Cuff-induced vascular intima thickening is influenced by titration of the Ace gene in mice

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Lacchini S, Heimann AS, Evangelista FS, Cardoso L, Silva GJ, Krieger JE. Cuff-induced vascular intima thickening is influenced by titration of the Ace gene in mice. Physiol Genomics 37: 225–230, 2009. First published March 3, 2009; doi:10.1152/physiolgenomics.90288.2008.—We tested the hypothesis that small changes in angiotensin I-converting enzyme (ACE) expression can alter the vascular response to injury. Male mice containing one, two, three, and four copies of the Ace gene with no detectable vascular abnormality or changes in blood pressure were submitted to cuff-induced femoral artery injury. Femoral thickening was higher in 3- and 4-copy mice (42.4 ± 4.3% and 45.7 ± 6.5%, respectively) compared with 1- and 2-copy mice (8.3 ± 1.3% and 8.5 ± 0.9%, respectively). Femoral ACE levels from control and injured vessels were assessed in 1- and 3-copy Ace mice, which represent the extremes of the observed response. ACE vascular activity was higher in 3- vs. 1-copy Ace mice (2.4-fold, P < 0.05) in the control uninjured vessel. Upon injury, ACE activity significantly increased in both groups [2.41-fold and 2.14-fold (P < 0.05) for 1- and 3-copy groups, respectively] but reached higher levels in 3- vs. 1-copy Ace mice (P < 0.05). Pharmacological interventions were then used as a counterproof and to indirectly assess the role of angiotensin II (ANG II) on this response. Interestingly, ACE inhibition (enalapril) and ANG II AT1 receptor blocker (losartan) reduced intima thickening in 3-copy mice to 1-copy mouse values (P < 0.05) while ANG II treatment significantly increased intima thickening in 1-copy mice to 3-copy mouse levels (P < 0.05). Together, these data indicate that small physiologically relevant changes in ACE, not associated with basal vascular abnormalities or blood pressure levels, do influence the magnitude of cuff-induced neointima thickening in mice. 

vascular injury; angiotensin I-converting enzyme; gene titration; transgenic mice

VASCULAR STRUCTURE AND FUNCTION derangements are common phenotypes underlying cardiovascular diseases such as atherosclerosis, in which a complex interplay between circulating blood cells and vascular wall resident cells appears to play a role (18, 26). Intima thickening is an early event in the development of atherosclerotic lesions, and several experimental models have been established to study the components of this pathological condition and to assess the efficacy of therapeutic strategies. The cuff-induced intima thickening model is a well-established intervention in which inflammation appears to play an important role (7, 14, 19).

Although a number of factors have been postulated to participate in the development and maintenance of vascular derangements, the isolated contribution of specific genetic or environmental factors to this process remains largely elusive. Components of the renin-angiotensin system (RAS) have always been considered good candidates since a large body of data indicates that angiotensin II (ANG II) is associated with the production of reactive oxygen species and inflammatory cytokines and activation of adhesion molecules (33). This is also consistent with the observation that angiotensin I-converting enzyme (ACE) is activated in inflammatory cells present in vascular lesions and that RAS components are activated during differentiation of monocytes to macrophages (32). ACE activity is also increased in the neointima associated with vascular injury in the rat (24), in human carotid artery plaques (11), and in stent-induced inflammatory processes (25).

In contrast, genetic evidence obtained from the use of the ACE I/D polymorphism, which is associated with changes in plasma and tissue ACE, is less clear (1, 6). Meta-analysis based on a large number of studies has indicated that the D allele that confers increased ACE levels may not be clinically relevant in the general population but may play a role in specific subgroups of individuals (28). Recently, the use of platforms that enable simultaneous test of multiple single nucleotide polymorphisms (SNPs) per locus has provided additional evidence for positive associations between ACE and metabolic or vascular phenotypes, indicating that under certain conditions or genetic backgrounds the ACE locus may play a role (13). Altogether, these data highlight the difficulties in assessing the contribution of single factors to a complex phenotype.

To circumvent some of these limitations, we used genetically engineered mice that carried one, two, three, or four copies of the Ace gene at its endogenous locus, resulting in discrete lower and above normal ACE levels (16), to evaluate the influence of increasing Ace gene dosage on the magnitude of neointima thickening associated with cuff-induced vascular lesion while other genetic and environmental factors are controlled.

METHODS

Animals. Adult male genetically modified mice harboring one to four copies of the Ace gene were maintained in a light (12:12-h light-dark cycle)- and temperature (22°C)-controlled environment with free access to standard rodent chow and tap water. Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of University of São Paulo Hospital (no. 1018/02).

Generation of mice with one to four copies of the Ace gene. Genetically engineered mice carrying either inactivation (15) or duplication (16) of the Ace gene at its endogenous locus on chromosome 11 were used. Heterozygous mice of each strain were intercrossed to generate the experimental groups with one, two, three, and four copies of the Ace gene. Genotyping of each animal was performed by PCR amplification of DNA isolated from ear biopsies, as described previously (15). Mice carrying one (1-copy; +/+), two (2-copy; ++/+), three (3-copy; ++/+ ), or four (4-copy; ++/+ + ) functional Ace gene copies were studied. These mice present no detectable vascular abnormalities (e.g., femoral and carotid arteries).

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Direct hemodynamic measurements. Blood pressure was directly monitored in mice harboring one to four copies of the Ace gene. Polyethylene cannulas (PE-08) were implanted under anesthesia (pentobarbital sodium, 40 mg/kg ip) into both left carotid and femoral arteries and tunneled to the back of the mice. Blood pressure was monitored 24 h later in awake animals. The analog signal from the strain gauge transducer (Statham P23Dd; Hato Rey, Puerto Rico) was amplified (GPA-4 model, Stemtech), converted to digital at 10 bytes (DataQ Instruments), and further recorded at 2,000 Hz.

Femoral artery cuff placement. The surgical procedure for the cuff-induced femoral artery injury model was performed according to previously described methods (19). Briefly, under anesthesia (90 mg/kg ketamine + 10 mg/kg xylazine ip) and with body temperature maintained at 38°C by placing the animals on an operating table with a servo-controlled heating plate, the left femoral artery was isolated from surrounding tissues, loosely sheathed with a 2.0-mm polyethylene cuff (inner diameter 0.56 mm, outer diameter 0.965 mm; Becton Dickinson), and closed with sutures. The cuff is larger than the vessel and does not obstruct blood flow. The right femoral artery was used as a control. The surgical site was then closed with a 6-0 polypropylene suture. After recovery from anesthesia, the animals were given a standard diet and water ad libitum.

Carotid cuff placement. The surgical procedure for the cuff-induced vascular injury model in the carotid artery was performed according to previously described methods (23). Briefly, the left carotid artery was surgically exposed via a midline incision over an area from the chin to the sternum. The salivary gland was separated, and the artery was dissected proximal to the bifurcation. A 2.0-mm polyethylene cuff was placed around the periphery of the artery proximal to the bifurcation. Similar to that used for the femoral artery, the cuff is larger than the vessel and does not obstruct blood flow. The right carotid artery was used as a control. The surgical site was then closed with a 6-0 polypropylene suture, and the mice were allowed to recover. After recovery from anesthesia, the animals were given a standard diet and water ad libitum.

Tissue harvesting and histological staining. Fourteen days after implantation, mice were killed with an intraperitoneal injection of pentobarbital sodium (100 mg/kg), concomitantly receiving heparin sodium (50 U) and subsequently perfused with 0.9% NaCl solution at constant pressure (80–90 mmHg) and then with buffered 4% formalin solution. The tissues were maintained in formalin for 24–48 h to complete the fixation process. The arteries were studied within the adjacent tissues to preserve their integrity. Tissues were processed and paraffin embedded for histological evaluation. Hematoxylin and eosin stained sections from each cuff were investigated, as is commonly done in this type of study. To minimize variation, we analyzed activity in a different group of 1- and 3-copy Ace mice. Total protein was extracted from the tissues with detergent (0.01 M HEPES, 0.3 M KCl, 0.5% NP-40) at 4°C overnight and then centrifuged at 4°C and 19,000 g for 90 min to recover the supernatant. Supernatants were frozen at −80°C. ACE activity was measured by a continuous recording assay using internally quenched fluorescent peptide with the sequence [Abz-FRK(Dnp)P-OH; Abz = ortho-aminobenzoyl; Dnp = dinitrophenyl] that was cleaved with the highest catalytic efficiency by purified rabbit lung ACE (K_m/Kcat = 45.4 μM−1s−1) (5). The hydrolysis of Abz-FRK(Dnp)P-OH in the artery supernatant was abolished by 0.5 μM captopril. Enzymatic activity was continuously followed in a fluorimeter by measuring the fluorescence at emission wavelength (λ_em) = 420 nm and excitation wavelength (λ_ex) = 320 nm. The slope was converted into micromoles of hydrolyzed substrate per minute. Twenty microliters of carotid supernatants was used for each assay. The supernatants were incubated with 15 μM Abz-FRK(Dnp)P-OH in 0.1 M Tris-HCl buffer containing 50 mM NaCl and 10 μM ZnCl2, pH 7.0 (200-μl final volume). Enzymatic activity was continuously followed by a fluorimeter measuring the fluorescence over 100 min for carotid arteries and over 50 min for femoral arteries. ACE activity was expressed as micromolar [1 μM = nmol of Abz-FRK(Dnp)P-OH hydrolyzed per minute in a final volume of 200 μl] normalized by protein sample content. All assays were performed in duplicate. The protein contents of the samples were measured by the method of Bradford (8), with bovine serum albumin as standard.

Sequential blockade of the renin-angiotensin system as a counter-proof and to assess indirectly the role of ANG II on this response. To counteract the effects of genetic ACE activation on neointima formation, 3-copy mice (n = 8) were treated with the ACE inhibitor enalapril maleate (100 mg/l to drink). The treatment started a day before cuff implantation and was maintained for the 14 days of vascular lesion. Two additional groups were prepared to verify the importance of ANG II in neointima formation: 1-copy mice (n = 5) received ANG II (100 ng·kg⁻¹·min⁻¹), and 3-copy mice (n = 6) received the ANG II AT1 receptor blocker (ARB) losartan (20 ng·kg⁻¹·min⁻¹) via subcutaneous osmotic minipump (Alzet). The doses of ARB and ANG II used resulted in no changes in blood pressure over the 2 wk period of observation. The minipumps were implanted 1 day before vascular injury by cuff, and the animals were maintained for 2 wk.

Statistical analysis. All values are expressed as means ± SD. Morphometric evaluations were first tested by two-way ANOVA, and ACE activity was tested by one-way ANOVA. When ANOVA demonstrated significant differences, Tukey’s post hoc analysis was used to compare groups. For all statistical analyses, P ≤ 0.05 was considered significant.

RESULTS

Hemodynamic measurements. Direct blood pressure measurements from cannulas placed in either carotid or femoral arteries showed that blood pressure and heart rate levels remained essentially the same in all groups regardless of the number of copies of the Ace gene (Fig. 1).

Morphometry, vascular ACE activity measurement, and pharmacological interventions were performed in three different sets of experiments, and the groups were always coded to prevent any bias until the end of the study.

For the evaluation of neointima thickening, mice harboring one, two, three, and four copies of the Ace gene were used. The femoral arteries, with and without intima injury, were investigated in these animals. In a pilot experiment (data not shown), we detected an important variation in the thickening areas among the animals when only a limited number of sections (5 sections from each cuff) were investigated, as is commonly done in this type of study. To minimize variation, we analyzed
a larger number of sections at fixed intervals, which enabled the estimation of the neointima area along the extent of the cuff. Special care was taken to always consider the same position with respect to the cuff so that the analysis started from the outer end toward the middle of the lesion. Body weight among the four groups remained unchanged [27.9 ± 3, 28.2 ± 2.9, 27.8 ± 2.9, and 28.3 ± 2.7 g (P > 0.5) for 1-, 2-, 3-, and 4-copy Ace groups, respectively; n = 9 or 10 for each group].

Neointima thickening in femoral artery. Upon cuff-induced injury, all mice developed vascular neointima thickening regardless of the Ace genotype. Figure 2A shows the relative area of neointima along 18 sections obtained every 50 μm for 1-, 2-, 3-, and 4-copy Ace groups, respectively.

Angiotensin I-converting enzyme activity. A different group of adult C57BL/6J mice was first used to standardize the method to quantify ACE activity in the short 2.0-mm vascular segment, where ANG II assessment cannot be easily done (data not shown).

We assessed femoral ACE activity in 1- and 3-copy mice based on the findings shown in Fig. 2 demonstrating that the maximal response is observed in the 3-copy mice and the fact that previous data from our lab and others have indicated that plasma and tissue ACE levels change 40–50% in mice harboring one and three copies of Ace gene compared with wild-type mice, whereas the response appears to level off in the 4-copy animals in some tissues. ACE vascular activity was higher in 3- vs. 1-copy Ace mice in the control uninjured vessel (2.4-fold, P < 0.05). Upon injury, ACE activity significantly increased in both groups [2.41-fold and 2.14-fold (P < 0.05) for 1- and 3-copy groups, respectively] but reached higher levels in 3- vs. 1-copy Ace mice (P < 0.05) as shown in Fig. 3.

Sequential blockade of the renin-angiotensin system. Cuff-induced injury in 1-copy mice exposed to higher ANG II levels while blood pressure remained unchanged resulted in neointima formation comparable to that observed in 3-copy mice (Fig. 4 and Supplemental Fig. S1).1 Similarly, when 3-copy mice were treated with ACE inhibitor or ARB, the neointima formation decreased its magnitude to the levels observed in 1-copy mice (Fig. 4 and Supplemental Fig. S1).

1 The online version of this article contains supplemental material.
Neointima thickening and sequential blockade of the renin-angiotensin system in carotid artery.

ACE activity levels in the carotid artery samples with cuff from the different groups could not be accurately estimated because of the large amount of perivascular tissue growth in these vascular segments. Overall, the neointima changes in the carotid arteries followed the same trend as observed in the femoral vessels, but were more discrete in magnitude, as shown in Supplemental Fig. S2. The neointima-to-media ratio in the 4-copy group was significantly different compared with the 1- and 2-copy groups (Supplemental Fig. S2). Again, the area of the media was not different among the groups both with and without neointima thickening (data not shown). Similarly, when 3-copy mice were treated with ACE inhibitor or ARB, the neointima formation decreased its magnitude to the levels observed in 1-copy mice (Supplemental Fig. S3).

DISCUSSION

The recognition that copy number polymorphisms represent a major source of genetic variation among individuals (17, 29) and potentially underlie phenotypical variation and disease susceptibility (2) has received enormous attention lately. The RAS is a point in case since it is a key cardiovascular control system and several functional gene variants in humans have
been identified. On the other hand, the relevance of these gene variants for assessment of individual risk remains controversial. Most likely, gene variants, associated with discrete alterations, may have important contributions for complex phenotypes that can only be properly uncovered in a context-dependent manner posing limitations for both human and experimental investigation and may explain, at least in part, the controversial issues.

The findings of the present study show that the magnitude of vascular intima thickening in response to a pathological stimulus was greater in animals harboring three or four copies of the Ace gene. The Ace genetic changes per se were not associated with vascular or blood pressure alterations. Kregel et al. (16) first demonstrated that serum ACE activity in the 1-copy mouse is 38% lower than in the 2-copy wild-type mouse and increases to 144% in the 3-copy mice, with no influence on blood pressure levels. Moreover, it appears that changes in Ace gene copy number from 1- vs. 3-copy mice also do not lead to changes in basal circulating ANG II levels, as shown by Alexiou et al. (4) for 1- and 2-copy animals, and also from 2- vs. 3-copy animals, which showed 2.90 ± 0.09 vs. 3.0 ± 0.1 pmol/ml (P < 0.05), respectively (personal communication from Dr. Dulce Casarini, Federal University of São Paulo, São Paulo, Brazil). One may assume that feedback control mechanisms take place, minimizing the impact of small perturbations, and then the issue becomes when the buffering capacity may reach a limit and what the consequences are for maintenance of normal homeostasis. Using this same experimental model, we previously demonstrated comparable exercise-induced cardiac hypertrophy in mice harboring different copy numbers of the Ace gene (1–4), despite the fact that cardiac ACE was proportional to Ace gene copy number. Most importantly, cardiac ANG II levels, which showed comparable values under basal conditions, increased to the same extent associated with exercise in mice harboring one and three copies of the Ace gene, which is consistent with the growth response observed. Although unexpected at first, this finding was in accordance with the reciprocal changes observed in plasma renin activity (PRA), that is, higher cardiac ACE associated with lower PRA and vice versa (10). In contrast, when a pathological stimulus was tested, pressure overload, a greater increase in cardiac mass and ANG II levels, was observed for the same increment in blood pressure in 3-copy vs. 1-copy mice, suggesting that perhaps the buffering capacity had reached a limit (30).

The findings of the present study lend support to the idea that compensation may be compromised by small changes in components of relevant control systems, such as the RAS. Mice harboring three copies of the Ace gene present no detectable vascular abnormality despite high vascular ACE levels, but upon injury ACE activity increased in both 1- and 3-copy mice, but the ACE levels continued to be higher in 3-copy vs. 1-copy mice, as illustrated in Fig. 3. ANG II was not assessed in these vessel segments because of technical limitations, but presumably the cuff-induced injury was accompanied by higher vascular ANG II, especially in the 3-copy group. This is supported by the fact that ARB treatment rendered the 3-copy mouse neointima response similar to that of 1-copy untreated mice and, the reciprocal, ANG II infusion, increased 1-copy mouse neointima area to the values observed in 3-copy untreated mice (Fig. 4 and Supplemental Fig. S2). These findings are in agreement with data indicating that ARBs effectively inhibit cell proliferation and restenosis after angioplasty or stenting (3, 9, 12, 20, 34). Given the fact that the ACE inhibitor was as effective as the ARB in reducing the neointima formation, ANG II appears to be the main modulator of this response. Less likely, one may still consider that accumulation of ANG-(1–7), in case of the ACE inhibitor group, or the preferential occupation of the ANG II AT2 receptor, in case of the ARB group, may play a role in this response. This would be consistent with data from Strawn et al. (31) on the antiproliferative actions of ANG-(1–7), from Okumura et al. (21), who provided evidence for an ANG II AT2 receptor vasoprotective role demonstrating that reduction in vascular injury was directly associated with the content of AT2 receptor in female mice, and from Sales et al. (27), who showed that the expression of ANG II AT2 receptor in atherosclerotic lesions might function as a modulator of atherosclerosis.

Neointima formation in general is a complex multifactorial process, but the RAS, and in particular ANG II, appears to play an important role since the peptide can influence key players of this process including cytokines, chemokines, and adhesion molecules, required for cell migration and proliferation in the vascular wall (33). More specifically, there is evidence implicating nitric oxide (19) with cuff-induced neointima formation and plasminogen activator inhibitor-1 (23) in a closely related model using a copper-induced cuff.

Changes in serum ACE associated with human ACE I/D polymorphism vary on the order of 20% below and above the heterozygous I/D genotype levels including different ethnic groups, as we have shown (22). Thus it is tempting to speculate that discrete single gene changes in major control systems may be relevant under certain conditions, underscoring the importance of developing more robust algorithms that take into account small gene effects in a context-dependent manner. Until then we may have to strive to obtain evidence that small gene effects affect complex phenotypes under well-controlled genetic and environmental settings, as we attempted to do in the present study.

Collectively, our data indicate that isolated small increases in ACE can increase the magnitude of vascular response to injury in mice.

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