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The angiotensin type II receptor tonically inhibits angiotensin-converting enzyme in AT2 null mutant mice

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Background. Pharmacologic inhibition of the angiotensin-converting enzyme (ACE) limits angiotensin II (Ang II)-induced vasoconstriction and cellular proliferation. There is emerging evidence that some of the beneficial effects of ACE inhibitors may be endogenously available through the angiotensin receptor type 2 (AT2).

Methods. To evaluate whether AT2 modulates ACE activity, we used an high-performance liquid chromatography (HPLC)-based enzymatic assay in tissues from AT2 knockout mice (*Agtr2-/y*) and cultured cells. These studies were complimented by physiologic studies of pharmacologic inhibition of AT2.

Results. Circulating (C) and tissue ACE activities in heart (H), lung (L), and kidney (K) were doubled in *Agtr2-/y* mice compared with wild-type mice [162.9 ± 17.6 mU/mL (C), 97.7 ± 20.7 (H), 6282.1 ± 508.3 (L), and 2295.0 ± 87.0 (K) mU/g tissue for *Agtr2-/y* vs. 65.3 ± 35.4 mU/mL (C), 44.5 ± 8.7 (H), 3392.4 ± 495.2 (L), and 1146.1 ± 217.3 (K) mU/g tissue for wild-type mice, $P \leq 0.05, 0.025, 0.002$, and 0.0001, respectively]. Acute pharmacologic inhibition of AT2 [PD123319 (PD), 50 μg/kg/min, i.v.] significantly increased ACE activity in kidneys of wild-type mice (1591.2 ± 104.4 vs. 1233.6 ± 88.0 mU/g tissue in saline-infused mice, $P < 0.05$; $P < 0.01$ vs. uninfused, wild-type mice). Moreover, ACE activity increased in A10 cells exposed to PD (10^{-6} mol/L) together with Ang II (10^{-7} mol/L), but not with an AT1 antagonist (losartan, 10^{-6} mol/L). This heightened ACE activity appears functionally relevant because infusion of angiotensin I caused more prompt hypertension in *Agtr2-/y* mice than in wild-type littermates. Likewise, infusion of bradykinin, also a substrate for ACE, caused significantly less hypotension in *Agtr2-/y* mice than controls.

Conclusions. These studies indicate that AT2 functions to decrease ACE activity tonically, which may, in part, underlie AT2's increasingly recognized attenuation of AT1-mediated actions.

Key words: ACE inhibition, AT2 receptor, bradykinin, renin-angiotensin system, progressive renal disease.

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The renin-angiotensin system (RAS) is an important modulator of cardiovascular and renal functions under normal conditions, as well as contributing substantially to pathophysiologic processes within the heart, blood vessels, and kidneys [1–4]. Angiotensin-converting enzyme (ACE) is recognized as having a substantial role in regulating this system. As such, ACE has become the central target to modify activity of the RAS through pharmacologic ACE inhibition, in order to ameliorate hypertension, ischemic and hypertrophic cardiac disease, and progressive renal damage [5–7]. The circulating level of endogenous ACE in humans is, in part, under the control of an intronic insertion/deletion polymorphism in the *ACE* gene such that the deletion variant is associated with the highest ACE levels [8]. Endogenous ACE is also up-regulated by glucocorticoids, fibroblast growth factor, endothelin, and hypoxia [9–12]. Recently, elevation in ACE activity has been documented in several pathophysiologic conditions, including atherosclerotic vascular disease, failing hearts, and neointimal formation after vascular injury [13–15]. Notably, a local increase in ACE activity, even in the absence of injury or systemic alterations, is associated with adverse consequences. Thus, transfection of the human *ACE* gene, which increases ACE activity in mouse hearts, leads to cardiac hypertrophy (abstract; Aoki et al, *Hypertension* 30:484a, 1997), whereas transfection into intact rat carotid arteries increases the vessel wall to lumen ratio through cellular hypertrophy [16].

It is notable that more pronounced parenchymal fibrosis is seen in the kidneys of mice with a disruption of the gene for the angiotensin type 2 receptor (AT2) following obstructive injury [17, 18]. These observations are particularly interesting because the AT2 receptor has been shown to have vasodilative, antiproliferative, antihypertrophic, and proapoptotic functions, which have been proposed to exert a brake on the damaging processes driven by the RAS [19–21]. For example, overexpression of the AT2 receptor, by transfection into bal-

loon-injured rat carotid artery, attenuates neointimal formation [22]. Conversely, antagonizing the AT2 receptors in hamsters with heart failure significantly heightens the level of cardiac fibroblast collagen production and worsens the extent of cardiac interstitial fibrosis [23]. Notably, these disease settings are among the conditions that have been characterized by elevated ACE activity and also show a characteristic benefit from ACE inhibition [14, 15, 24, 25]. Therefore, to examine the possibility that the AT2 receptor curbs the well-recognized actions of Ang II by modulating ACE activity, we examined the circulating and tissue ACE in mice with genetically disrupted AT2 receptor.

METHODS

Agtr2-/y animals

We studied male mice aged 12 to 14 weeks genetically engineered with a disruption of the AT2 receptor gene, located on the X chromosome (*Agtr2-/y*). These mice were the offspring of *Agtr2* deletion mutants produced in our laboratories by homologous recombination in embryonic stem cells derived from strain 129/Ola [19]. Chimeric males were mated with C57BL/6J females such that the genetic background of the mutants consisted of 129/Ola and C57BL/6J. Wild-type male littermates served as the controls. Southern blot analysis of tail DNA was used to screen for the *Agtr2* genotype, as previously described [19]. Briefly, 10 μg of tail DNA were digested with BamHI and then hybridized with a [α-^{32}P]dATP-labeled 1.5 kb BamHI-EcoRI fragment of the *Agtr2* genomic DNA (5′-flanking region) as a probe. Using this probe, which is outside the 5′ end of the targeting vector, a 9.5 kb band represents the wild-type allele, and a 4.5 kb band represents the mutant allele.

Cell culture

A10 rat embryonic aortic smooth muscle cells were purchased from the American Type Culture Collection cell repository (Rockville, MD, USA). Our binding studies in these cells reveal the presence of the AT2 receptor, as well as the AT1 receptors, and reiterate previous observations in this cell line, which show expression of these receptor subtypes [26]. Cells were cultured at 37°C, under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated (55°C for 30 min) fetal bovine serum (FBS) and 1% penicillin G (10^4 U/mL)/streptomycin (10^4 μg/mL). Cell lines were trypsinized [0.25% trypsin-1 mmol/L ethylenediaminetetraacetic acid (EDTA); GIBCO BRL, Life Technologies, Grand Island, NY, USA], passaged twice weekly, and studied at passage 16 to 18. Confluent cell cultures, in 100 mm dishes, were serum deprived for 48 hours before study. Cells were then exposed to angiotensin II (Ang II; 10^{-7} mol/L; Sigma Chemical Co., St. Louis, MO, USA), PD 123319 (PD, 10^{-6} mol/L; Research Biochemicals International, Natick, MA, USA), or losartan (Los; 10^{-6} mol/L; Merck, Piscataway, NJ, USA) for 48 hours. Cells were then harvested and homogenized in 0.1 mol/L potassium phosphate, pH 8.3, and frozen at −20°C until assay for ACE activity. This dose of Ang II has previously been shown to induce Ang II-dependent mRNA levels maximally; likewise, this dose of Los eliminated that response [27]. Similarly, the dose of PD123319 was shown to eliminate the response induced by 10^{-7} mol/L Ang II in AT2-bearing cells [28].

Protein determination

Protein concentration of each cell suspension was determined spectrophotometrically after solubilization in 1% Triton X (Sigma) by the modified Lowry assay (Bio-Rad, Melville, NY, USA). Standard solutions of bovine serum albumin (Sigma) were assayed at each determination to generate a standard curve from which the sample protein concentration was calculated.

In vivo response of wild-type mice to exogenous AT2 receptor antagonism

Wild-type C57BL/6J male mice were anesthetized with a mixture of ketamine (100 mg/kg body wt, i.p.; Parke-Davis, Morris Plains, NJ, USA) and xylazine (15 mg/kg body wt, i.p.; Bayer Corporation, Shawnee Mission, KS, USA). The left jugular vein was cannulated with a polyethylene cannula (PE-10 fused to PE-50 tubing; Becton Dickinson, Parsippany, NJ, USA) containing 50 U/mL heparin in saline, which was passed under the skin and out through the nape of the neck and fixed in place with bonding adhesive (Permabond International, Englewood, NJ, USA). The mice were placed in individual cages for two hours of recovery from anesthesia before infusions were begun. PE tubing was connected to a swivel to allow free mobility. PD123319 (50 μg/kg body wt/min in normal saline) or normal saline was infused. This dose of PD123319 has previously been shown to exert physiologic effects *in vivo* in rodents [29, 30]. After eight hours, animals were anesthetized with methoxyflurane (Mallinkrodt Veterinary, Inc., Mundelein, IL, USA), and organs were harvested and immediately frozen in liquid nitrogen and stored at −70°C until assay.

In vivo response of *Agtr2-/y* animals to exogenous angiotensin I and bradykinin

Agtr2-/y animals and wild-type male littermates were anesthetized with Inactin (10 mg/100 g body wt, i.p.; Byk Pharmaceutical, Konstanz, Germany). The carotid artery and jugular vein were cannulated with polyethylene cannulae (PE-10 fused to PE-50 tubing; Becton Dickinson) containing 50 U/mL heparin in saline. Ang I (Sigma) at 0.1 and 1.0 μg/kg body wt and bradykinin (BK; Sigma) at 100 μg/kg body wt were dissolved in

saline immediately before each experiment [31]. The total volume of each injection was adjusted to deliver 15 μL/10 g body wt. Peptides were injected through the jugular vein, and blood pressure was measured through the carotid arterial line connected to a pressure transducer and blood pressure data collection system (Micro-Med, Inc., Louisville, KY, USA, and Gould Inc., Valley View, OH, USA).

Determination of angiotensin-converting enzyme activity

Serum and tissue ACE activities were measured by high-pressure liquid chromatography (HPLC), as described by Meng et al [32]. Briefly, tissue samples were immediately frozen in liquid nitrogen and stored at −70°C. Tissue was homogenized using a Tempest Virtishear tissue homogenizer (Virtis, Inc., Gardiner, NY, USA) in 0.02 mol/L potassium phosphate buffer (pH 8.3) and then centrifuged at 40,000 *g* for 20 minutes, followed by two additional cycles of homogenization and centrifugation, after which the tissue pellet was resuspended in 0.1 mol/L potassium phosphate buffer (pH 8.3). An aliquot of tissue homogenate was incubated at 37°C with 500 μL of 0.3 mol/L NaCl, 0.01% Triton X-100 (Sigma), 10^{-4} mol/L $ZnCl_2$, and 5 mmol/L hippuryl-histidine-leucine (HHL; Sigma) in 0.1 mol/L potassium phosphate buffer (pH 8.3). A second aliquot of tissue homogenate was incubated at room temperature for 30 minutes with 10^{-4} mol/L captopril before incubation with HHL, to determine any non-ACE degradation of HHL to hippuric acid (HA). The formation of HA from HHL by ACE was terminated by the addition of 1 N HCl. Residual tissue was pelleted by centrifugation at 14,000 *g* for one minute, and 500 μL of supernatent was removed, to which 1.5 mL of ethyl acetate was added. After vigorous vortexing to transfer HA from the aqueous to the organic phase, a 1 mL aliquot of ethyl acetate was transferred to a new tube and dried using a Speed Vac SC100 solvent evaporation apparatus (Savant Instruments, Holbrook, NY, USA). Dried samples were then dissolved into 15% aceteonitrile in 0.1 mol/L ammonium phosphate buffer (pH 6.8) and applied to an Alltima Phenyl HPLC column (4.6 × 250 mm; Alltech, Deerfield, IL, USA). Standard solutions of HA (Sigma) in the HPLC mobile phase were prepared and applied daily to the HPLC column for calculation of HA generation rates by ACE. ACE activity is expressed as nmol/L of HA formed per minute (mU) per gram of tissue, mU per mL of serum, or μU per mg protein of cultured cell suspensions as previously defined [32, 33].

Northern analysis

Total RNA was isolated from tissue using a modified acid guanidium isothiocyanate extraction with RNAzolB (Tel.:Test, Inc., Friendswood, TX, USA). The amount and purity of RNA were assessed by spectrophotometry. Ten micrograms of total RNA were electrophoresed in 1.2% agarose gels containing 6.6% formaldehyde in 20 mmol/L 3-(N-morpholino)propanesulfonic acid (MOPS), 5 mmol/L sodium acetate, and 1 mmol EDTA, pH 7.0. The RNA was then blotted to a Hybond N^+ nylon nucleic acid transfer membrane (Amersham, Arlington Heights, IL, USA) by overnight capillary transfer and was cross-linked to the membrane by ultraviolet irradiation. The probe used for these studies was a mouse cDNA probe, ACE.31, which encodes the N-terminal 332 amino acids of mouse ACE [34]. The probe was labeled with [α-^{32}P]dATP by random labeling (Stratagene, La Jolla, CA, USA). Prehybridization and hybridization were performed in QuickHyb hybridization buffer (Stratagene) at 68°C. After hybridization, membranes were washed twice for 15 minutes at room temperature in 0.1% sodium dodecyl sulfate (SDS)/2 × standard saline citrate (SSC) and once for 10 minutes at 55°C in 0.1% SDA/0.1 × SSC. The results were visualized by autoradiography with X-Omat AR film (Eastman Kodak, Rochester, NY, USA). Loading of RNA was assessed by rehybridization of the same membrane with a cDNA probe for cyclophillin, a gene that is highly conserved and ubiquitously expressed throughout rodent tissues [35]. Relative amounts of mRNA were quantitated by densitometric scanning of the autoradiograph with an image analysis system (GelPro; Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Results of each parameter within a group are expressed as mean ± SEM. For intergroup comparisons, statistical significance was assessed using the unpaired Student's *t*-test. All statistical analyses were performed using the computer software Statview 4.51 (Abacus Concepts, Inc., Berkeley, CA, USA). Statistical significance was taken as $P < 0.05$.

RESULTS

Circulating and tissue angiotensin-converting enzyme activities

Angiotensin-converting enzyme activity was measured by a recently described, sensitive assay of ACE activity that uses a synthetic ACE substrate and HPLC-based quantitation of the formed product [32]. In *Agtr2-/y* mice, serum ACE activity was 162.9 ± 17.6 versus 65.3 ± 35.4 mU/mL in age- and sex-matched wild-type mice ($P < 0.05$). Because ACE exists predominantly as a membrane-bound ectoenzyme expressed largely in the endothelium and certain epithelia, we also investigated whether this relatively larger pool of membrane-bound ACE is also different in the AT2 mutant mice (Fig. 1) [36, 37]. ACE activity in tissue homogenates from the

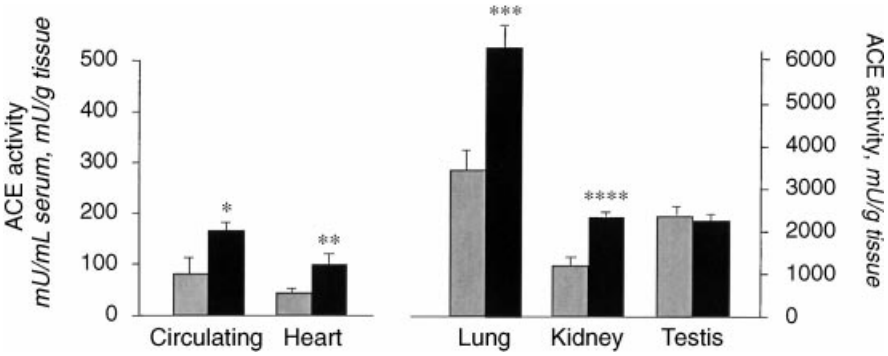

Fig. 1. Circulating and tissue angiotensin converting enzyme (ACE) activities in *Agtr2-/y* (■) and wild-type mice (▩). Each bar represents mean ± SE. *$P < 0.05$; **$P < 0.025$; ***$P < 0.002$; ****$P < 0.0001$.

lung of wild-type mice was 3392.4 ± 495.2 mU/g tissue. ACE activity in *Agtr2-/y* mice lung was markedly elevated at 6282.1 ± 508.3 mU/g of tissue ($P < 0.002$). A similarly striking elevation was observed in the kidney of *Agtr2-/y* mice in which ACE activity was more than doubled at 2295.0 ± 87.0 mU/g tissue versus 1146.1 ± 217.3 mU/g tissue in wild-type mice ($P < 0.0001$). Cardiac ACE is known to be an order of magnitude lower than tissue ACE in the lung and kidney, and other enzymes have been shown to be capable of the cleavage of Ang I to Ang II in the heart, especially in the interstitium [38]. However, again, a marked elevation in cardiac tissue ACE was seen for *Agtr2-/y* animals (97.7 ± 20.7 mU/g tissue vs. 44.5 ± 8.7 in wild-type mice, $P < 0.025$). Notably, ACE activity in testes of wild-type and *Agtr2-/y* mice did not differ (2319.4 ± 200.2 vs. 2202.3 ± 169.5 mU/g tissue, respectively; P = NS).

Effects of AT2 receptor blockade on angiotensin-converting enzyme in wild-type mice

The AT2 receptor was pharmacologically antagonized in normal wild-type mice to address the potential compensation in the null mutant mice to fetal AT2 disruption and the relevance of the observed *Agtr2-/y* phenotype to adult animals. ACE activity was assayed in wild-type mice after eight hours of continuous intravenous infusion of either PD123319, a selective AT2 receptor antagonist, or normal saline while the mice were conscious. ACE activity in the kidneys of saline infused mice was 1233.6 ± 88.0 mU/g tissue, which is similar to the value obtained from unmanipulated, baseline wild-type mice kidneys (1146.1 ± 217.3 mU/g of tissue). By contrast, kidney ACE activity in PD-treated mice increased to 1591.2 ± 104.4 mU/g of tissue ($P \leq 0.05$ vs. saline infused wild-type mice, Fig. 2; $P < 0.01$ vs. uninfused wild-type mice, Fig. 1). A Northern blot of kidney mRNA showed that the ACE mRNA to cyclophillin ratio of 0.14 ± 0.08 in saline-infused mice tended to increase to an average of 0.22 ± 0.06 in PD-infused mice.

Effects of AT2 receptor blockade on angiotensin-converting enzyme in cultured cells

Mutational disruption of the AT2 receptor or pharmacological inhibition of AT2 increases ACE activity *in vivo*. To evaluate whether the AT2 receptor indeed responds at the cellular level by modulating ACE activity, we evaluated cells known to have the AT2 receptor, namely A10 cells [26]. In control A10 cells, ACE activity was 187.9 ± 31.4 μU/mg protein. In A10 cells treated with Ang II, ACE activity was markedly reduced to 85.9 ± 24.0 μU/mg protein ($P < 0.025$ vs. control; Fig. 3). In A10 cells treated with Ang II together with the selective AT2 receptor antagonist, ACE activity was restored to control values, at 185.9 ± 38.1 μU/mg protein ($P < 0.05$ vs. Ang II-treated cells, P = NS vs. control cells; Fig. 3), indicating that antagonism of the AT2 receptor prevented the Ang II-induced decrease in ACE activity. By contrast, cells exposed to Ang II together with the selective AT1 receptor antagonist, Los, such that only AT2 receptors were available for interaction with Ang II, had an ACE activity that was comparable to cells treated solely with Ang II, 93.4 ± 23.7 μU/mg protein (pNS vs. Ang II-treated cells; $P < 0.025$ vs. control cells; $P < 0.05$ vs. Ang II + PD-treated cells). PD alone or Los alone did not affect ACE activity in these cells.

Blood pressure response to exogenous angiotensin I and bradykinin

To assess whether the different levels of ACE impart functional significance, we provided exogenous substrates for ACE metabolism to wild-type and AT2 null mutant mice (Fig. 4). Infusion of exogenous Ang I (0.1 mg/kg body wt, i.v.) increased the mean arterial pressure (MAP) of wild-type controls by a maximum of 14.0 ± 3.1 mm Hg, which occurred at 52.0 ± 10.6 seconds after the injection. Although the same dose of Ang I caused a similar increase in MAP in the AT2 null mutant mice (16.0 ± 2.3 mm Hg), the peak increase occurred much faster at 28.0 ± 2.0 seconds ($P < 0.05$; Fig. 4A). Infusion of a tenfold higher dose of Ang I (1.0 mg/kg

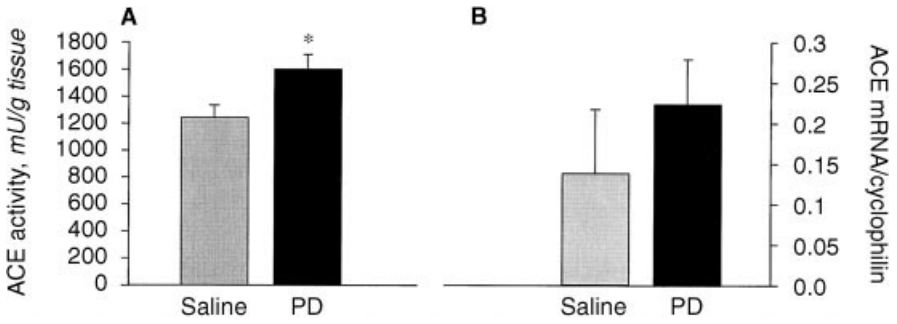

Fig. 2. ACE activity in kidney tissue after antagonism of the AT2 receptor in wild-type mice. (*A*) Saline infusion (▩) versus infusion of PD123319 for eight hours (■). (*B*) Northern blot data of ACE to cyclophilin ratio from mRNA extracted from saline- and PD-infused kidneys. $*P < 0.05$.

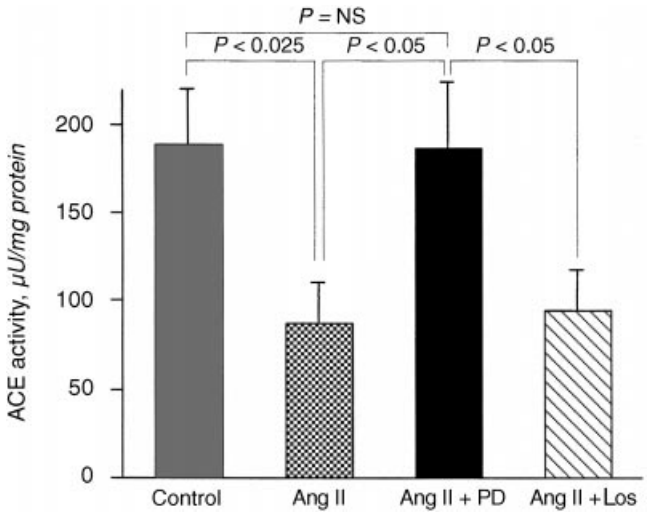

Fig. 3. Effects of antagonizing either the Ang II type 1 or 2 receptor on cellular ACE activity. Cultured A10 cells were incubated with vehicle (▩) or Ang II (10^{-7} mol/L, ▨) in the presence of either AT2 receptor antagonists PD123319 (PD, 10^{-6} mol/L, ■) or AT1 receptor antagonist, losartan (Los, 10^{-6} mol/L, ▧). Each bar represents mean ± SE.

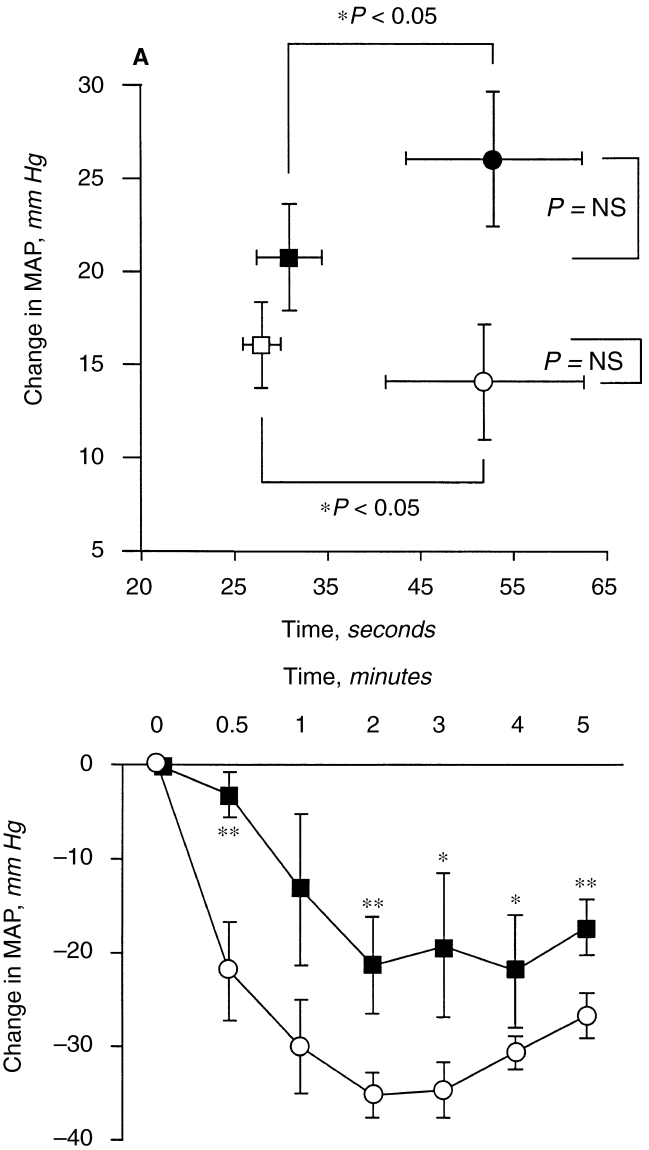

Fig. 4. Blood pressure response and time course following exogenous Ang I and bradykinin infusions. (*A*) Effects of Ang I (0.1 μg/kg body wt, □, and 1.0 μg/kg body wt, ■) in *Agtr2-/y* as well as in wild-type mice (0.1 μg/kg body wt, ○, and 1.0 μg/kg body wt, ●). (*B*) Effects of bradykinin (100 μg/kg body wt) in *Agtr2-/y* (■) and wild-type mice (○). $*P < 0.05$; $**P < 0.025$.

body wt, i.v.) caused a maximum increase in MAP of 26.0 ± 3.6 mm Hg at 53.0 ± 9.5 seconds in control mice. Again, although the maximum effect on MAP was similar in AT2 null mutant mice (20.7 ± 2.9 mm Hg), the effect was more prompt, occurring at 31.0 ± 3.6 seconds ($P < 0.05$).

Bradykinin is another endogenous substrate for ACE, which normally degrades BK to inactive metabolites. In these studies, AT2 null mutant mice showed an attenuated vasodilator response after the injection of exogenous BK (Fig. 4B). Wild-type mice decreased their MAP by 22.0 ± 5.3, 29.3 ± 5.3, 34.7 ± 2.7, 34.0 ± 3.5, 30.7 ± 1.8, and 26.7 ± 2.4 mm Hg at 0.5, 1, 2, 3, 4, and 5 minutes, respectively. At the same time points, AT2 null mutant mice decreased their MAP by only 3.3 ± 2.4, 13.3 ± 8.1, 21.3 ± 5.2, 19.3 ± 7.7, 22.0 ± 6.6, and 17.3 ± 2.9 mm Hg ($P < 0.025$ for 0.5, 2, and 5 min; $P < 0.05$ for 3 and 4 min).

DISCUSSION

Angiotensin II transduces its effects through the AT1 and AT2 receptor subtypes. Most of the recognized effects of Ang II such as vasoconstriction, cellular proliferation, and matrix deposition occur through the AT1 receptor [1–4]. The role of the AT2 receptor has recently been characterized rather to counter AT1 actions, encompassing antipressor, antichronotropic, and proapoptotic functions [17–23]. Thus, *in vivo* cardiac overexpression of the AT2 receptor dampens Ang II-induced pressor effects, whereas *in vitro* AT2 overexpression dampens vascular smooth muscle cell proliferation [22, 39]. Mice lacking the AT2 receptor gene manifest more parenchymal fibrosis in the kidney following injury, suggesting that lack of AT2 facilitates detrimental processes [17, 18]. Such parenchymal damage can be ameliorated by inhibiting ACE, which lessens vasoconstriction, cellular hypertrophy, and proliferation [3–7]. Given that the emerging function of the AT2 receptor appears to recapitulate the effects of ACE inhibition and because preliminary studies indicate that ACE activity might be modulated by Ang II receptors (abstract; Berecek et al, *FASEB J* 6:1578, 1992), we investigated whether or not the AT2 receptor specifically impacts ACE.

We studied mice devoid of the AT2 receptor and found the circulating ACE activity to be uniformly higher in *Agtr2-/y* mice, being twice that of wild-type littermates. ACE exists, however, predominantly as a membrane-bound ectoenzyme in the endothelium, and some epithelia and releasing mechanisms from this relatively larger pool of membrane-bound ACE into the circulation are poorly understood [36, 37]. We therefore examined ACE activity in tissue compartments of *Agtr2-/y* mice. Although ACE activity in the lung of *Agtr2-/y* mice and controls was markedly and significantly higher than circulating ACE, the remarkable step up in *Agtr2-/y* mice persisted.

The importance of increased ACE activity is evidenced by observations that ACE is dynamically regulated and is induced in several settings, including pressure overload ventricular hypertrophy, hypoxia, and atherosclerotic coronary artery disease [12–14, 25, 40, 41]. Even in the absence of other injurious stimuli, Morishita et al recently showed that doubling vascular ACE activity per se in the carotid artery fosters vessel hypertrophy [16]. This occurred without an increase in systemic ACE or blood pressure, reiterating the potential importance of local ACE as a pathophysiologically relevant effector. Genetically driven increase in ACE activity, such as that found in patients homozygous for the intronic deletion of the human ACE gene, has also been shown to facilitate more aggressive cardiac and renal damage [42–44]. It is interesting that increased fibrosis/damage after renal injury characterizes the AT2 null mutant mice, and we now show that these mice are characterized by a widespread increase in ACE activity [17, 18]. Conversely, it is interesting that local overexpression of the AT2 receptor in rat carotid arteries attenuated neointimal formation following balloon injury, a perturbation known to induce ACE expression [15, 22]. Taken together, it appears that a decrease in AT2 unleashes ACE, whereas enhancement of AT2 limits cellular proliferation, which, at least in part, may be due to AT2 limitation of ACE.

To evaluate whether the AT2 effect on ACE persisted in normal mice, we pharmacologically inhibited AT2 receptors in wild-type animals. AT2 blockade significantly increased renal tissue ACE activity, which rose by some 30% above baseline levels. This increase was not seen in saline-infused control mice. ACE mRNA showed a similar tendency. We also studied cells bearing both the AT1 and AT2 receptors. Exposing these cells to Ang II caused a decrease in ACE activity, which was reversed following concomitant treatment with the AT2 receptor antagonist. By contrast, this reversal in ACE activity was not seen after antagonizing the AT1 receptor. Collectively, these *in vivo* and *in vitro* studies suggest that AT2 tonically decreases ACE activity. Recent studies in rats with pressure overload cardiac hypertrophy [40] and myocardial infarction [25, 41] complement our findings. In this setting, increased ACE activity is lessened by antagonizing the AT1 receptor, raising the possibility that AT1 antagonism channels more Ang II to AT2, which, in turn, down-regulates ACE [25, 40]. It remains possible, however, that this ACE decrease reflects loss of a direct AT1-mediated increase in ACE, although there is little support for this notion.

Notably, although ACE activity in testes was also remarkably high, no difference was seen between *Agtr2-/y* and wild-type mice. It is possible that AT2 is not sufficiently expressed in testicular tissue and/or that it does not contribute to regulation of ACE activity in this organ. Importantly, ACE exists in two predominant isoforms, somatic and testicular. Somatic ACE (sACE) comprises the bulk of membrane-bound and circulating ACE and consists of two enzymatic domains. Testicular ACE (tACE), on the other hand, is limited to the testis and consists of a single enzymatic domain identical to the carboxy-terminal domain of sACE. In fact, tACE is encoded within the last half of the sACE gene, being regulated by a separate testis-specific promoter in intron 12 of the sACE gene [45, 46]. The sACE-determined ACE activities in human serum, heart, and circulating mononuclear cells appear to be regulated by the insertion/deletion polymorphism in intron 16 of human sACE, whereas no correlation is found between that locus and tACE-determined ACE activity in human spermatozoa, despite the fact that tACE also encompasses the same region of the intron [8, 33, 47, 48]. This study comple-

ments the idea that tACE is under a distinct and unique regulation; moreover, these results show this regulation to be independent of the AT2 receptor.

To evaluate the functional importance of the baseline increase in ACE activity in *Agtr2-/y* mice, we studied the consequences of providing exogenous ACE substrates to these and wild-type mice. Infusion of Ang I, which requires metabolism to Ang II for full vasoconstrictor activity, caused a similar increase in systemic blood pressure in both groups. However, Ang I-induced vasoconstrictive effects occurred significantly faster in *Agtr2-/y* mice than in wild-type littermates, suggesting that higher ACE *in vivo* facilitates more efficient conversion to Ang II. It remains possible that this functional phenotype is linked to ACE effects on other vasoactive agents and/or the AT1 receptor. Nonetheless, we propose that increased ACE activity in *Agtr2-/y* mice likely has a pivotal role. In this regard, computer modeling by Smithies predicts that ACE gene dose in ACE null mutant and transgenic mice is proportional to the rates of disappearance of Ang I and appearance of Ang II [49, 50]. This study also found that infusion of BK, which requires no activation for its vasodepressor effects but rather is degraded by ACE to inactive metabolites, caused hypotension in wild-type mice. By contrast, the hypotensive effect in *Agtr2-/y* mice was significantly blunted, with the fall in systemic blood pressure only a fraction of that seen for wild-type mice, suggesting facilitated degradation of BK by ACE. It is noteworthy that BK levels have recently been shown to be inversely proportional to ACE activity in humans [51]. Individuals homozygous for the deletion allele in intron 16 of the ACE gene (DD), who have the highest ACE levels, also have the lowest circulating levels of BK. The opposite is true in individuals homozygous for the insertion allele (II); these subjects have the lowest ACE and the highest BK levels. Thus, these physiological parameters appear to be relevant to the baseline hemodynamics of the *Agtr2-/y* mice and extend our original characterization in that disruption of the AT2 receptor increases ACE activity and facilitates production of Ang II and degradation of BK, promoting higher systemic blood pressure in the conscious state, and relative resistance of blood pressure to ACE inhibition [19].

In summary, we show that *Agtr2-/y* mice have heightened circulating as well as tissue ACE activity. Thus, genetically engineered disruption or pharmacological inhibition of AT2 increases ACE *in vivo* and in cultured cells, suggesting that this receptor tonically decreases ACE activity. These studies suggest that the vasodilatory and antiproliferative actions ascribed to the AT2 receptor may be linked to curbing the known actions of ACE, including synthesis of Ang II and degradation of BK.

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