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Effects of angiotensin receptor blockers on endothelial nitric oxide release: the role of eNOS variants

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Angiotensin II receptor blockers improve endothelial cell-dependent vasodilation in patients with hypertension through suppression of angiotensin II type 1 receptors but may have additional and differential effects on endothelial nitric oxide synthase (eNOS) function.

WHAT THIS STUDY ADDS

- The key finding from this study is that angiotensin II receptor blockers (ARBs) differentially enhanced nitric oxide (NO) release in a manner influenced by certain genetic variants of eNOS. This finding provides new insights into the effects of ARBs on endothelial cell-dependent vasodilation and eNOS function that are of high importance in vascular medicine and clinical pharmacology.

AIM

Angiotensin II receptor blockers (ARBs) improve endothelial cell (EC)-dependent vasodilation in patients with hypertension through suppression of angiotensin II type 1 receptors but may have additional and differential effects on endothelial nitric oxide (NO) synthase (eNOS) function. To investigate this question, we tested the effects of various ARBs on NO release in ECs from multiple donors, including those with eNOS genetic variants linked to higher cardiovascular risk.

METHODS

The effects of ARBs (losartan, olmesartan, telmisartan, valsartan), at 1 μM, on NO release were measured with nanosensors in human umbilical vein ECs obtained from 18 donors. NO release was stimulated with calcium ionophore (1 μM) and its maximal concentration was correlated with eNOS variants. The eNOS variants were determined by a single nucleotide polymorphism in the promoter region (T-786C) and in the exon 7 (G894T), linked to changes in NO metabolism.

RESULTS

All of the ARBs caused an increase in NO release as compared with untreated samples ($P < 0.01$, $n = 4$–5 in all eNOS variants). However, maximal NO production was differentially influenced by eNOS genotype. Olmesartan increased maximal NO release by 30%, which was significantly greater ($P < 0.01$, $n = 4$–5 in all eNOS variants) than increases observed with other ARBs.

CONCLUSIONS

The ARBs differentially enhanced NO release in ECs in a manner influenced by eNOS single nucleotide polymorphisms. These findings provide new insights into the effects of ARBs on EC-dependent vasodilation and eNOS function.

Introduction

The vascular endothelium regulates vascular motion while providing a non-thrombogenic surface and macromolecular barrier between blood elements and the underlying vessel wall. Vascular endothelial cells (ECs) also play an important role in regulating vascular tone, largely through the constitutive release of nitric oxide (NO). Under pathophysiologic conditions, an imbalance occurs between the endothelial release of this powerful vasodilator and the systemic release of vasoconstrictive peptides, such as angiotensin II and endothelin, resulting in increased vascular resistance [1, 2]. Beyond abnormal vasodilation, the loss of NO bioavailability with endothelial dysfunction contributes to various atherogenic processes, including activation of the renin-angiotensin system, thrombus formation and inflammation [3].

In clinical studies, angiotensin II receptor blockers (ARBs) demonstrated a significant improvement in endothelium-dependent vasodilation in patients with hypertension as compared with placebo or other antihypertensive agents [4–7]. Losartan significantly improved endothelial function in patients with atherosclerosis and coronary heart disease (CHD) while candesartan improved forearm blood flow in patients with hypercholesterolaemia-associated endothelial dysfunction [8–10]. Comparative studies have shown similar effects of ARBs and angiotensin converting enzyme (ACE) inhibitors on endothelial function in patients with hypertension and CHD [9, 11]. The pharmacologic basis for enhanced NO release with ARB treatment is attributed to reduced activity of angiotensin II, similar to the mechanistic effects of ACE inhibitors, leading to anti-oxidant protection and increased NO bioavailability [9, 12]. Unlike ACE inhibitors, ARBs do not enhance concentrations of bradykinin, a stimulus for NO release; but they may enhance eNOS expression [3, 13–15].

This study was conducted to compare the effects of multiple ARBs (losartan, olmesartan, telmisartan, valsartan) on stimulated NO release from healthy human ECs with certain endothelial nitric oxide synthase (eNOS) genetic variants as determined by single nucleotide polymorphism (SNP) genotyping analysis. In addition to the normal eNOS genotype, we evaluated the effects of treatment in ECs consisting of two well-characterized eNOS polymorphisms, G894T, a SNP in the exon 7 of the human eNOS gene (located at 7q35-36), which results in a Glu298Asp substitution in the eNOS sequence and T–786C, a SNP at position –786, in the promoter region of the eNOS gene, which results in decreased expression of the eNOS enzyme. These eNOS polymorphisms have been linked, albeit with some inconsistencies, to changes in NO metabolites (nitrites, nitrates), arterial and EC function, as well as susceptibility to hypertension [16–21]. While ARBs improve vasodilation in patients with hypertension through suppression of angiotensin II, these agents may have additional effects on eNOS function. To understand these processes, we measured directly the effects of ARBs on NO release in ECs from donors with normal eNOS genotypes as well as those having eNOS polymorphisms linked to enhanced cardiovascular risk.

Methods

Subjects and cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated into primary cultures from Caucasian female donors (18 subjects) by Clonetics (San Diego, California) and purchased as proliferating cells. HUVECs pooled from the same 18 Caucasian donors were also purchased from Clonetics. All cell culture donors were healthy, with no pregnancy or prenatal complications. Cells were cultured in the recommended complete endothelial cell growth medium and maintained at 37°C in a 5% CO_2 humidified incubator. Cells were supplied with fresh medium every other day and propagated using an enzymatic (trypsin) procedure, for a maximum of 16 population doublings.

Preparation of nanosensors for NO detection

Measurements of NO were carried out with an electrochemical nanosensor with a total diameter of 200–500 nm and length of 4–5 μm. Nanosensor design was based on previously developed chemically modified carbon-fibre technology [22, 23]. Each of the nanosensors was made by depositing a sensing material on the tip of a carbon fibre. We used polymeric nickel (II) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin for the NO sensor.

Measurement of NO

The confluent cells (4 to 5 × 10^5 cells/35 mm dish) were incubated with ARBs in endothelial basal medium for 6 h. The ARBs were dissolved in 18 μl of dimethy sulfoxide (DMSO) and further diluted in endothelial basal medium (1 ml) to obtain a final concentration of 1 μM. Further experiments were performed to rule out the influence of the small concentration of DMSO on endothelial NO release.

NO release was stimulated by calcium ionophore (CaI), A23187, at a concentration of 1 μM, injected with a nanoinjector that was positioned by a computer-controlled micromanipulator. Four to five measurements of NO were performed per group.

An NO nanosensor with a platinum wire (0.1 mm) counter electrode and saturated calomel reference electrode was applied. Differential pulse voltammetry (DPV) and amperometry were performed using a computer-based Gamry VFP600 multichannel potentiostat. DPV was used to measure the basal NO concentrations and amperometry was used to measure changes in NO concentrations from its basal level with time. The DPV current at the peak potential characteristic for NO oxidation (0.70 V) was

directly proportional to the concentrations of this compound in the immediate vicinity of the sensor. Linear calibration curves (current *vs.* concentration) were constructed for each sensor from 10 nM to 2 μM before and after measurements with aliquots of NO standard solution. The detection limit of the sensors was 1 nM. At a constant distance of the sensors from the surface of an endothelial cell, the reproducibility of measurements was relatively high (5–12%). The position of the nanosensors (x, y, and z coordinates) *vs.* the ECs was established with the help of a computer controlled micromanipulator. The sensor was moved horizontally (x, y coordinates) and positioned above the surface of a randomly chosen single EC.

Determination of eNOS variants

Maximal generation of NO was correlated with eNOS variants (G894T and T–786C) linked to changes in NO metabolism. These genotypes were determined by polymerase chain reaction (PCR) using a SNP genotyping assay kit from Applied Biosystems Inc. (Foster City, California). Between three and six Caucasian donors with specific eNOS variant combinations were assigned to each group (n = 3–6).

Statistical analyses

In order to control intra-experimental variations, measurements of endothelial NO were performed using a single cell randomly selected from 4–5 different cell culture dishes. The significance of differences between results from independent experimental conditions was tested using either the two-tailed Student's *t*-test (measurements of NO from various treatments) or one-way analysis of variance (ANOVA) with Student–Newman–Keuls multiple comparisons *post hoc* analysis. Data were presented as mean ± standard deviation (SD). Mean values were considered significantly different at $P < 0.05$.

Results

NO release in human endothelial cells with different eNOS gene polymorphisms

We measured NO release from ECs as a function of the G894T and T–786C genotypes. As shown in Figure 1, there was no significant difference in NO release among these variants following Cal stimulation. The average level of NO release (585 ± 34 nM) was consistent with previous studies in ECs from Caucasian donors treated with a similar receptor-independent stimulus like Cal [24].

Effect of ARBs on NO release from human endothelial cells with different eNOS gene polymorphisms

We compared NO release from HUVECs, pooled from all 18 Caucasian donors carrying all available eNOS variants, in the absence and presence (6 h) of various ARBs (losartan, olmesartan, telmisartan, valsartan) at a concentration of

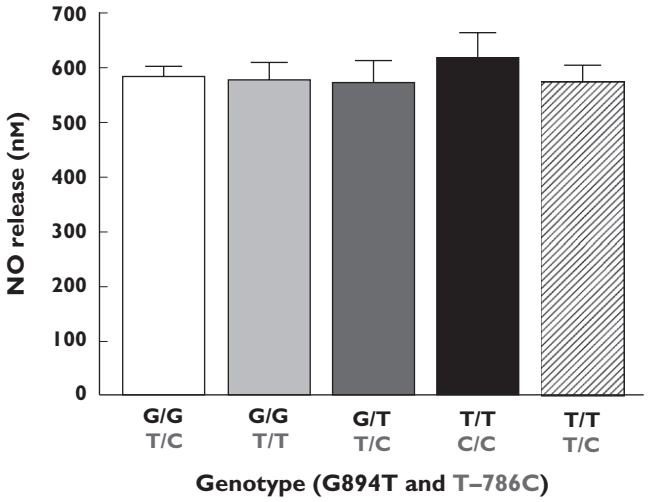

Figure 1

Release of NO from a single HUVEC cell with eNOS gene polymorphisms at positions 894 (G894T) and/or –786 (T–786C). Values are mean ± SD (n = 4–5 measurements of NO per donor, 3–6 donors per group)

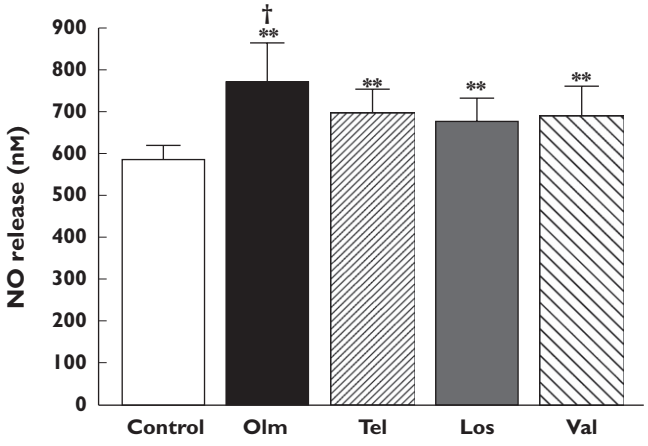

Figure 2

Comparative effects of losartan (Los), olmesartan (Olm), telmisartan (Tel), valsartan (Val) on Cal (1 μM)-induced NO release from HUVECs (pooled from all 18 Caucasian donors carrying all available eNOS variant combinations) following treatment for 6 h at 1 μM. Values are mean ± SD (n = 4–5 measurements of NO per group). $^{**}P < 0.01$ *vs.* control; $^{\dagger}P < 0.01$ *vs.* all other treatments (Student–Newman–Keuls multiple comparisons test; overall ANOVA: $P < 0.0001$, $F = 17.336$)

1 μM (Figure 2). Each ARB caused an increase in NO release as compared with untreated samples ($P < 0.01$, n = 4–5 measurements of NO per group). The greatest effect was observed with olmesartan, which increased NO release by 30% (from 585 ± 34 nM to 768 ± 98 nM) and was significantly greater ($P < 0.01$, n = 4–5 measurements of NO per group) than any increases observed with the other ARBs. Olmesartan also increased NO release in most eNOS variant combinations (homozygous and heterozygous) as

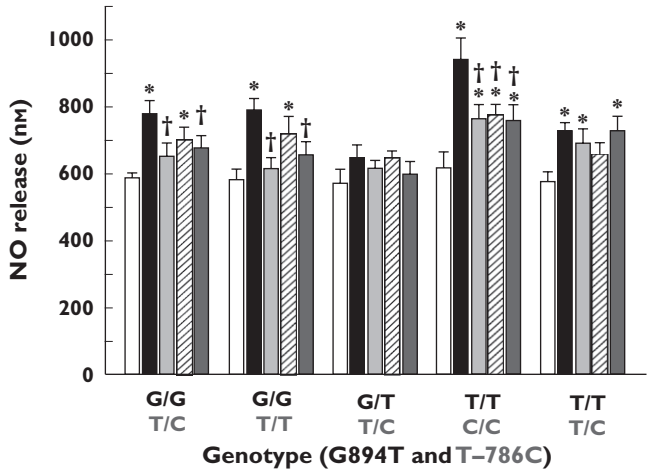

Figure 3

Effect of ARB treatment (1 μM, 6 h treatment) on NO release from HUVECs obtained with eNOS gene polymorphisms at positions 894 (G894T) and/or −786 (T–786C). Values are mean ± SD (n = 4–5 measurements of NO per donor, 3–6 donors per group). *P < 0.05 *vs.* cognate control; †P < 0.01 *vs.* cognate olmesartan treatment (Student–Newman–Keuls multiple comparisons test; overall ANOVA: P < 0.0001, F = 18.109). Control (□); Tel (▭); Val (▭); Olm (■); Los (▨)

compared with untreated cells (Figure 3). The most apparent difference in potency between olmesartan and the other ARBs was observed in ECs homozygous for both the 894T and −786C alleles.

Discussion

The key finding from this study is that treatment of human ECs with ARBs differentially enhanced NO release in a manner influenced by certain genetic variants of eNOS. All of the ARBs tested (losartan, olmesartan, telmisartan, valsartan) caused an increased in endothelial NO release compared with untreated cells. Olmesartan had the greatest effect on NO release as it enhanced NO concentrations by 30%. The eNOS polymorphisms were not associated with differences in stimulated NO release except in the presence of the different ARBs. Olmesartan increased NO release in the various eNOS genotypes with the most apparent difference in ECs from donors homozygous for both G894T and T–786C single nucleotide mutations. This finding indicates agent-specific effects of ARBs on eNOS function that are influenced, in turn, by single nucleotide substitutions that are linked to changes in NO metabolism and increased cardiovascular risk, including hypertension [16–21]. The explanation for the relative contribution of the different variants to the responses of the ARBs is complex and may require further investigation.

The eNOS genotype may be linked to changes in the homeostatic balance between concentrations of NO and reactive oxygen species, such as superoxide anion, that regulate the cellular redox state essential for normal endothelial function. Endothelial dysfunction and impairment in the capacity of the vessels to dilate are directly linked to enhanced catabolism of NO by superoxide anions [3, 13, 14]. Loss of normal NO bioavailability, coupled with increased oxidative stress, is causally related to various diseases, including atherosclerosis, hypertension and diabetes mellitus [25, 26]. Reductions in NO release have also been associated with eNOS uncoupling, an effect that may be reversed with certain therapies [3, 13, 14]. The differential effects of the ARBs on NO release with these eNOS variants may therefore be linked to changes in the homeostatic balance between NO and free radical generation in these cells.

Tissue angiotensin and its products have a direct role in the pathophysiology of vascular disease through enhanced oxidative stress, inflammation and other mechanisms [27]. Like ACE inhibitors, ARBs suppress the activity of angiotensin II, leading to antioxidant protection that promotes NO bioavailability [9]. Specifically, inhibitors of the renin-angiotensin system reduce NAD(P)H activity, which acts as an important enzymatic source of intracellular superoxide [9, 12]. Although ARBs do not affect bradykinin concentrations, these agents have been shown in bovine pulmonary artery endothelial cells to increase significantly levels of eNOS [15]. Thus, ARBs may promote NO release through the up-regulation of eNOS protein as well as antioxidant protection. Clinical studies have demonstrated that ARBs improve endothelium-dependent vasodilation in patients with hypertension, compared with placebo or comparator agents [4–7]. In comparison with ACE inhibitors, ARBs have similar effects on endothelial function in patients with hypertension and with CHD [9, 11].

ARBs inhibit the vasoconstrictor effects of angiotensin II by selectively and reversibly blocking its binding to the AT_1 receptor. The basis for observed differences in the endothelial effects of olmesartan may be related to various factors, including its chemical structure and receptor affinity. The covalent structure of olmesartan includes a hydroxyl group and an intermediate-sized alkyl substituent (isopropyl) at the 4-position, which undergoes hydrogen bonding with the carboxylate anion at the 5-position of the imidazole ring, allowing the isopropyl substituent to interact with a hydrophobic region on the AT_1 receptor. The other ARBs also interact with a hydrophobic region of the AT_1 receptor, so this factor alone is not the basis for uniqueness. The order of potency for the IC_{50} values (nM) has been reported as follow: olmesartan (0.78), telmisartan (0.88), valsartan (3.1) and losartan (11) [28]. These data indicate that the receptor binding characteristics for olmesartan to the human AT_1 receptor is slightly more potent than telmisartan and more potent when compared with the other ARBs, especially losartan. However, the relative differences in the potency of these agents for the AT_1 receptor alone failed to predict their complex effects on NO release

among the eNOS variants that were tested. Olmesartan may also have a differential effect on eNOS expression, although this mechanism is a less likely contributor to changes observed in this study, given the relatively short treatment periods. The presence of the hydroxyl group may contribute to free radical scavenging properties for olmesartan, including chain-breaking antioxidant activity. In particular, the hydroxyl group may provide proton donation and electron stabilization properties that reduce free radical propagation resulting in loss of NO bioavailability through formation of peroxynitrite ion. Also, it is possible that olmesartan attenuates the activation of NADPH oxidase and decreases superoxide generation in the absence of angiotensin II, leading to an increase in NO bioavailability [29]. These potential effects need to be explored further.

One of the most intriguing findings from this study was the ARB-specific changes in NO release among the various eNOS variants. On the other hand, there were no genotype-specific differences in NO release following stimulation with CaI. This may be due, in part, to the type of stimulus used in these studies. By using a CaI, we fully activated available cellular eNOS in a receptor-independent manner. If these variants influence receptor-dependent pathways related to eNOS activation, then these effects may be missed with the use of the CaI.

This is the first study to compare the direct effects of various ARBs on the NO release capacity of human endothelial cells with different eNOS variants. Additional studies are needed to elucidate the basis for ARB effects on eNOS function, including eNOS protein and co-factor levels, as well as changes in the redox state of the cell, all of which may be linked to their specific chemical structures. These studies also need to be extended to whole animals and human clinical evaluations with well-characterized genotype analyses to understand the relationships between selective AT_1 receptor inhibition and endothelial-dependent NO release in patients with cardiovascular risk.

An important limitation of this study was the number of patients with any particular eNOS variant. However, the reproducibility in the genotype-dependent response of these cells to stimulation indicates that this model serves as an important predictor of NO release as a function of treatment with these agents. It will be important to examine the effects of these pharmacologic agents on NO metabolism in a larger numbers of patients having these and other possible eNOS genotypes.

Competing Interests

Dr Mason has received independent research grants from Astra Zeneca, Forest Laboratories, Pfizer, Sanofi-Aventis and Daiichi Sankyo. All other authors have no conflicts of interest to disclose.

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