The angiotensin II receptor antagonist valsartan inhibits endothelin 1–induced vasoconstriction in the skin microcirculation in humans in vivo: Influence of the G-protein β_3 subunit (*GNB3*) C825T polymorphism

Objective: **We investigated the influence of angiotensin II receptor blockade on angiotensin II–induced, endothelin 1 (ET-1)–induced, and norepinephrine-induced vasoconstriction to further characterize interactions of the 3 major pressor systems. ET-1, angiotensin II, and norepinephrine act via G protein–coupled receptors with a possible involvement of the G-protein β_3 subunit (*GNB3*) C825T polymorphism. We studied the influence of this polymorphism on the responses to angiotensin II antagonism in the presence of ET-1, norepinephrine, and angiotensin II.**

Methods: **Twenty-five healthy men (mean age, 28.6 ± 4 years; n = 14 CC, n = 9 CT, and n = 2 TT) were included in a double-blind, randomized, placebo-controlled crossover study. We used a laser Doppler imager to evaluate skin perfusion changes after injection of ET-1, angiotensin II, and norepinephrine (10^{-18}, 10^{-16}, and 10^{-14} mol) after oral intake of the angiotensin II receptor antagonist valsartan (80 mg) or placebo. Data were analyzed with ANOVA or *t* test and are expressed as arbitrary perfusion units (PU) (mean ± SEM).**

Results: **Valsartan abolished angiotensin II–induced vasoconstriction and, more importantly, also ET-1–induced vasoconstriction in the skin microcirculation (ET-1 placebo versus valsartan, − 33 ± 10 PU versus +33 ± 21 PU for CC [$P = .02$] and −71 ± 25 PU versus +108 ± 21 PU for CT/TT [$P < .001$]). For both ET-1 and angiotensin II, valsartan effects were greater in *GNB3* 825T-allele carriers ($P = .007$ and $P = .03$ for ET-1 and angiotensin II, respectively, for CC versus CT/TT). Norepinephrine-mediated constriction was not influenced by valsartan. These effects were independent of blood pressure.**

Conclusion: **Our results indicate that the renin-angiotensin system may significantly contribute to ET-1–mediated microvascular responses. Valsartan inhibited local vasoconstriction to angiotensin II and ET-1 to a greater degree in carriers of the *GNB3* 825T allele, which adds to data from earlier studies implicating the C825T polymorphism as a pharmacogenetic marker for drug effects. (Clin Pharmacol Ther 2006;79: 274-81.)**

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A genetic predisposition is one of multiple factors contributing to the development of hypertension. Interactions between the blood pressure–regulating systems—the renin-angiotensin system (RAS), the sympathetic nervous system (SNS), and the endothelin system (ETS)—are also being implicated in the pathogenesis of a sustained increase in blood pressure.[1,2] Whereas the interactions of the RAS and SNS have been well characterized, knowledge about their respective interrelationships with the ETS is limited.[3,4]

In earlier studies aimed at identifying interactions between the RAS, SNS, and ETS, we have shown that endothelin 1 (ET-1) potentiates the vasoconstriction to angiotensin II and norepinephrine in the human skin microcirculation.[5] Endothelin A (ET_A) receptor–selective antagonism significantly inhibited vasoconstriction to ET-1, angiotensin II, and norepinephrine in the skin microcirculation but did not influence ET-1 and norepinephrine effects in human dorsal hand veins,[6] indicating that varying regulatory mechanisms may apply in different vessel types.

In vitro and animal studies have demonstrated multiple stimulatory effects of angiotensin II on ET-1 gene and messenger ribonucleic acid (mRNA) expression, endothelin-converting enzyme 1 activity, ET-1 secretion, and ET-1 binding.[7-10] In turn, antagonism at angiotensin II type 1 (AT_1) receptors inhibited ET-1 gene expression in rat vascular smooth muscle cells and reduced vascular ET-1 content and cardiac ET-1 mRNA expression in rats.[11-13]

Numerous studies have focused on the angiotensin II–mediated promotion of SNS activity at various levels. In animal models angiotensin II has been shown to enhance sympathetic neurotransmission, norepinephrine synthesis, and postsynaptic norepinephrine effects, to name but a few of the proposed interactions.[4,14,15] AT_1 antagonists effectively inhibited angiotensin II–induced facilitation of sympathetic neurotransmission in rats, but reports on the inhibition of vasoconstriction mediated by exogenous norepinephrine are conflicting.[15-17]

Data on the influence of AT_1 blockade on vasoconstriction induced by ET-1 or norepinephrine in humans are scarce. In hypertensive patients prolonged angiotensin II antagonism significantly reduced vasoconstriction mediated by endogenous ET-1,[18] but norepinephrine-induced vasoconstriction in healthy individuals was not inhibited by short-term angiotensin II receptor blockade.[19]

Genetic polymorphisms may influence vascular tone and vascular reactivity. The 825T allele of the C825T polymorphism, which was identified for the G-protein β_3 subunit (*GNB3*) gene, has been linked with hypertension and other risk factors for cardiovascular morbidity.[20-25] The mechanisms underlying these associations are not fully understood. In vitro the 825T allele is associated with increased intracellular signal transduction and increased cell responsiveness.[25-27] Receptors for both ET-1 and norepinephrine may activate G-protein heterotrimers involving the β_3 subunit. The ET_A receptor–mediated Ca^{++} release in rat portal vein myocytes is selectively transduced by a heterotrimeric G protein composed of α_{11}, β_3, and γ_5 subunits.[28] Antisense oligonucleotides directed against the mRNA coding for $G\alpha_q$, $G\alpha_{11}$, β_1, β_3, γ_2, and γ_3 subunits selectively inhibited the increase in Ca^{2+} activated by α_1-adrenergic receptors in these cells.[29] Earlier studies in our laboratory have indicated that the G-protein β_3 subunit and its splice variant $G\beta_3$-s may be involved in mediating vasoconstrictor effects of ET-1, norepinephrine, and indeed, angiotensin II in humans: vasoconstriction to injections of all 3 substances was enhanced in 825T-allele carriers.[6,30] In studies investigating systemic hemodynamics 825T-allele carriers have shown enhanced hemodynamic responses to various antihypertensive agents,[6,31,32] including an angiotensin-converting enzyme inhibitor (Schäfers RF, Oberhausen, Germany, unpublished data, 2003). Taken collectively, these results have suggested a generally increased response to vasoactive substances in carriers of the 825T allele, who might thus be expected also to respond more strongly to angiotensin II receptor antagonism.

In this study we have used the AT_1 receptor antagonist valsartan to further characterize the interactions between the major blood pressure–regulating systems while taking into account the influence of the *GNB3* C825T polymorphism. We hypothesized that, apart from inhibiting angiotensin II–mediated vasoconstriction, systemic AT_1 receptor blockade would reduce vasoconstriction to ET-1 and norepinephrine and that responses would differ with regard to *GNB3* C825T genotype.

METHODS

Design. We performed a placebo-controlled, randomized, crossover study. The investigator and subjects were blinded with regard to the drugs given orally (valsartan or placebo) and *GNB3* genotype. Subjects were also blinded with regard to the substances applied at each injection site.

Study population. Twenty-five young, white male volunteers (mean age ± SD, 28.6 ± 4 years; n = 9 CT, 2 TT, and 14 CC *GNB3* C825T genotype) were in-

Table I. Baseline characteristics of study population according to genotype at G-protein β_3 subunit C825T locus

	CT/TT (n = 11)	*CC (n = 14)*	*P value*
Age (y)	27.7 ± 3.5	28.9 ± 3.6	.41
Height (cm)	181 ± 2.8	181.4 ± 2.4	.89
Body mass index (kg/m^2)	22.02 ± 1.0	22.92 ± 1.3	.08
Systolic blood pressure (mm Hg)	114 ± 13	115 ± 8	.92
Diastolic blood pressure (mm Hg)	62 ± 5	67 ± 12	.27
Heart rate (beats/min)	62 ± 9	56 ± 6	.09

Data are shown as mean ± SD.

cluded in this study. All were healthy judged on the basis of medical history, physical examination, electrocardiogram, and routine laboratory screening. All subjects were nonsmokers and drug-free, and body mass index had to be 25 kg/m^2 or less. Each subject gave written informed consent before taking part in the study, which had been approved by the University of Duisburg-Essen Medical School Ethics Committee, Essen, Germany. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Genotyping. Genotyping with respect to the *GNB3* C825T polymorphism was performed as detailed previously.[25,33]

Drugs. The following drugs diluted in physiologic saline solution were used: ET-1 (Clinalfa, Läufelfingen, Switzerland), angiotensin II (Clinalfa), and norepinephrine (Arterenol; Aventis Pharma, Frankfurt am Main, Germany). To prevent adsorption of ET-1 to the syringe surface, 5% albumin was added to the ET-1 solution. Valsartan (Diovan) was provided by Novartis Pharma (Nürnberg, Germany).

Laser Doppler imaging. A laser Doppler imager scanner (Moor LDI; Moor Instruments, Axminster, Devon, United Kingdom) was used to measure skin perfusion. We have previously described the principles of laser Doppler image scanning.[34]

Study protocol. Experiments were performed on 2 study days, at least 7 days apart. Measurements were performed in a quiet, temperature-controlled room. Volunteers reported to the laboratory at between 8 and 9 AM in the fasting state. During the investigation, subjects remained in the supine position. Assessment of the skin microcirculation in response to ET-1, angiotensin II, and norepinephrine was started 2 hours after ingestion of either valsartan or placebo, in line with the pharmacokinetic profile of valsartan.[35,36] Injection sites were selected on the volar surfaces of both forearms, with avoidance of skin areas with visible veins. Care was taken to use the same injection sites for each agonist dose on the 2 study days. Skin temperature was monitored continuously. Before injections, the total volar surface of the arm was scanned to assess resting blood flow at each injection site. Then, 0.01 mL of saline solution was injected strictly intradermally, followed by ET-1, norepinephrine, or angiotensin II (10^{-18}, 10^{-16}, and 10^{-14} mol/0.01 mL) or a second injection of saline solution. Injections were made serially over a period of 2 minutes, starting proximally and moving distally on the forearm. To allow for assessment of drug effects over time, 12 images were collected per arm at 2.5-minute intervals. The double-injection technique has been applied in several studies and shows a high interday reproducibility.[6,30,34,37-39] Coefficients of variation for interday variability were 0.13 ± 0.09 (mean ± SD), 0.25 ± 0.02, and 0.07 ± 0.02 for ET-1, angiotensin II, and norepinephrine, respectively.

Data and statistical analysis. Data from the laser Doppler imager scanner were analyzed offline by use of Moor-Software V.3.01 (Moor Instruments). Resting blood flow was calculated from the flux values of the image taken before injection. The double injections with saline solution were used to control for the effects of injection trauma, carrier, and local volume changes. Saline solution–induced vasodilation was used as the baseline for the effects of the vasoconstrictors. To assess the net effects of ET-1, angiotensin II, and norepinephrine, the values for resting blood flow and saline solution at each injection site were subtracted from the values obtained for the agonists. All values were calculated as mean arbitrary perfusion units (PU) ± SD of the 12 measurements. The values for each dose were used for description of the dose-response curves to ET-1, angiotensin II, and norepinephrine.

Data from in vitro experiments and indirect evidence from population studies[25,40] demonstrate that the presence of the 825T allele, whether homozygous or heterozygous, changes cardiovascular and neurohumoral regulation relative to the 825CC genotype. Therefore CT and TT subjects were pooled for analyses.

The vascular responses to ET-1, angiotensin II, and norepinephrine were analyzed by 2-way ANOVA with

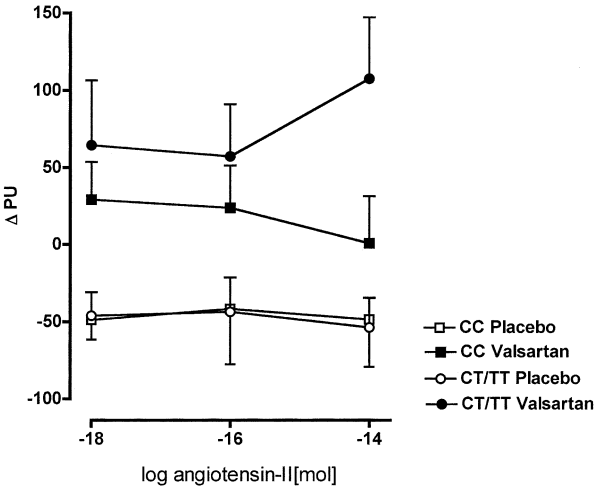

Fig 1. Mean changes ± SEM in skin perfusion (expressed as arbitrary perfusion units [PU]) in response to angiotensin II after ingestion of either placebo or valsartan. Valsartan abolished vasoconstriction to angiotensin II and instead induced vasodilation ($P = .018$ and $P < .001$ for CC and CT/TT versus placebo). This effect was more pronounced in carriers of the G-protein β_3 subunit (*GNB3*) 825T allele ($P = .03$ for CC versus CT/TT).

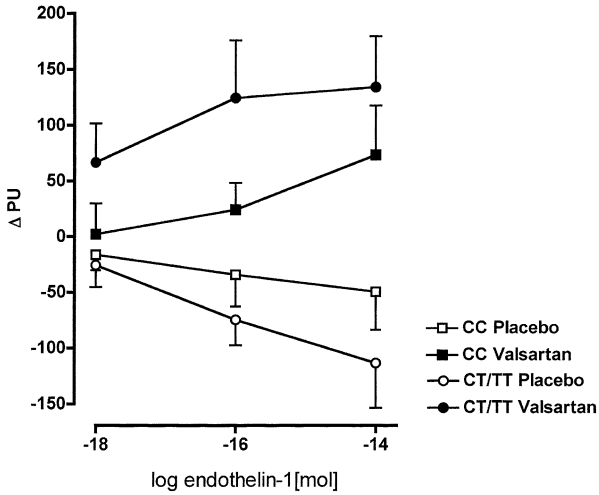

Fig 2. Mean changes ± SEM in skin perfusion in response to endothelin 1 after ingestion of either placebo or valsartan. Valsartan abolished vasoconstriction to endothelin 1 and instead induced vasodilation ($P = .02$ and $P < .001$ for CC and CT/TT versus placebo). This effect was more pronounced in carriers of the *GNB3* 825T allele ($P = .007$ for CC versus CT/TT).

the factors genotype and drug dose; anthropometric parameters were compared by use of 2-way unpaired *t* test. Results are expressed as mean ± SD unless stated otherwise. Significance levels were set at $P < .05$. Statistical analyses were performed with Prism V4.0 software (GraphPad Software, San Diego, Calif).

RESULTS

Anthropometric variables and baseline hemodynamic parameters did not differ by genotype (Table I). Skin perfusion before injections was similar in both groups and was not changed by angiotensin II antagonism (128 ± 28 PU versus 144 ± 49 PU for CC versus CT/TT on placebo day, $P = .16$; 151 ± 39 PU versus 142 ± 41 PU for CC versus CT/TT on valsartan day, $P = .48$; and $P = .10$ and $P = .83$ for CC and CT/TT, respectively, for valsartan versus placebo). Baseline perfusion after saline solution injections was also similar on both study days and did not depend on genotype (301 ± 199 PU versus 301 ± 113 PU for CC versus CT/TT on placebo day, $P = .99$; 279 ± 158 PU versus 260 ± 62 PU for CC versus CT/TT on valsartan day; and $P = .59$ and $P = .19$ for CC and CT/TT, respectively, for valsartan versus placebo).

On the placebo day, angiotensin II caused similar vasoconstriction at all doses without any difference according to genotype (−46 ± 4 PU versus −47 ± 5 PU for CC versus CT/TT, $P = .94$) (Fig 1). After ingestion of valsartan, angiotensin II–induced vasoconstriction was abolished and was instead turned into vasodilation (+17 ± 15 PU versus +76 ± 27 PU for CC versus CT/TT and $P = .018$ and $P < .001$ for CC and CT/TT, respectively, for valsartan versus placebo) (Fig 1). This effect was significantly greater in 825T-allele carriers (mean net difference in angiotensin II–mediated vascular responses of placebo versus valsartan day ± SD, +50 ± 11 PU for CC and +140 ± 15 PU for CT/TT; $P = .03$).

ET-1 caused dose-dependent vasoconstriction on the placebo day with a tendency toward a more pronounced response in 825T-allele carriers; however, this was not statistically significant (−33 ± 17 PU versus −71 ± 44 PU for CC versus CT/TT, $P = .14$) (Fig 2). After valsartan, ET-1–mediated vasoconstriction not only was abolished but was replaced by ET-1–mediated vasodilation (+33 ± 36 PU versus +108 ± 36 PU for CC versus CT/TT and $P = .02$ and $P < .001$ for CC and CT/TT, respectively, for valsartan versus placebo) (Fig 2). This effect was also significantly greater in 825T-allele carriers (mean net difference in ET-1–mediated vascular responses of placebo versus valsartan day ± SD, +66 ± 52 PU for CC and +182 ± 86 PU for CT/TT; $P = .007$).

Norepinephrine induced only minor vasoconstriction on the placebo day, which did not differ according to genotype (−9 ± 46 PU versus −29 ± 11 PU for CC versus CT/TT, $P = .56$). Valsartan had no significant effect on norepinephrine-mediated constriction (−10 ± 42 versus +17 ± 29 PU for CC versus CT/TT [$P = .26$] and $P = .94$ and $P = .15$ for CC and CT/TT, respectively, for valsartan versus placebo).

During the 4-hour investigation period, blood pressure and heart rate were similar on both study days and did not differ according to genotype (data not shown).

DISCUSSION

To our knowledge, we are the first investigators to show that short-term angiotensin II antagonism with valsartan prevents local vasoconstriction to exogenous ET-1 in healthy men. This effect, as well as inhibition of angiotensin II–mediated vasoconstriction by valsartan, was more pronounced in carriers of the *GNB3* 825T allele.

We found that after ingestion of valsartan, instead of vasoconstriction, angiotensin II induced vasodilation. Angiotensin II as the principal effector of the RAS acts at 2 cell membrane receptors, AT_1 and angiotensin II receptor type 2 (AT_2). Activation of AT_1 receptors on vascular smooth muscle cells elicits vasoconstriction. A wealth of animal studies have shown that, in contrast, activation of AT_2 receptors on endothelial cells induces vasodilation mediated by nitric oxide (NO) through both bradykinin-dependent and bradykinin-independent pathways.[41,42] AT_2 receptors contribute to the hypotensive and vasodilating effects of AT_1 antagonists in rats.[43,44] The role of AT_2 receptors in humans is much less well defined. Because of a lack of heuristic tools, so far only 3 studies have directly investigated the relevance of AT_2 receptors for the regulation of vascular tone in humans in vivo.[45-47] From this limited database, it seems that in the presence of an activated RAS or AT_1 receptor blockade (or both), AT_2 antagonism increases vascular resistance, which indirectly confirms a vasodilatory role for AT_2 receptors in humans.[45,47] Although determining the influence of AT_2 receptors on angiotensin II–mediated responses in the skin microcirculation was not the focus of our current investigations, our experimental condition, that is, local activation of the RAS by angiotensin II injections during AT_1 blockade, would make it plausible that the angiotensin II–induced vasodilation that we observed was a result of AT_2 receptor stimulation.

Data from animal and in vitro studies and, indeed, our own investigations in the human microcirculation suggest that angiotensin II contributes to ET-1–mediated vasoconstriction. In this study AT_1 antagonism not only abolished ET-1–induced vasoconstriction but induced vasodilation. At present, our interpretations of these results must remain hypothetical. So far, only 1 study in humans has provided evidence that AT_1 antagonism affects ET-1–mediated vascular responses: in hypertensive patients but not in healthy control subjects, the vasoconstricting effects of endogenous ET-1 were reduced after prolonged treatment with an AT_1 antagonist.[18] In that study it could not be determined whether blood pressure reduction or lowering of ET-1 plasma concentrations (or both) may have contributed to the results and whether, therefore, the results were specific to AT_1 blockade. Our study differs from this previous investigation in many respects. Nevertheless, the fact that our results were obtained in the absence of any hypotensive valsartan effects and with identical local ET-1 doses on both study days supports the hypothesis of a specific influence of AT_1 antagonism on ET-1–mediated local vascular responses. We have recently seen that angiotensin II potentiates ET-1–mediated vasoconstriction in the skin microcirculation of young, healthy men (Mitchell A, unpublished data, 2005). Reversal of this potentiation may also partly explain our results.

In patients with atherosclerosis AT_1 antagonism improved endothelial function on a short-term basis.[48] An increase in NO bioavailability by either an AT_1 receptor–mediated reduction in oxidant stress or an AT_2 receptor–mediated increase in NO synthesis was suggested as the underlying mechanism.[48] NO and ET-1 interact importantly in the control of vascular tone, and NO has been seen to directly inhibit ET-1–mediated contractions, possibly via the ET_A receptor.[49-52] Albeit speculatively, it would seem feasible that an increase in NO due to AT_1 antagonism with valsartan inhibits ET_A receptor–mediated vasoconstriction, leaving endothelial endothelin B receptors unchallenged, which may further increase the secretion of NO and vasodilatory prostaglandins, resulting in net ET-1–induced vasodilation.

Contrary to our working hypothesis, vasoconstriction to exogenous norepinephrine was not influenced by AT_1 antagonism. In rats high doses of AT_1 antagonists inhibited vasoconstriction to exogenous norepinephrine, and it is possible that valsartan would affect norepinephrine-mediated constriction at higher doses.[15] However, our data are in agreement with the single study that has previously investigated the effects of an AT_1 antagonist on norepinephrine-mediated vasoconstriction in healthy men with negative results.[19]

We did not reproduce our earlier observations in the skin microcirculation and the dorsal hand vein of a significantly greater vasoconstriction to ET-1, angiotensin II,

and norepinephrine in carriers of the *GNB3* 825T allele. This may be a result of the lower doses of the agonists applied in our study, which might also account for the fact that there was no dose-response relationship for angiotensin II–induced vasoconstriction.

Valsartan effects on both ET-1–mediated and angiotensin II–mediated local vascular responses were significantly greater in carriers of the *GNB3* 825T allele, whereas the lack of effect on norepinephrine-induced vasoconstriction was similar in CC homozygous subjects and carriers of the 825T allele. Previous data from our own laboratory and those of other authors suggest that 825T-allele carriers may respond more strongly to various antihypertensive/vasodilating agents.[31,32,53] In addition, this latest study indicates that, as a consequence of antihypertensive treatment, local hemodynamic control may differ significantly between genotypes even in the absence of differences in systemic hemodynamics. Although, at present, we cannot conclude which mechanisms underlie our results, we will offer some suggestions: In an earlier study investigating the vasoconstrictor effects of angiotensin II, ET-1, and norepinephrine in the skin microcirculation, we found evidence that the vasoconstriction in 825T-allele carriers may be partially antagonized by an enhanced release of endothelium-derived vasodilators via α_2-adrenergic receptors.[30] That study did not address the role of angiotensin II receptors, and so far, data on putative differences in endothelial function dependent on *GNB3* C825T genotype are not conclusive; other investigators have reported that acetylcholine-induced venodilation is similar in healthy CC homozygous individuals and 825T-allele carriers.[54] On the other hand, we have shown that venodilation to nitroglycerin is enhanced in 825T-allele carriers.[53] Although this might indicate an endogenous NO deficit in carriers of the 825T allele, an enhanced signal transduction via cyclic guanosine monophosphate pathways in the presence of elevated NO concentrations is another attractive concept. Altogether, it is tempting to assume that, as a result of AT_1 antagonism with valsartan, angiotensin II may stimulate NO secretion via AT_2 receptors and that this stimulation may result in either higher local NO concentrations or enhanced intracellular cyclic guanosine monophosphate activation in 825T-allele carriers.

Of note, in healthy individuals NO contributes to the intradermal vasodilation induced by saline solution injections,[55] which served as the baseline for assessing the vasoconstrictor effects in our study. Other mediators, such as prostaglandins, may also be involved. Thus we cannot exclude that our investigational approach may have altered the physiologic baseline. However, it seems unlikely that this would have influenced our results, because the injection protocols were the same for all individuals on both study days. The fact that saline solution injections produced similar intradermal vasodilation in both study groups may indicate that the mechanisms underlying saline solution–induced increases in skin perfusion do not differ according to genotype at the *GNB3* C825T locus.

In conclusion, our study, which was designed to further explore interactions of the major blood pressure–regulating systems in vivo, suggests that AT_1 antagonism with valsartan interferes with vascular responses to the main effectors of the RAS and ETS in the skin microcirculation of healthy men. The differences that we observed according to *GNB3* C825T genotype add to data from earlier studies that have implicated this polymorphism as a pharmacogenetic marker for drug responses.

A note of caution must be added to the interpretation of our data: Although the skin microcirculation contributes to total peripheral resistance,[56] vascular responses in the skin may be unique to this vascular bed and cannot be regarded as representative of the resistance vasculature. Investigations in other vascular beds will have to be performed, and the role of endothelial vasodilators for the effects of AT_1 antagonism on angiotensin II– and ET-1–mediated vasoconstriction will have to be clarified. Further perspectives include investigations in women and patients with cardiovascular disease with a view to determining the physiologic and, indeed, pathophysiologic relevance of our results.

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