGP 42112 labels exclusively AT₂, and not AT₁, receptors [53]. To determine specific binding to AT₂ receptors, consecutive sections were incubated in the presence of 10⁻⁶ M of PD 123319 to selectively displace binding to AT₂ sites. Non-specific binding was determined by incubating consecutive sections with 5 $\times$ 10⁻⁶ M of Ang II (Peninsula).

Quantitative receptor autoradiography

We exposed dry sections to Hyperfilm-³H (Amersham Co., Arlington Heights, IL) along with 16 μ m sections of autoradiographic [14 C] micro scales (Amersham) at 4°C. Films were developed in ice-cold D-19 developer (Eastman Kodak, Rochester, NY) for 4 min, fixed in Kodak rapid fixer for 4 min at 22°C, and rinsed in water for 15 min. We measured optical densities in the autoradiograms by computerized micro densitometry using the NIH Image 1.6 analysis system (NIMH, Bethesda, MD). For quantitative autoradiography, we measured the optical densities separately in lung parenchyma, red pulp of the spleen, and liver parenchyma. The optical densities were related to the concentration of radioactivity present in the sections by comparison with the [14 C] micro-scales, and transformed to corresponding values of fmol/mg protein [36,54].

Emulsion autoradiography

To obtain more precise localization of AT₁ and AT₂ receptors, we used adjacent sections incubated with [125 I]Sar¹-Ang II or [125 I]CGP 42112 as described above. Adjacent sections were stained with hematoxylin and eosin. After binding experiments sections were fixed for 60 min in paraformaldehyde vapors at 80°C and dipped in photo emulsion (Eastman-Kodak). After exposure for two to three weeks, sections were developed in Kodak D-19 developer (Eastman-Kodak) for three minutes at 15°C, fixed for four minutes in Kodak rapid fixer,

counter stained with hematoxylin and eosin, and the silver grains visualized with a Zeiss microscope.

In situ hybridization histochemistry

A full length cDNA clone of the mouse AT_{1A} receptor (I.M.A.G.E clone ID 3497420) was obtained from Invitrogen (Carlsbad, CA). From the sequence of the clone, we selected primers for PCR (Forward -5'TTGAAGAAGTAATTATGCAAAGCTG3'; Reverse -5'GACACGTGGGTCTCCATTG 3') of a unique 280bp region within the 3' untranslated region of the cDNA. After amplification, we used a TOPO cloning kit (Invitrogen, Carlsbad, CA) to clone the region into the *Eco* RI site of the vector pCR®II-TOPO®. The clones containing plasmids were linearized with *Hind*III for sense or *Xba*I for antisense template, respectively. Radiolabeled riboprobes were prepared by *in vitro* transcription in the presence of [³⁵S]-UTP (Amersham, Arlington Heights, IL), 1µg of linearized plasmid and T7 (sense probe) or SP6 (antisense probe) RNA polymerase, using a MAXIScript® T7/SP6 kit (Ambion, Austin, TX) according to the protocol of the manufacturer. Unincorporated nucleotides were removed by centrifugation through ProbeQuant™ G-50 Micro Columns (Amersham Biosciences, Little Chalfont Buckinghamshire, England). The labeling of riboprobes was monitored with liquid scintillation counting and the specificity verified by a preliminary experiment with adrenal gland. In preliminary experiments, we verified the specificity of the riboprobe for the AT_{1A} receptor by performing in situ hybridization in brain sections of wild-type mice. Specific signals for AT_{1A} receptor mRNA were present in hypothalamic paraventricular nucleus, expressing AT_{1A} receptors in the rat, but not in hippocampus, expressing AT_{1B} receptors in the rat [9] (results not shown).

Prior to use, the sections were warmed in a desiccator at room temperature and then fixed in 4% formaldehyde in PBS for 10 min. After two washes in PBS, they were acetylated for 10 min in 0.1M triethanolamine HCl, 0.9% NaCl, containing 0.25% acetic anhydride, delipidated in ethanol and chloroform, and air-dried. Each slide-mounted section was covered with 150 µl hybridization buffer containing 50% formamide, 0.3 M NaCl, 1mM EDTA, 20mM Tris, pH 7.5, 1x Denhardt's solution, 10% dextran sulfate, 100 µg/ml salmon testes DNA, 250 µg/ml yeast RNA, 250 µg/ml yeast tRNA, 150 mM DTT, 0.2% SDS, 0.2% sodium tiosulfate (NTS) and 2×10⁷ cpm/ml sense or antisense probe, and the slide was covered with a glass cover slip. After hybridization for 18 hrs at 54°C, coverslips were removed and sections were rinsed several times in 4X standard saline citrate (SSC). Non-hybridized probes were digested by incubation with 40 µg/ml RNase A (Sigma Chemical, St. Louis, MO) for 30 min at 37°C. After a final high stringency wash in 0.1X SSC at 65°C for 60 min, sections were dehydrated in graded ethanols containing 0.3M ammonium acetate, air dried and exposed to Biomax MR film (Kodak, Rochester, NY) for 9 days. Films were developed as described above. The mRNA expression was analyzed by measuring optical film densities using the Scion Image Program 4.0.2 (Scion Corporation) based on the NIH Image Program. The intensities of hybridization signals were expressed as nCi/g tissue equivalent after calibration with [¹⁴C]-microscales [55,56], and subtraction of the values obtained in the same areas of adjacent sections hybridized with sense (control) probes, which represent nonspecific hybridization. Data were expressed as means ± SEM, for groups of five animals, measured individually.

Statistical analysis

Results were expressed as means ± standard error of the mean (SEM), calculated and analyzed using GraphPad Prism (version 2.00) and Microsoft Excel (version 7.0a), and compared for significance using unpaired Student's t-test.

RESULTS

Ang II receptors in spleen

We detected high AT_{1A} mRNA expression in the spleen of wild type mice, primarily localized to the red pulp (Figure 1). This pattern correlated well with high losartan-sensitive [¹²⁵I]-Sar¹-ang II binding, corresponding to AT₁ receptors, highly expressed in the red pulp (Figure 2 and 3). Conversely, AT₂ receptor binding was not detected in spleen from control wild-type mice (Figure 2).

AT₂ ^{-/-} mice expressed significantly higher AT_{1A} mRNA and AT₁ binding in the spleen when compared to AT₂ ^{+/+} mice, and both AT_{1A} mRNA and AT₁ binding showed a similar distribution to that present in the wild type mice (Figures 1 and 2). AT₂ ^{-/-} mice did not express AT₂ receptor binding in the spleen (Figure 2).

Ang II receptors in lung

In wild type mice, we found significant AT_{1A} mRNA expression uniformly distributed throughout the lung parenchyma (Figure 4). There was no AT_{1A} mRNA expression associated with lung bronchia (Figure 4). High [¹²⁵I]Sar¹-Ang II binding expression was detected throughout the lung, both in lung parenchyma and bronchia. All binding corresponded to Ang II receptors, because incubation in the presence of Ang II completely prevented binding of [¹²⁵I]-Sar¹-ang II to both parenchyma and bronchia (Figure 5). In wild type mice, high losartan-sensitive binding to [¹²⁵I]Sar¹-Ang II, indicative of AT₁ receptor expression was uniformly distributed throughout the lung parenchyma (Figure 5). There were no AT₁ receptors in the lung bronchia (Figure 5). Conversely, small and large size bronchia expressed large numbers of [¹²⁵I]Sar¹-Ang II binding sensitive to displacement by PD123319, indicative of the presence of AT₂ receptors (Figure 5), with no AT₂ receptor present in the lung parenchyma. Ang II-sensitive binding to CGP 24112, indicative of AT₂ receptor expression, was also present in lung bronchia, and was undetectable in the lung parenchyma (Figure 6). In bronchia, binding to [¹²⁵I]-CGP42112 was associated with smooth muscle and epithelial cells as revealed by emulsion autoradiography (Figure 7).

In AT₂ ^{-/-} mice, there was a significant increase in AT_{1A} mRNA and AT₁ receptor binding expression restricted to the lung parenchyma, when compared to wild type mice (Figures 4 and 5). As it was the case in wild type mice, no AT_{1A} mRNA or AT₁ binding were found in lung bronchia of AT₂ ^{-/-} mice (Figures 4 and 5). No AT₂ receptor binding was detected in the lungs of AT₂ ^{-/-} mice (Figures 5 and 6).

Ang II receptors in liver

In wild type mice, we detected significant AT_{1A} receptor mRNA and AT₁ receptor binding (Figure 8), and no AT₂ receptor binding (not shown). In the liver, there were no significant differences in expression of AT_{1A} receptor mRNA and AT₁ receptor binding between AT₂ ^{+/+} and AT₂ ^{-/-} mice (Figure 8).

DISCUSSION

The novel finding in our study is the demonstration of a significant increase, in adult AT₂ ^{-/-} mice, in the expression of AT_{1A} receptor mRNA and AT₁ receptor binding in tissues and cells where AT₂ receptors are never expressed, the red pulp of the spleen and the lung parenchyma [9,11,12,37,38,47,48,49,50,51,52].

Although upregulation of AT₁ receptor transcription and translation was reported earlier in other tissues such as the aorta, kidney, adrenal gland and hypothalamic paraventricular nucleus,

the possibility remained that these tissues may express low levels of AT₂ receptors [33,42, 44,45,46]. In the present study, we focused on structures where AT₂ receptors were unequivocally demonstrated to be absent [9,11,12,37,38,47,48,49,50,51,52]. Thus, the main implication of our findings is that life-long gene-disruption of one Ang II receptor type, the AT₂ receptor, coincides, in different cell populations, with life-long enhanced transcription and overexpression of the main physiological Ang II receptor, the AT₁ receptor.

Analysis of Ang II receptor binding in lung, spleen and liver of AT₂ ^{+/+} type mice confirmed expression of AT₁ receptors in lung parenchyma, red pulp of the spleen and liver. Conversely, AT₂ receptor binding was only present in lung bronchia, and was absent in lung parenchyma, spleen or liver. Expression of AT₁ and AT₂ receptors in the mouse is identical to that of the rat, confirming previous reports [9,11,12,38,41,48,49,50,51,52]. Our results reveal that in the mouse, like in the rat, AT₁ and AT₂ receptors were expressed in different cells not anatomically associated.

Our results are an extension of previous work reporting increased AT₁ receptor mRNA and binding in the hypothalamic paraventricular nucleus, kidney glomeruli, adrenal gland and aorta [29,33,42,43]. These studies, however, utilized a riboprobe directed to the translated region of the rat AT₁ receptor, with very high homology to the translated region of the mouse AT₁ receptor [33,42,43]. Although this probe successfully demonstrated mouse AT₁ receptor transcription, its use did not clarify the AT₁ receptor subtype involved. In the mouse and rat liver and lung, and in the rat spleen, AT₁ receptors have been characterized as of the AT_{1A} subtype [7,8,57,58]. For this reason we constructed a riboprobe selective for the mouse AT_{1A} receptor. We took advantage of the low homology in the untranslated regions of the mouse AT_{1A} and AT_{1B} receptors, and we designed a subtype-specific riboprobe complementary to the 3' noncoding region of the AT_{1A} receptor mRNA [5]. With the use of this riboprobe, we now demonstrate mRNA expression for AT_{1A} receptors in lung parenchyma, the red pulp of the spleen and the liver, what is in agreement with previous reports [7,8,57, 58,59]. Although we did not study AT_{1B} mRNA, this AT₁ receptor subtype has a very restricted expression in adult male rodents, it is not present in the lung or the liver [7,8,51,57,58,59] and its presence has not been reported in spleen.

Our combined autoradiography and in situ hybridization results reveal that AT_{1A} mRNA expression and AT₁ receptor binding are very significantly increased in spleen and lung parenchyma of AT₂ ^{-/-} mice. These results and our previous observations [29,33,42,43] suggest a revised mechanism for the AT₁/AT₂ cross-talk hypothesis. We postulate that, instead of intracellular, autocrine interactions, AT₂ receptor expression physiologically limits and controls AT₁ receptor transcription and expression, and therefore AT₁ receptor activity in cells devoid of AT₂ receptor expression. As a consequence, disruption of the AT₂ receptor should be expected to promote enhanced AT₁ receptor activity. Increased activity of AT₁ receptors, as a result of their transcription and translation upregulation, explains the AT₂ ^{-/-} receptor phenotype of increased anxiety, response to stress, vasoconstriction and hypertension [25,26, 27,28,29,30,31,32,33,34,35,42,43].

Accordingly, AT₂ ^{-/-} mice would be expected to present a phenotype consistent with AT₁ receptor overactivity in the lung and the spleen. Basal lung functions were not altered in AT_{1A} or AT₂ gene-deleted mice [11]. However, indirect evidence strongly suggest a vulnerability to inflammatory responses when AT₂ receptor activity is decreased, and, reciprocally, diminished inflammatory responses when AT₁ receptor activity is reduced. For example, the inflammatory response of the lung to bacterial endotoxin was decreased in AT_{1A} ^{-/-} mice [12]. Inactivation of the AT₂ receptor but not AT₂ receptor blockade aggravates acute lung inflammatory injury, indicating that the role of the AT₂ receptor was indirect, possibly through AT₁ receptor activation [11]. In addition, both gene deletion of the AT_{1A}

receptor and AT₁ receptor blockade protected against lung injury [11] and AT₁ receptor blockade reduces inflammatory responses in the lung [60]. These findings demonstrate that AT₁ receptor activity contributes to pulmonary vulnerability to injury and inflammation, and may be best explained by our present finding of enhanced AT_{1A} receptor transcription and expression in AT₂^{-/-} mice. Increased AT₁ receptor expression in the spleen, in turn, may contribute to explain the enhanced inflammatory responses in AT₂^{-/-} mice, because spleen AT₁ receptor blockade decreases inflammatory responses to bacterial endotoxin [61].

Spontaneous, life-long, complete absence of AT₂ receptor activity has not been reported and probably represents a condition not existing in nature. This questions the physiological significance of findings in this model. For this reason it is of interest that pharmacological inhibition of AT₂ receptors in adult rats enhances AT₁ receptor expression in the brain [62]. Moreover, cardiac-specific over-expression of AT₂ receptors attenuated AT₁ receptor-mediated pressor and chronotropic effects [23,63] a finding indicative of decreased AT₁ receptor function. This indicates that AT₂-dependent modulation of AT₁ receptor activity does not only occur after complete AT₂ gene disruption, but also after partial AT₂ receptor blockade or organ-selective over-expression. Moreover, it appears that the expression of AT₁ and AT₂ receptors is reciprocally regulated, since AT₁ receptor blockade enhances AT₂ receptor expression in selected brain areas [64,65,66] and in peripheral tissues such as the muscular gastric layer and adipose tissue [67,68]. The conclusion is that while AT₂ receptor activity may limit and control AT₁ receptor expression, AT₁ receptor activity, in turn, limits the expression of AT₂ receptors.

The reciprocal influence of AT₁ and AT₂ Ang II receptors is not universal, since there were no alterations in liver AT_{1A} receptor transcription and expression in AT₂^{-/-} mice. This is not surprising, because it is well known that AT₁ receptor transcription is regulated independently in a cell and tissue-specific manner [69,70]. In the liver, increased AT₁ receptor stimulation is associated with fibrosis [71], and fibrosis as a consequence of bile duct ligation is reduced in AT_{1A}^{-/-} mice [51].

The question remains as to the mechanism of the reciprocal regulation of AT₁/AT₂ receptor expression. Our present results appear to rule out intracellular (intracrine) and close-range intercellular (paracrine) mechanisms to explain AT₁/AT₂ receptor cross-talk. Instead, two possible mechanisms of interregulation should be considered. AT₂/AT₁ regulation may occur through factors acting at a distance of their site of production, a hormonal mechanism. For example, the increased sensitivity to Ang II in AT₂^{-/-} mice has been explained by a deficiency of AT₂ receptor-mediated local generation of bradykinin and nitric oxide [28,72]. Expression of endothelial nitric oxide synthase was lower and nitric oxide synthesis was decreased in AT₂^{-/-} mice [28,73,74], and the endothelial nitric oxide synthase^{-/-} phenotype is consistent with AT₁ receptor upregulation [72].

Alternatively, we must consider the possibility of genomic regulatory mechanisms. Genomic regulations are complex and poorly understood. However, it is not unreasonable to suggest that expression of genes belonging to a given system may influence each other. Multiple epistatic interactions between genes regulating blood pressure, and a major influence of the genetic background, are being demonstrated with the use of genetically hypertensive congenic rat strains [75]. The genetic background may be of fundamental importance for AT₂ receptor function and its effects on AT₁ receptor expression. For example, AT₂^{-/-} mice were generated in strains with different genetic background by two independent groups [26,31]. While one of the strains, studied here, expressed a phenotype consistent with AT₁ receptor stimulation [31] the other strain did not [26].

In conclusion, the results presented here indicate that AT₂ receptor activity regulates AT₁ receptor transcription and expression at a distance, in cells not expressing AT₂ receptors. These observations clearly demonstrate that the interaction between Ang II receptor types is of an indirect nature, possibly involving still undetermined hormonal and/or genomic mechanisms.

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Figure 1.

Representative film autoradiograms and quantitative analysis of AT_{1A} mRNA in spleen of wild-type (control) and AT₂ ^{-/-} mice detected by *in situ* hybridization. Note that the amount of AT_{1A} mRNA is significantly higher (*p<0.05) in AT₂ ^{-/-} mice as compared with wild-type control. Bars are means ± SEM for group of five animals, measured individually. Wp – white pulp, Rp – red pulp.

Figure 2.

Angiotensin II receptor binding in spleen of wild-type and $AT_2^{-/-}$ mice. Autoradiograms represent [125 I]-Sar¹-Ang II binding in the absence of selective ligands, in the presence of PD123319, losartan or Angiotensin II to visualize total binding, AT_1 receptors, AT_2 receptors and nonspecific binding as described in Material and Methods. Quantitative data are means $\pm$ SEM for group of five animals, measured individually. Note that AT_1 receptor binding is significantly higher (* $p < 0.05$) in $AT_2^{-/-}$ mice and is restricted to the red pulp. Wp – white pulp, Rp – red pulp.

Figure 3. Hematoxylin-eosin staining, bright and dark field emulsion autoradiography of ^{125}I -Sar¹-Ang II binding to spleen of wild type mouse. Silver grains over red pulp represent Ang II receptor binding. Magnification 400x. Wp – white pulp, Rp – red pulp.

Figure 4.

Representative film autoradiograms and quantitative analysis of AT_{1A} mRNA in lung of wild-type (control) and AT₂ ^{-/-} mice detected by *in situ* hybridization. Note that the amount of AT_{1A} mRNA is significantly higher (*p<0.05) in AT₂ ^{-/-} mice as compared with wild-type control. Bars are means ± SEM for group of five animals, measured individually.

Figure 5.

Hematoxylin-eosin staining, representative film autoradiograms of Angiotensin II receptor binding in lung of wild-type and AT₂-null mice. Autoradiograms of [¹²⁵I]-Sar¹-Ang II binding in the presence of selective ligands as described in Material and Methods. Results are means ± SEM for group of five animals, measured individually. AT₁ receptor binding is significantly higher (*p<0.05) in lung parenchyma (asterisks) of AT₂ -/- mice as compared with wild-type control. AT₂ receptor binding in bronchia (arrows) is present in wild-type, but absent in AT₂ -/- mice. Bars are means ± SEM for group of five animals, measured individually.

Figure 6.

Hematoxylin-eosin staining, representative autoradiograms of ^{125}I -CGP42112 receptor binding in lung of wild-type and $\text{AT}_2^{-/-}$ mice. Incubation of consecutive sections with ^{125}I -CGP42112 and displacement with Angiotensin II confirmed the presence of AT_2 receptors in bronchia (arrows) of wild-type animals, and absence in $\text{AT}_2^{-/-}$ mice. Asterisk represents parenchyma.

Figure 7. Hematoxylin-eosin staining, bright and dark field emulsion autoradiography of ^{125}I -CGP42112 binding to lung bronchia of wild type mouse. Silver grains over bronchia represent AT_2 receptor binding. Magnification 400x.

Figure 8.

Quantitative autoradiography of the AT_{1A} mRNA and receptor binding in liver of wild-type and AT₂ $-/-$ mice. Bars are means $\pm$ SEM for group of five animals, measured individually. Non significant differences were found between wild-type and AT₂ $-/-$ mice.