Tubuloglomerular feedback in ACE-deficient mice

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1Departments of Physiology and Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109; 2Department of Pathology, University of North Carolina, Chapel Hill, North Carolina 27599; 3Guilford Medical Associates, Greensboro, North Carolina 27405; and 4National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892

Traynor, Timothy, Tianxin Yang, Yuning G. Huang, John H. Krege, Josie P. Briggs, Oliver Smithies, and Jürgen Schnermann. Tubuloglomerular feedback in ACE-deficient mice. Am. J. Physiol. 276 (Renal Physiol. 45): F751–F757, 1999.—In these experiments, we used a strain of angiotensin converting enzyme (ACE) germline null mutant mice, generated by J. H. Krege and co-workers (J. H. Krege, S. W. M. J ohn, L. L. Langenbach, J. B. Hodgin, J. R. Hagaman, E. S. Bachman, J. C. J ennette, D. A. O’Brien, and O. Smithies. Nature 375: 146–148, 1995), to examine the effect of chronicACE deficiency on the magnitude of tubuloglomerular feedback (TGF) responses. The genotype was determined by PCR on DNA extracted from the tail and was verified after each experiment by assessment of the blood pressure response to an injection of ANG I. To assess TGF responsiveness, we determined the change in stop-flow pressure (Psf) caused by increasing NaCl concentration at the macula densa by using micropuncture techniques. When loop of Henle flow rate was increased from 0 to 40 nl/min, Psf fell from a mean of 42.3 ± 1.95 to 33.6 ± 2.09 mmHg (n = 6, P = 0.005) in wild-type mice (+/+) or fell from 40.6 ± 2.35 to 38.6 ± 1.93 mmHg in heterozygous (+/−) mice (n = 7, P = 0.014), and did not change in homozygous ACE (−/−) mice (36.7 ± 2.02 mmHg vs. 36.4 ± 2.01 mmHg; n = 4, P = not significant (NS)). During an infusion of ANG II at a dose that did not significantly elevate blood pressure (70 ng·kg−1·min−1), TGF response magnitude (Psfs0 − Psfs40) increased from 6.5 ± 1.4 to 9.8 ± 1.19 mmHg in+/+ (P = 0.006), from 1.14 ± 0.42 to 4.6 ± 1.3 mmHg in +/− (P = 0.016), and from 0.42 ± 0.25 to 4.02 ± 1.06 in −/− mice (P = 0.05). Absence of TGF responses in ACE null mutant mice and restoration of near-normal responses during an acute infusion of ANG II supports previous conclusions that ANG II is an essential component in the signal transmission pathway that links the macula densa with the glomerular vascular pole.

transgenic mouse; gene knockout; micropuncture; angiotensin converting enzyme; stop-flow pressure

AN INCREASE in NaCl concentration at the macula densa cells causes a prompt increase in the resistance of afferent arterioles and a subsequent decrease in glomerular capillary pressure and glomerular filtration rate (3). There is abundant evidence to indicate that the effect of NaCl on vascular tone is linked to Na-K-2Cl cotransport activity in macula densa cells and that an alteration in transport causes the formation of a paracrine mediator capable of interacting with the vascular smooth muscle cells of the afferent arterioles (3). Identification of this flow-dependent mediator is still incomplete, mainly because interference with the generation or action of a number of potential vasoactive compounds has been demonstrated to affect the tubulovascular response magnitude. Results from a variety of technical approaches lend support to the notion that the renin-angiotensin system is intimately involved in determining the magnitude of the vascular response. Pharmacological inhibition of ANG II type I (AT1A) receptors and of converting enzyme has been shown to reduce the magnitude of the tubulovascular response (8, 16, 18, 22, 25, 28), whereas peritubular and systemic infusion of ANG II was found to enhance the vascular response magnitude (13, 19). The physiological implication of the dependence of feedback regulation on ANG II levels is that a change in the activity of the renin-angiotensin system may be responsible for the alteration in macula densa-dependent responses seen during changes in extracellular fluid volume (3, 21).

The generation of transgenic mice with null mutations in components of the renin-angiotensin system offers a new way to examine the role of ANG II in tubuloglomerular feedback (TGF) coupling. Previous results have shown that the effect of changes in macula densa NaCl concentration on afferent arteriolar tone is virtually abolished in mice with a knockout mutation in the AT1A receptor gene (24). Two laboratories have independently generated mice with a targeted disruption of the angiotensin converting enzyme (ACE) gene that interferes with the production of functional ACE enzymes of both the somatic and testicular isoforms (5, 10). ACE enzyme activity in plasma and tissues such as lung, kidney, heart, and brain, as well as ACE mRNA and protein in various tissues, was virtually undetectable in homozygous ACE-knockout mice (5, 26). The current experiments in one of these ACE-knockout strains were performed to examine whether interference with the renin-angiotensin system by an independent intervention results in the same alteration of the tubulovascular signaling pathway previously found in the AT1A receptor knockout animals (24). The second aim of these studies was to test whether an acute administration of ANG II can restore the ability of increases in distal NaCl concentration to cause afferent vasoconstriction.

METHODS

Experiments were performed in a strain of ACE germline null mutant mice that has been generated as described by

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Krege et al. (10). All animals used in this study [wild-type ACE (+/+), heterozygous ACE (+/-), and homozygous ACE (-/-)] were derived from a heterozygous breeder pair from the original North Carolina colony. At weaning, animals were ear-tagged and a short piece of the tail was clipped off. Genomic DNA was extracted from the tail samples with a standard procedure involving digestion with proteinase K and purification of DNA with ethanol. We determined the genotype of each DNA sample by testing for the presence of wild-type or modified ACE sequence using PCR. To identify the presence of the wild-type gene, ACE gene-specific primers upstream and downstream of the Hind III site used for the insertion of the neomycin resistance gene (Neo') construct were used to amplify a 443-bp product that included a 207-bp intronic sequence but that was identical in its exon sequences to the published ACE cDNA (2). Primer sequences are sense 5'-AAGCACGAGTATCCATACACC-3' (bp 1086–1100) and antisense 5'-AATGGCACCATTGTCGCCCTGGAA-3' (bp 1297–1321). We detected the mutant ACE gene with primers amplifying a 457-bp product of the Neo' by using previously published oligonucleotide sequences (1). An example of PCR genotyping is shown in Fig. 1. A third PCR for β-globin served as control to validate similar amounts of DNA in each sample. Amplification was carried out for 30 cycles (denaturation at 94°C for 40 s, annealing at 58°C for 40 s, and extension at 72°C for 40 s, followed by a final extension at 72°C for 8 min). After genotyping, animals were separated according to genotype and gender.

Animals were maintained on a standard rodent chow and tap water. Male mice (23–30 g) were anesthetized with 100 mg/kg xactin ip and 100 mg/kg ketamine im. Body temperature was maintained at 38°C by placing the animals on an operating table with a servo-controlled heating plate. The trachea was cannulated and a stream of 100% oxygen was blown toward the tracheal tube throughout the experiment. The femoral artery was cannulated with hand-drawn polyethylene tubing for measurement of arterial blood pressure. The femoral vein was cannulated for an intravenous maintenance infusion of 2.25 g/100 ml BSA in saline at a rate of 0.35 ml/h. The perfusion fluid contained (in mM) 136 NaCl, 4 NaHCO3, 4 KCl, 2 CaCl2, 7.5 urea, and 100 mg/100 ml FD&C green (Keystone). At the end of each experiment, we tested the blood pressure responses to intravenous ANG I (10, 50, and 100 ng) and ANG II (1, 3, 5, and 10 ng) to functionally verify the genotype of the animal.

Micropuncture data are expressed as experimental, not tubule, means. Statistical comparisons were made with ANOVA, in association with the Scheffe test or the Student's t-test, using paired or unpaired comparisons, as appropriate.

RESULTS

Experiments were performed in six +/+ mice, seven +/- mice, and four -/- mice. Average body and kidney weights, respectively, were 27.7 ± 0.6 g and 168 ± 3.8 mg in wild-type, 27.6 ± 2 g and 149 ± 14.7 mg in heterozygous mice, and 30.2 ± 0.95 g and 173 ± 12.8 mg in homozygous mice, values not significantly different between genotypes. Mean arterial blood pressure of the anesthetized mice was 99.3 ± 5.4 mmHg in wild-type (78.5–110 mmHg), 90.5 ± 2.9 mmHg in heterozygous (83–102 mmHg), and 79.5 ± 5.9 mmHg in homozygous mice (73–97 mmHg; P = 0.057 compared with wild-type). To determine whether elevated bradykinin levels may contribute to the low blood pressure in ACE-knockout mice, two ACE -/- and two ACE +/- mice were injected intravenously with 10 μg of the bradykinin B2 receptor agonist HoE-140 immediately after cannulation of the femoral vein. No sustained blood pressure effects were observed in HoE-140-pretreated mice. Blood pressure responses to exogenous...
ANG I were similar between ACE +/+ and ACE +/− mice but were markedly blunted in ACE −/− animals, providing an independent verification of the ACE-null genotype (Fig. 2). Blood pressure responses to ANG II were not significantly different between ACE +/+ , +/− , and −/− animals (Fig. 2).

In response to an increase in loop of Henle flow rate from 0 to 40 nl/min, Psf fell from a mean of 42.3 ± 1.95 to 33.6 ± 2.09 mmHg (mice/tubules = 6/23; P = 0.005) in wild-type mice, and from 40.6 ± 3.25 to 38.6 ± 1.93 mmHg in ACE +/− mice (mice/tubules = 7/16; P = 0.004). In homozygous ACE mutant mice, Psf averaged 36.7 ± 2.02 at 0 loop flow and 36.4 ± 2.01 mmHg at a flow of 40 nl/min, indicating abolished TGF responsiveness (mice/tubules = 4/15; NS). Examples of original recordings obtained in mice with different ACE genotypes are shown in Fig. 3.

Maximum Psf responses during both control and ANG II infusion periods were obtained in six nephrons of wild-type, seven nephrons of heterozygous, and nine nephrons of homozygous ACE transgenic mice. Because results were obtained in the same nephrons, comparison between control and angiotensin infusion periods was done by paired t-test. Data are shown in Fig. 4. It can be seen that ANG II infusion caused a significant increase in the TGF response magnitude in all three groups of animals (3.3 ± 0.63 mmHg in +/+ , 3.4 ± 1.03 mmHg in +/− , and 3.6 ± 1.1 mmHg in −/− animals), implying that the degree of increase in responsiveness was greater in the transgenic than in the control mice (1.68 ± 0.18-fold in +/+ , 2.7 ± 1-fold in +/− , and 4.7 ± 1.5-fold in −/− mice). Even though TGF responses in heterozygous or homozygous mice during ANG II infusion tended to be somewhat lower than those measured during control in wild-type mice, the difference did not achieve statistical significance, suggesting that angiotensin infusion restored responsiveness to near normal levels. During ANG II infusion, Psf, in the absence of loop perfusion, averaged 46.3 ± 2.2 mmHg in +/+ mice, 39.8 ± 2.2 mmHg in +/− mice, and 41.7 ± 2.07 mmHg in −/− mice, values not significantly different from each other (as determined by ANOVA). Mean arterial blood pressure during ANG II infusion averaged 101 ± 5.6 mmHg in +/+ mice (vs. 99.2 ± 5.6 mmHg before the infusion), 89.5 ± 3.1 mmHg in +/− mice (vs. 88.4 ± 3.3 mmHg before ANG II), and 78.7 ± 2.8 mmHg in −/− mice (vs. 77.1 ± 2.8 mmHg before ANG II). Blood pressures were not significantly elevated by ANG II in any of the three groups of mice.

DISCUSSION

Previous studies from our laboratory have shown that TGF responses are essentially absent in mice homozygous for a null mutation in the AT1A receptor gene (24). The present experiments in ACE transgenic mice complement these previous studies by showing that both homozygosity and heterozygosity for the disrupted ACE gene are associated with a marked reduction in the efficiency of macula densa-mediated feedback control of vascular resistance. The observation that interference with the normal operation of the renin-angiotensin system by two independent genetic interventions causes the same change in the physiological endpoint studied is strong evidence for ANG II being an integral and necessary component of the TGF pathway. It is unlikely that genetic background effects, which would be expected to be different between animals of the AT1A and the ACE mutant strains, are responsible for the strong and systematic influence of functional ANG II deficiency in the juxtaglomerular...
signaling pathway. Abolition of TGF-mediated effects of luminal NaCl concentration on glomerular hemodynamics in mice homozygous for the disruption in the ACE gene is paralleled by undetectable levels of ACE activity in plasma and by tissue ACE activities in lung, kidney, heart, and brain that approach zero (5, 10, 26). In the same tissues, ACE mRNA expression was found to be extremely low (26). Virtual absence of plasma and tissue ACE activity is supported at the functional level by the almost complete absence of blood pressure responses to intravenously injected ANG I in both the present study and previous observations (26).

Even though only a small number of homozygous ACE mutant animals were studied successfully, we believe that our conclusion of complete TGF blockade in these animals is justified. Results from 16 nephrons in four mice were remarkably homogeneous, with average responses in the four mice being between 0 and 0.3 mmHg. Statistical analysis in this small group of animals is, therefore, not skewed by abnormal responses in one of the mice. Micropuncture experiments in homozygous ACE mutants proved difficult for two main reasons. Consistent with previous observations in conscious mice, most of the −/− animals we attempted to study had blood pressures lower than 65–70 mmHg (5, 10, 11, 26). The reliable use of the P_{SF} method, which requires multiple punctures of the same nephron, is virtually impossible at these low pressure levels. Furthermore, previous results in normal rats, as well as in mice, have shown that the TGF response magnitude is directly dependent on blood pressure, with responses becoming essentially undetectable at a level of ~60 mmHg (20, 27). Thus the effects of pressure per se and of the genetic intervention would be indistinguishable. Preliminary studies with the bradykinin B2-receptor blocker Hoe-140 did not yield evidence that the low blood pressure was due to a tonic blood pressure-lowering effect of elevated bradykinin levels, which one may expect in ACE−/− mice. Enhanced and prolonged blood pressure-lowering effects of bradykinin have been reported in ACE−/− animals in a previous study, but the converse, a blood pressure increase with BK-receptor blockade, was not studied (26). The second problem was related to choosing tubules for study in view of visible inhomogeneities in the appearance of the renal surface. Consistent with the structural abnormalities described earlier (5, 7), the surface of ACE−/− mice showed regions with dilated tubules and cystic tubular enlargements. Although micropuncture was restricted to areas that appeared largely normal, infusion of the perfusate sometimes indicated delays in the clearance of the stained fluid. Thus we cannot exclude
the possibility that the absence of feedback-induced vasomotion is, in part, related to the structural abnormalities that have been observed in the renal vasculature (5, 7). Restoration of TGF responses during ANG II infusion to values not significantly different from control would suggest that afferent arterioles have maintained at least some capacity to regulate their tone.

Our present results are in general agreement with several previous studies showing that acute administration of a number of different ACE inhibitors, including captopril, enalapril, teprotide, and CGS-14824, caused a consistent attenuation of TGF responsiveness (8, 18, 22, 25, 28). Nevertheless, the degree of TGF inhibition in all of these studies was more modest, with TGF responses being reduced to only 25–50% of control. Even with very high doses of captopril, TGF inhibition did not reach 100% (22). It is likely that the doses utilized in most cases reduced plasma ACE activity to very low levels for the duration of the experiment (4, 9, 12). For example, a single oral dose of 10 mg/kg enalapril has been shown to reduce plasma ACE activity in rats to undetectable levels for longer than 24 h (12). Furthermore, blood pressure responses to ANG II during ACE inhibition were tested in most of the studies referred to above and were found to be largely abolished (8, 18, 25, 28). Because acute and complete ACE inhibition is usually paralleled by a reduction of plasma ANG II concentration to low levels (4, 9), TGF responsiveness does not appear to correlate tightly with plasma ACE activity and plasma ANG II levels. However, modulation of TGF responses by ANG II may primarily depend on the formation of the peptide in the juxtaglomerular interstitium and, therefore, on the activity of renal tissue ACE rather than plasma ACE. It is conceivable that acute administration of ACE inhibitors may inhibit tissue ACE and deplete tissue ANG II levels to a lesser degree than chronic ACE deficiency. Thus it appears that the chronicity of complete ACE inhibition distinguishes the current studies in ACE-knockout mice from the previous studies with pharmacological blockers. Whether long-term adaptations outside the renin-angiotensin system take place that contribute to TGF inhibition remains to be determined. It is possible that chronic ACE deficiency is associated with increases in bradykinin or angiotensin-(1–7) levels in the juxtaglomerular interstitium that may affect TGF responses by nonspecific vasodilatation (4, 29). Furthermore, the contribution of the structural alterations in the homozygous knockout mice to overall vascular reactivity is an issue that requires further exploration.

In view of the inability of even high doses of ACE inhibitors to induce complete inhibition of TGF responses when given acutely, it was somewhat surprising that TGF responses were found to be markedly reduced in heterozygous ACE mutants. In fact, even though loop of Henle flow elevation still caused a significant reduction of P_{SF}, the inhibition of TGF responses in the heterozygous ACE mice appeared greater than that previously observed in mice heterozygous for the AT_{1A} knockout mutation (24). In agreement with earlier observations, blood pressure levels in the +/− animals were not significantly different from those of +/+ animals, even though they tended to be slightly lower (5, 11, 26). Thus heterozygous animals appear to be able to generate sufficient amounts of ANG II to fulfill its role in sustaining blood pressure. Largely normal blood pressure responses to exogenous ANG I in heterozygous animals support the notion of a well-maintained ACE enzymatic activity in plasma and/or endothelial cells. Because blood pressure sustenance may be primarily dependent on plasma ACE, whereas TGF support may mainly come from kidney tissue ACE, it is conceivable that this difference in susceptibility to an ACE reduction in the heterozygous animals may signify a greater suppression of tissue ACE than plasma ACE. However, available data do not strongly support this possibility. Plasma ACE activity has been found to change in a linear fashion when ACE copy number was reduced to 1 or increased to 3 by gene knockout or gene duplication strategies, respectively (11). In agreement with this finding, ACE plasma activity was reduced by ~50% in the heterozygous ACE animals (5, 10, 26). Similarly, kidney tissue ACE activity, kidney ACE mRNA, and kidney ACE protein expression were also reduced to ~40–60% of control in ACE mutant heterozygotes (5, 26). It appears that, despite identical reductions in ACE activity, ANG II levels in renal tissue are affected to an extent that limits TGF responsiveness, whereas plasma ANG II is sufficient to support blood pressure. In fact, there is previous evi-
dence to show that the decrease in ANG II levels after the administration of low doses of converting enzyme inhibitors is markedly greater in plasma than in kidney tissue (4, 6). For example, a dose of the ACE inhibitor perindopril, which caused a 50% reduction in ACE activity, did not affect plasma ANG II while it reduced renal tissue ANG II to 40% of control (4). Measurements of ANG II levels in plasma and renal tissue of heterozygous ACE-knockout mice are required to determine if ANG II generation in the kidney is reduced in proportion to plasma ANG II. Because the appearance of the kidneys in +/– mice was indistinguishable from that of +/- animals, the reduced TGF activity does not seem to be explainable by changes in renal vascular structure.

The major additional finding in these experiments is the observation that administration of exogenous ANG II caused a restoration of TGF responsiveness to 71% of control in heterozygous and to 62% of control in homozygous ACE mutant mice. Even though TGF responses in the mutant mice during ANG II infusion were not significantly different from those observed in control mice before angiotensin infusion, it appears that full restoration of TGF responsiveness was not achieved. Because arterial blood pressure did not significantly change during the angiotensin infusion period, restoration of TGF responses does not appear to be explainable by the hypertensive action of ANG II. Our observations are consistent with previous studies showing that infusion of ANG II in rats treated acutely with enalapril or captopril tended to restore TGF responses without reaching 100% normalization (8, 17). Furthermore, in volume-expanded rats, in which TGF responses were markedly reduced in parallel to a reduction in plasma ANG II concentration, infusion of ANG II caused a restoration of TGF responses to levels normally seen in hypodinamic rats (21). It was concluded from these and other results that ANG II sensitizes the TGF target vessels to the constrictor effect of the flow-dependent TGF-mediating agent (3, 17, 21). Although in the current studies, the effect of angiotensin was highly significant and consistent, it is noteworthy that full normalization of TGF responses was not achieved. The reason for the tendency of an acute ANG II infusion to not fully correct the response deficit associated with acute or chronic ACE deficiency is unclear. Based on previous determinations of the relation between ANG II infusion rate and plasma ANG II, it seems unlikely that response restoration was limited by insufficiently high plasma ANG II levels (15, 21). Furthermore, the rate of infusion in the present study, as well as in previous studies, was higher than needed to restore TGF responses in the volume-expanded rats (21). These data suggest that the TGF-sensitizing effect of ANG II is only in part dependent on plasma ANG II and needs to be complemented from an extravascular source. In fact, there is strong evidence to suggest that marked differences exist in ANG II levels between the plasma and tissue compartments in the kidney and, most likely, between different regions of the kidney as well (14). The inability to quantitatively restore the functional consequences of chronic ANG II depletion with an acute and short-term infusion may be a consequence of this highly compartmentalized distribution of the renin-angiotensin system in the kidney. It is conceivable that, despite restoration of plasma ANG II concentrations, normalization of peptide levels was not achieved at the site where ANG II interacts with the TGF system, presumably in the juxtaglomerular interstitium.

In summary, marked blunting of TGF responses in mice homozygous or heterozygous for a null mutation of the ACE gene, and restoration of responsiveness in these animals by ANG II, supports the notion that the renin-angiotensin system is a necessary component of macula densa control of renal vascular tone.

This work was supported by National Institutes of Health (NIH) Grants DK-37448 and DK-40042 (to J. Schnermann and J. Briggs), HL-49277 and GM-20609 (to O. Smithies), and HL-03470 (to J. H. Krege). J. H. Krege was a Howard Hughes Postdoctoral Fellow. Support was provided, in part, by the General Clinical Research Center at the University of Michigan, funded by National Center for Research Resources Grant M01-RR-00042. Present address and address for reprint requests and other correspondence: J. Schnermann, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bldg. 10, Room 4D 51, 10 Center Dr. MSC 1370 (E-mail: J. urgen@ntra.niddk.nih.gov).

Received 20 October 1998; accepted in final form 23 February 1999.

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