The Renin-Angiotensin-Aldosterone System as a Therapeutic Target for Endothelial Dysfunction

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Abstract: Endothelial dysfunction has been characterized by decreased nitric oxide (NO) synthesis or reduced NO bioavailability, which relates to inflammation, proliferation of smooth muscle cells, deposition of extracellular matrix, vasoconstriction, and a prothrombotic state within the vessel lumen. The endothelium is the site of the final step of synthesis of both NO and angiotensin II (Ang II) and is a major site for their countervailing interaction. Evidence suggests that renin-angiotensin system (RAS) blockade may have an impact on early mechanisms of vascular disease, such as endothelial dysfunction and vascular remodeling that underlie clinical manifestations of cardiovascular disease. This article reviews the current views on the biologic organization of RAS. Evidence supports a pathologic role of RAS activity in promoting endothelial dysfunction characterized by the impairment of NO bioavailability, and provides the basis for considering inhibition of RAS activity as a major target for therapeutic intervention.

Key words: Nitric oxide, renin-angiotensin-aldosterone system, vascular disease prevention.

INTRODUCTION

The endothelium is a dynamic autocrine and paracrine organ that regulates anti-inflammatory, mitogenic, and contractile activities of the vessel wall, as well as the haemostatic process within the vessel lumen. Nitric oxide (NO) produced by endothelial cells is a highly reactive molecule with many biologic effects [1]. A dysfunctional endothelium, characterized by decreased NO synthesis and/or NO bioavailability, facilitates inflammation, smooth muscle cell proliferation and extracellular matrix deposition, vasoconstriction, as well as a prothrombotic state within the vessel lumen [2, 3]. In addition, the long-term follow-up of patients with endothelial dysfunction suggests that reduced bioavailability of NO may even have prognostic implications and contributes to the progression of coronary atherosclerosis, because impaired endothelium-mediated vasomotion is associated with future cardiovascular events [4-6].

The endothelium is the site of the final step of synthesis of both NO and angiotensin II (Ang II) and is a major site for their countervailing interaction [7, 8]. NO exerts vasoprotective effects that maintain important physiological functions such as vasodilatation, anticoagulation, antioxidative capacity, and inhibition of leukocyte adhesion and smooth muscle proliferation. On the other hand, Ang II *via* the Ang II type1 (AT_1) receptors exerts vasoconstrictor, thrombotic, inflammatory, and fibrotic effects. The balance between Ang II and NO appears crucial for maintaining the homeostasis of the cardiovascular systems, particularly for regulation of vascular tone and modulation of inflammatory and growth-related pathological processes [7, 8]. During the pathogenesis of hypertension and atherosclerosis in both animal models and humans, this homeostatic balance becomes perturbed so that the actions of Ang II predominate over those of NO.

This article reviews the current views on a pathophysiological role of renin-angiotensin system (RAS) activity in promoting endothelial dysfunction and vascular disease, and provides the basis for considering inhibition of RAS activity as a major therapeutic target for endothelial dysfunction from bench to bedside.

NOVEL MECHANISTIC INSIGHT INTO ENDOTHELIAL DYSFUNCTION

NO is produced in the blood vessel wall mainly by eNOS, and may be scavenged reactive oxygen species (ROS) [9]. Thus, the availability of NO to dilate blood vessels depends on the balance between ROS in the vascular wall and the production of NO. Physiologically, vessels are generally maintained in NO-mediated quiescent and dilated state. In pathological situations, NO is quenched by excess ROS, generated in large part in blood vessels by vascular nicotinamide adenine dinucleotide (NADPH) oxidase [10]. In the endothelium, ROS formation is highly complex because not superoxide formation by activated NADPH oxidase, but eNOS also the potential to generate ROS. In fact, eNOS may produce both NO, *via* its oxygenase function, and superoxide through its reductase function, the latter dependent on NADPH. In oxidative states, reduction in tetrahydrobiopterin (BH_4) results in uncoupling of eNOS [11], resulting in production of superoxide by the eNOS monomer whereas the dimmer, in the presence of abundant BH_4, produces mainly NO (Fig. **1**). Thus, eNOS may have 2 faces in atherosclerosis

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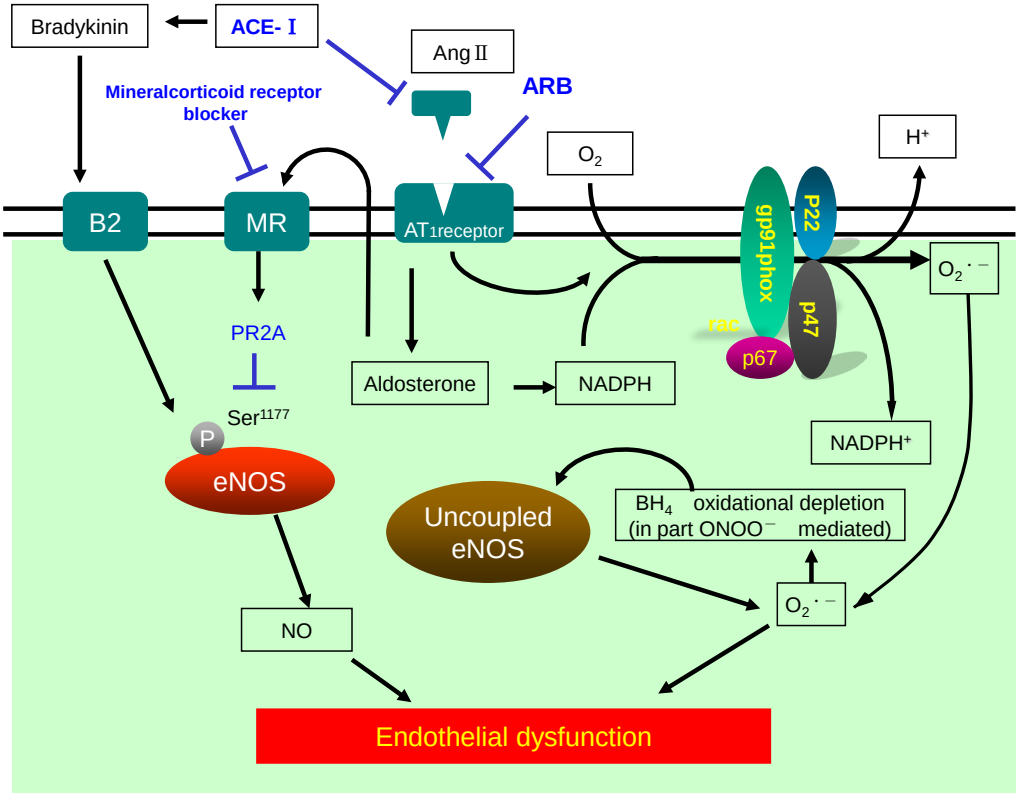

Fig. (1). Proposed mechanism explaining the beneficial effects of inhibition of renin-angiotensin-aldosterone system in Ang II-induced endothelial dysfunction. Ang II, stimulate superoxide ($O_2^{\cdot -}$) formation from the vascular NADPH oxidase *via* AT_1 receptor. This results in oxidation of the eNOS cofactor tetrahydrobiopterin (BH_4). Both peroxynitrite ($ONOO^-$) and $O_2^{\cdot -}$ contribute to BH_4 oxidation. Cofactor-deficient eNOS or eNOS with oxidized forms of BH_4 produces large amounts of $O_2^{\cdot -}$ upon stimulation of the enzyme. Both ACE inhibitor and ARBs reduce Ang II-stimulated NADPH oxidase activation and increased $O_2^{\cdot -}$ generation by the AT_1 receptor. An effect unique to ACE inhibitors is the increase in eNOS expression, which may be mediated *via* a bradykinin-mediated mechanism. Mineralocorticoid receptors inhibit aldosterone-induced reduced NADPH oxidase-mediated increase in $O_2^{\cdot -}$ generation and increases eNOS phosphorylation at Ser1177, an effect that may be mediated *via* inhibition of protein phosphatase 2A activation [121].

depending on tissue BH_4 metabolism. Uncoupled eNOS has been associated with accelerated atherosclerotic lesion formation, which could be prevented by BH_4 treatment [12]. Increased endothelial BH_4 synthesis, by targeted transgenic guanosine triphosphate-cyclohydrolase I overexpression has been shown to reduce endothelial dysfunction and atherosclerosis in apolipoprotein E knockout mice [13]. Collectively, increased ROS production and a shift in balance from NO to ROS signaling represent common characteristics in vascular disease.

Lavi *et al.* [14] reported in a human study that endothelial function in the left anterior descending territory of the coronary circulation as evaluated during coronary angiography, in early atherosclerosis, and in the absence of obstructive coronary artery disease, coronary artery diameter in response to cholinergic stimuli is related not to the activity of eNOS and generation of NO but rather to the bioavailability of NO. The latter depends on the amount of ROS present that can transform NO to peroxynitrite ($ONOO^-$) and as well oxidize BH_4 to dihydrobiopterin (BH_2) leading to eNOS uncoupling and more ROS production. Dissecting the mechanisms that contribute to reduced bioavailability of NO would facilitate novel therapeutic approaches that could allow increasing NO bioavailability and combating local ROS generation.

The association of heat shock protein (hsp90) with eNOS is a universal mechanism among species for increasing NO generation. The importance of this interaction to endothelial biology was confirmed by studies showing that hsp90 increased the efficiency of Akt-dependent phosphorylation of eNOS and that specific domains of hsp90 were responsible for delivering and directing Akt to serine 1179 on bovine eNOS [15]. The possibility that a direct protein interaction between hsp90 and eNOS preserves enzyme activity is supported by Song *et al.* [16, 17]. Using purified recombinant neuronal NOS (nNOS) and hsp90 and spin-tapping with electron spin resonance to quantify NO production, they showed that activation of nNOS in the presence of hsp90 increased NO generation [16, 17]. Another mechanism by which hsp90 might modulate eNOS function is by protecting sites of phosphorylation of eNOS. Using Western analysis, Wu *et al.* [18] found that vascular endothelial growth factor (VEGF) increases phosphoserine residues on eNOS by a protein kinase C (PKC)-dependent mechanism that directly correlated with increased NO production and activity. These observations reveal how important it is for hsp90 to associate with eNOS when phosphorylation of eNOS at serine 1177 levels is increased. Failure to increase hsp90 interactions with eNOS results in an inefficient coupling of enzyme ac-

tivity to L-arginine metabolism and in an increase in eNOS-dependent superoxide generation.

PATHOPHYSIOLOGIC EFFECTS OF ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction has been proposed to be an early event of pathophysiologic importance in the atherosclerotic process [19]. The impairment of NO bioavailability can upregulate vascular cell adhesion molecule (VCAM)-1 in the endothelial cell layer *via* induction of NF-κB expression [20]. The expression of VCAM-1, intracellular adhesion molecule (ICAM)-1 and E-selectin plays a role in the initiation of the inflammatory process. VCAM-1 binds to monocytes and T lymphocytes, the first step of invasion of the vessel wall by inflammatory cells [21]. NO inhibits leukocyte adhesion [22]. Reduction in NO results in induction of monocyte chemoattractant protein 1 (MCP-1) expression, which recruits mononuclear phagocytes [23]. Monocytes are transformed to lipid-loaded foam cells. Oxidized LDL (Ox-LDL), for example, is scavenged through lectin-like Ox-LDL receptors (LOX-1) [24], which is highly expressed in blood vessels in hypertension, diabetes and hyperlipidemia [25]. Ox-LDL uptake by LOX-1 triggers a variety of actions: It reduces eNOS expression [26] and further stimulates adhesion molecule expression [27]. LOX-1 expression can be stimulated by Ang II and endothelin-1 [28, 29]. As the atherosclerotic plaques progresses, growth factors secreted by macrophages in the plaque stimulate vascular smooth muscle cell growth and interstitial collagen synthesis [21]. The event that initiates a majority of myocardial infarction is the rupture of the fibrous cap of the plaque, inducing thrombus formation. Decreased NO and oxidative stress may activate matrix metalloproteinases (MMP) [30, 31], namely MMP-2 and MMP-9, which weaken the fibrous cap. Because NO inhibits platelet aggregation [32], reduced NO contributes to thrombogenicity and to the severity of the event. Thus, endothelial dysfunction with reduced NO bioavailability, increased oxidant stress, and expression of adhesion molecules contributes not only to initiation but also to progression of atherosclerotic plaque formation and triggering of cardiovascular events.

INTERACTION BETWEEN NO AND ANG II

Ang II regulates the expression of eNOS and NO production, whereas NO down-regulates the AT_1-receptor [33, 34]. Treatment of rat vascular smooth muscle cells with NO donors inhibited Ang II binding to cells, without altering receptor affinity [33, 34]. Treatment of the cells with cyclic guanosine monophosphate (cGMP) analogs, however, had no significant effect on Ang II receptors through a cGMP-independent mechanism [33, 34]. In vascular smooth muscle cells, inhibition of Ang II binding by NO is caused by decreased AT_1-receptor mRNA expression and occurs as the transcriptional level [35]. In addition, downstream effectors of Ang II and NO signaling pathways also interact with each other. For example, the inhibitory effects of Ang II on cGMP accumulation, elicited by a NO donor or atrial natriuretic peptide in vascular smooth muscle cells and vessels, are likely to be mediated by activation of Ca^{2+}/calmodulin-stimulated phosphodiesterases, which are known to play critical roles in controlling intracellular cGMP levels by converting cGMP to 5-GMP [36].

Mollnau *et al.* [37] have shown that NO in the vascular segments from the aorta, assessed with electron paramagnetic resonance, was markedly reduced in the Ang II-infusion rat model. They have proposed a mechanism where NADPH oxidase-induced superoxide production may trigger eNOS uncoupling, leading to impaired NO/cGMP signaling and to endothelial dysfunction in this animal model. We also showed how Ang II may alter plasma NO concentration in anesthetized genetically hyperlipidemic rabbits using a catheter-type NO sensor [38]. That is, short-term infusion of Ang II elicited the basal and ACh-induced increase of plasma NO concentration [38]. In contrast, chronic Ang II infusion elicited the impairment of the basal and ACh-induced decrease of plasma NO concentration possibly through a decrease in NO synthesis or an increase in NO inactivation owing to locally enhanced production of ROS [38].

ENDOTHELIAL DYSFUNCTION IN ACTIVATION OF RENIN-ANGIOTENSIN SYSTEM

Ang II and Oxidative Stress

Ang II increases oxidative stress, inflammation, and alters endothelial function [39, 40]. The principle sources of ROS in the human vasculature is NAD(P)H oxidase, activated by a number of proatherogenic stimuli including Ang II. Overall NAD(P)H oxidase activity in cells is achieved by several components. These included cell-membrane-associated p22phox and gp91phox (or gp91phox [nox2] homologues, nox1 and nox4), as well as cytosolic p47phox and p67phox [40]. Vascular NAD(P)H oxidase is upregulated by vasoactive factors, stretch, shear stress and pulsatile strain [40]. At low concentrations ROS serve a physiological role as signaling molecules involved in endothelial function and vascular contractility [39]. However, pathological increases in ROS results in a plethora of effects that damage the vessel wall [41]. Moreover, O_2^- anions generated by NAD(P)H oxidase may contribute to the initiation and maintenance of hypertension [42].

Ang II and Endothelial Progenitor Cells Dysfunction

The identification of endothelial progenitor cells (EPCs) has led to a significant paradigm in the field of vascular biology and opened a door to the development of new therapeutic approaches [43, 44]. Circulating EPCs may represent an important endogenous repair mechanism to maintain the integrity of the endothelial monolayer and promote ischemia-induced neovascularization. The mobilization of EPCs by VEGF, statins, estrogen, or physical exercise has been shown to be dependent eNOS [45, 46]. Moreover, EPCs from eNOS-deficient mice had a reduced neovascularization capacity in the hind limb ischemia, suggesting that eNOS-dependent signaling is important for EPCs function [46].

We showed that in animal models of hypertension, as well as in subjects with essential hypertension, EPCs become precociously senescent and dysfunctional [47]. Higher blood pressure (BP) levels are associated with lower EPCs levels in the general population [48], and in diabetic subjects [49].

Hyperreactivity of the renin-angiotensin-aldosterone system (RAAS) as been recognized as one link between hypertension and altered EPCs biology. Bahlmann *et al.* [49] documents that angiotensin receptor antagonists increase the number of EPCs in patients with type II diabetes mellitus. This effect seems to be a class effect, because they have demonstrated it with standard doses of 2 long acting ARBs (olmesartan or irbesartan). In contrast, in patients treated with standard antihypertensives, they did not observe any effects of EPCs. Ramipril is an angiotensin-converting enzyme (ACE) inhibitor that is used to reduce RAAS activation in patients with stable coronary artery disease. Min *et al.* [50] showed that increased numbers of EPCs could be cultured from ramipril-treated patients with stable coronary artery disease and that ACE inhibition resulted in improved functional properties like adhesion, proliferation, migration, and *in vitro* vasculogenesis assay, independent of any impact on BP. These results show that EPCs are sensitive to Ang II signaling and that this should indeed impact on number and function. Our group showed that Ang II increases the rate of senescence of EPCs and that this appears to be a consequence of its ability to stimulate expression of gp91phox and thus O_2^- formation [51]. In addition, we demonstrated that the ability of Ang II to induce senescence also involves the suppression of telomerase [51]. Our group also noticed an expected increase in formation of a marker of oxidative stress-peroxynitrite, which is formed of O_2^- with NO in the Ang II-treated EPCs [51]. Increased O_2^- production is a feature of Ang II-dependent hypertension [42]. Thus, under conditions of Ang II excess, Ang II most likely contributes to the decline in formation of the vascular endothelium at least in part by the mechanisms *via* Ang II-induced EPCs senescence. On the other hand, it has been demonstrated that Ang II and angiotensin peptides promote hematopoietic progenitor cell proliferation and hematopoietic recovery after radiation therapy and chemotherapy [52]. Murohara et al reported that the Ang II-AT_1 receptor pathway plays an important role in angiogenesis associated with ischemia and tumor [53, 54]. These results appear to the contradictory to protective effects of RAS suppression on EPCs function. Recent studies suggested that the intracellular redox state is a critical modulator of the balance between self-renewal and differentiation in dividing precursor cells and that anti-oxidant may preserve their stemness [55]. It is plausible that a reduction in oxidative stress resulted in restoration of the impaired faction of EPCs in spontaneous hypertensive rats as well as patients with metabolic disorders, although it remains to be determined whether RAS inhibition stimulates EPCs function under physiological conditions in healthy subjects.

ENDOTHELIAL DYSFUNCTION AND ATHEROSCLEROSIS

Accumulating clinical studies suggest an important pathophysiological role of endothelial dysfunction, as determined by impaired endothelium-dependent vasodilation, by demonstrating a close association of the degree of coronary or peripheral endothelial dysfunction with cardiovascular events [56]. Recently, endothelial dysfunction has been shown to predict future cardiovascular events in patients who have had an acute coronary syndrome [57]. In addition, impaired flow-dependent, endothelium-mediated vasodilation predicted the occurrence of in-stent restenosis in patients undergoing percutaneous coronary intervention in a prospective study [58]. Moreover, in women with typical angina and evidence of myocardial ischemia but angiographically normal coronary arteries, Burgiardini *et al.* [59] have shown that after a long-term (10-year) follow-up, endothelial dysfunction was associated with the development of coronary disease and persistence of chest pain. Interestingly, in a recent large-scale study of more than 2000 healthy young adults, the number of cardiovascular risk factors was correlated with increased carotid intima-media thickening in subjects with an impaired endothelium-dependent vasodilation, but not in subjects with an enhanced endothelium-dependent vasomotion [60], suggesting that the status of systemic endothelial function may modify the association between coronary risk factors and atherosclerosis. The clinical evidence of impaired eNOS function is also suggested by studies analyzing eNOS gene polymorphisms. Cattaruzza *et al.* [61] provided evidence that an eNOS polymorphism, the CC type of the 786C/T single-nucleotide polymorphism in the promoter region of eNOS casing impaired shear stress sensitivity of eNOS expression, is more frequent in patients with coronary disease, providing evidence that reduced shear stress-dependent eNOS activation may contribute to coronary disease.

ASSESSMENT OF ENDOTHELIAL DYSFUNCTION

The usual parameter assessed when testing endothelial function is endothelium-dependent vasodilation. In coronary arteries, this is performed angiographically by Doppler flow measurements, assessing the effect of endothelium-dependent agonists, mainly acetylcholine (ACh) [62]. Endothelial function may be assessed on forearm resistance arteries by forearm blood flow measurement using strain-gauge plethysmography [63]. ACh or methacholine is administered through an intra-arterial catheter. Because shear stress is a stimulus to the endothelium to release NO, a noninvasive technique consists of inducing increased shear stress during hyperemia to assess flow-mediated vasodilation of the brachial artery by ultrasound [64]. However, the methodology is associated with a relatively poor signal-to-noise ration, which reflects variability in brachial artery size and the current resolution of vascular ultrasound [65]. Given the limitations of ultrasound-determined flow-mediated dilation, there is continuing interest in the development of better approaches to test endothelial function.

Direct Measurement of Circulating NO

Previously, it had been considered that NO, once released from vascular endothelial cells into the bloodstream, is immediately oxidized or inactivated by dissolved oxygen, oxyhemoglobin and/or oxygen radical species [66, 67]. However, growing experimental and clinical evidence suggests that NO remains active in the bloodstream, causing remote vasodilatory responses [68, 69]. As a high-temporal resolution method, electrochemical measurement methods for NO, i.e. NO sensors, have been developed by several groups [70, 71]. These sensors enable us to evaluate dynamic changes in NO concentration in solutions and tissues in response to agonists, NO-generating reagents and physical stimuli [72,

73]. However, electrical interference vibration, poor durability of the sensor-tip coatings and other factors has made *in vivo* NO measurement very difficult. To overcome these drawbacks, a new NO sensor, which encloses both the working and reference electrodes within a highly gas-permeable and robust enclosure, has been developed [74-76]. In addition, we have recently developed a catheter-type NO sensor [75, 76] (Fig. **2**). Using this sensor, we could evaluate the dynamic state of NO in a canine model [76], and in the aorta in a rabbit model [38]. More importantly, we succeeded in the first-in-human measurement of real-time plasma NO concentration in the coronary sinus (unpublished data).

INHIBITION OF RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM AND NO BIOAVAILABILITY

Large clinical trials demonstrate that RAS blockade protects against cardiovascular events and reduces mortality. Pharmacologic interruption of the RAS is possible at 5 major sites: release of renin from juxtaglomerular cells of the kidney, renin-catalyzed cleavage of angiotensinogen, conversion of angiotensin I (Ang I) to Ang II by ACE, binding of Ang II to the AT_1 receptor, and binding of aldosterone to the mineralocorticoid receptor (MR) (Fig. **3**). Evidence suggests that RAS blockade may have an impact on early mechanisms of vascular disease, such as endothelial dysfunction and vascular remodeling that underlie clinical manifestations of cardiovascular disease.

Angiotensin-Converting Enzyme Inhibitor and Angiotensin Type 1 Receptor Blockade

The clinical benefits of both ACE inhibitors and ARBs extend beyond BP reduction to improve endothelial function in patients with cardiovascular risk factors or coronary artery disease [77-79]. However, these effects are achieved by different mechanisms. ACE inhibitors reduce circulating and tissue Ang II levels and potentiates the beneficial effects of bradykinin-dependent NO production [80]. By contrast, the protective effects of ARBs are owing to the blockade of the AT_1 receptors and possibly also to the stimulation of Ang II type 2 (AT_2) receptors [81], again resulting in NO release. In fact, Yagi *et al.* demonstrated that combined treatment with an ARB, valsartan, and an ACE inhibitor benazepril, had an additive effect on inhibiting neointima formation *via* improvement of NO production and suppression of oxidative stress in a rat vascular endothelial injury model [82]. We also showed using genetically hyperlipidemic rabbits that chronic administration of either enalapril or losartan increased both ACh-induced and basal plasma NO concentration by using a catheter-type NO sensor [83]. More intriguingly, the combined chronic treatment with enalapril and losartan increased ACh-induced as well as basal plasma NO concentration significantly more than either enalapril or losartan alone [83]. Taken together, the complementary mechanisms of action of ACE inhibitors and ARBs might create a rationale for combination therapy in high-risk patients.

The pharmacological differences in the mechanisms by which ARBs and ACEIs suppress the renin-angiotensin system may have important clinical implications. In terms of heart failure, the combined treatment with both drugs has clinical evidence. In the Valsartan Heart Failure Trial (Val-HeFT), in which some 93% of patients received ACE inhibitors, valsartan 160 mg/day reduced hospital admission for congestive heart failure by 27.5% compared with placebo [84]. Some assessments of the relative merits of treatment with an ACEI, an ARB, and or both will be provided by the Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial (ONTARGET) Trial Programme [85].

Mineralocorticoid Receptor Blocker

Blockade of the RAS with ACE inhibitors has beneficial effects in high-risk patients, but cardiovascular events are not always appropriately controlled [86]. Although early after initiation of therapy ACE inhibition reduces plasma levels of

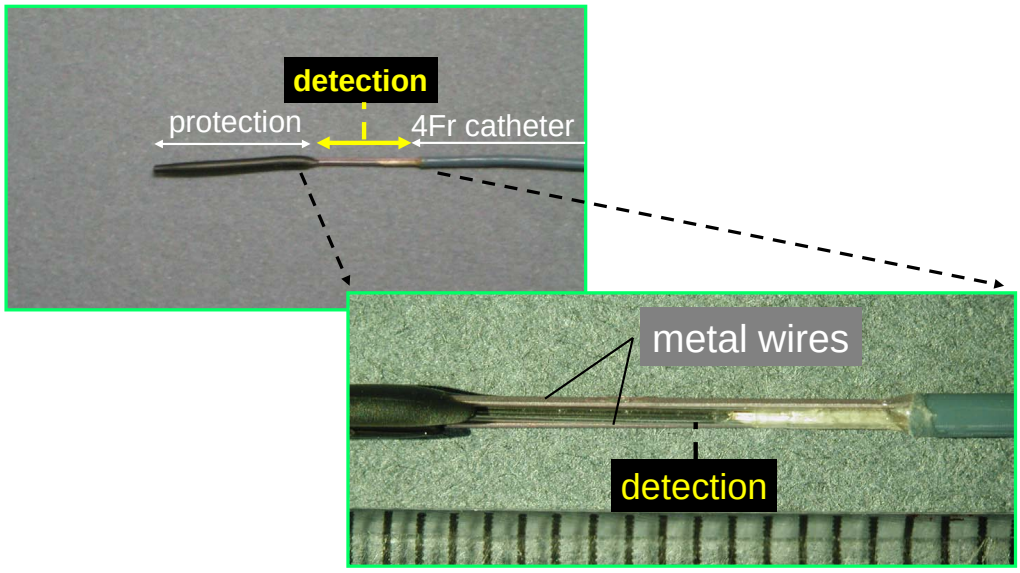

Fig. (2). Catheter-type NO sensor. A catheter-type NO can measure intra-aortic NO concentration *in vivo*. The NO sensor was encased and fixed in a 4-Fr catheter. This system has high specificity to NO and the monitored current reflected the changes in plasma NO concentrations of NO released from the endothelium by ACh infusion [122].

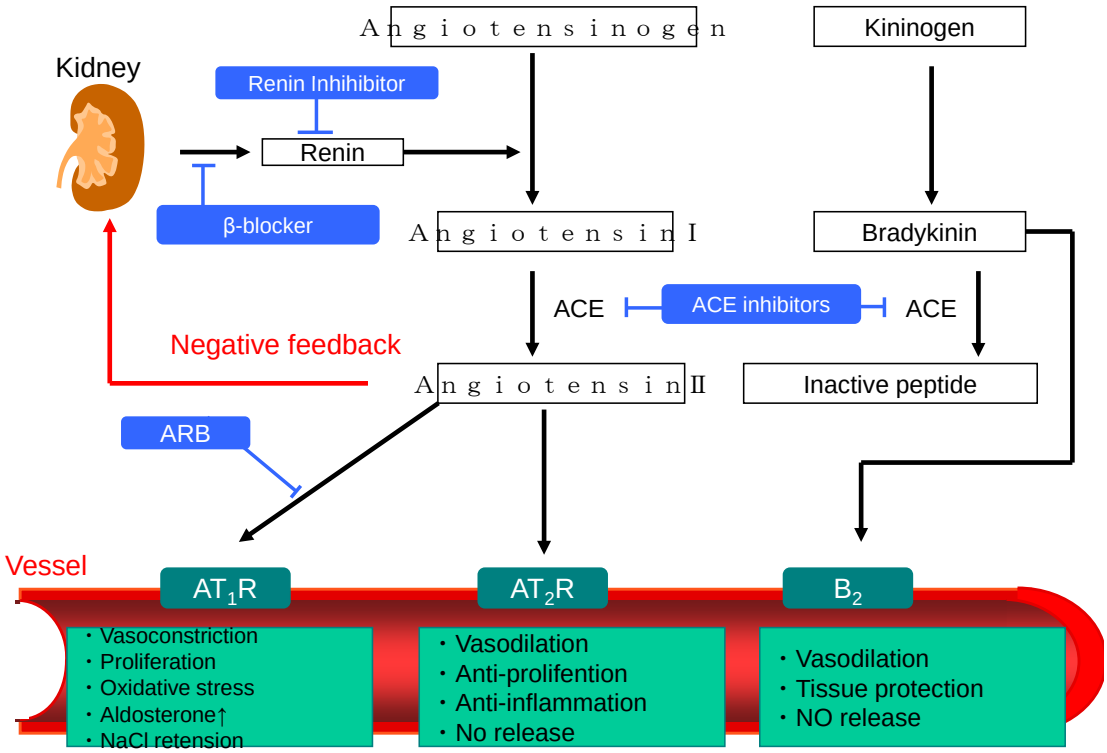

Fig. (3). The pharmacological modulations for renin-angiotensin system. Angiotensin II (Ang II) is formed from angiotensin I by angiotensin-converting enzyme (ACE) and acts *via* 2 receptor populations, AT_1 and AT_2, to produce a variety of effects on the heart, vasculature, and kidneys. The inhibition of the production or action of Ang II by ACE inhibitors or angiotensin receptor blockers (ARBs), respectively, prevents stimulation of the AT_1 receptor and disturbs the negative feedback loop through which Ang II normally inhibits renin release by the kidney. ACE inhibitors and ARBs thus stimulate a compensatory increase in renin release from the kidney, which ultimately leads to increased levels of Ang I and Ang II. A renin inhibitor could inhibit the reactive rise in renin activity that occurs with ACE inhibitors or ARBs. B_2 = bradykinin B_2 receptor.

both Ang II and aldosterone, during prolonged ACE inhibition aldosterone levels may increase the so-called aldosterone escape. Aldosterone levels independently predict cardiovascular risk [87], and direct detrimental effects of aldosterone on the vascular wall have been described [88]: mineralocorticoid receptor (MR) activation stimulates the formation of ROS in endothelial cells and limits NO generation and bioavailability [89]. Ang II-mediated ROS formation in the vascular wall may be mediated in part by MR activation [90], and aldosterone potentiates Ang II-induced signaling processes in vascular smooth muscle cells [91]. MR blockade reduces ROS formation and improves left ventricular remodeling, as well as endothelial function, when added to ACE inhibition in heart failure. We very recently showed that additional impact of eplerenone and ACE inhibition on atherosclerotic changes in genetically hyperlipidemic rabbits and dissected several potential mechanisms [92]. ACE inhibition and MR blockade displayed sustained vascular protection by complementary and additive effects on the balance between NO and ROS in the vascular walls. We also observed, in sharp contrast to enalapril, eplerenone markedly increased eNOS phosphorylation at Ser^{1177} [92]. A potential mechanism for eplerenone-induced upregulation of eNOS phosphorylation is through inhibitory effects of eplerenone on aldosterone-induced activation of protein phosphatase 2A (PP2A). Michell *et al.* [93] have shown previously that PP2A is responsible for dephosphorylation of eNOS Ser^{1177}, because pretreatment with okadaic acid selectively blocked protein kinase C-mediated dephosphorylation of Ser^{1177}. Taken together, improvement in NO bioavailability with eplerenone in hyperlipidemic rabbits may be due to reduced O_2^- production and increased eNOS phosphorylation, an effect that may be mediated by the prevention of MR-driven activation of protein phosphatase 2A. Site-specific dephosphorylation of eNOS at Ser^{1177} represents an important mechanism modulating eNOS enzyme activity and NO bioavailability in the vasculature by aldosterone. Improvement of endothelial dysfunction by normalization of reduced eNOS phosphorylation at Ser^{1177} in response to selective MR blockade *in vivo* has recently also been observed early after experimental myocardial infarction [94]. This experimental evidence may stimulate clinical trial using MR blockade to retard atherosclerosis progression in patients with atherosclerotic disease who are at high risk for cardiovascular events even when treated with ACE inhibitors.

Renin Inhibitor

Recent studies have provided additional evidence that plasma renin activity (PRA) is a predictor of cardiovascular risk. Alderman *et al.* [95] have demonstrated that the organ damage caused by the RAS may be independent of BP; increased PRA in patients with hypertension is associated with an increased risk of myocardial infarction, even when BP levels are effectively controlled by antihypertensive therapy. Although these findings are likely to be at least in part be-

cause of Ang II-mediated effects, increased renin or prorenin could also directly increase cardiovascular risk through enhanced stimulation of the newly discovered renin/prorenin receptor [96]. Given that overall RAS activity is regulated by the activity of renin, and that the effects of ACE inhibitors and ARBs are potentially attenuated by the increased renin, inhibiting the action of renin stands out as a logical approach to achieve the completeness of RAS suppression.

Aliskiren, the first in a new class of orally effective renin inhibitors for the treatment of hypertension, is a potent and specific inhibitor of human renin *in vitro* with an IC_{50} in the low nanomolar range [97, 98]. Studies in healthy volunteers showed that treatment with aliskiren caused a dose-dependent reduction in PRA and Ang II levels [99]. Clinical trials in patients with hypertension showed that this drug provided anti-hypertensive efficacy comparable to that of the ARBs losartan and irbesartan [100, 101].

Statin and RAS Inhibition

An activated renin-angiotensin system and hypercholesterolemia are well-established risk factors for coronary artery disease [102]. Approximately 40% of patients with hypertension have hypercholesterolemia [103]. However, hypertension is an important risk factor in patients with increased cholesterol [104]. Pharmacological treatment of both risk factors by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) and ARBs reduce end-organ damage and cardiovascular mortality and morbidity [105, 106]. However, beyond cholesterol and blood pressure lowering, additional mechanisms underlying these protective effects of both therapies including NO availability, and reduced oxidative stress are proposed.

Tao *et al.* have demonstrated that hypercholesterolemia induces endothelial dysfunction by increased O_2^- generation rather than decreased NO production [107]. Interestingly, Warnholtz *et al.* [108] have demonstrated using WHHL rabbits that hypercholesterolemia is associated with AT_1-receptor upregulation and increased O_2^- production secondary to an activation of vascular NADPH oxidase. We also showed that both pitavastatin and valsartan inhibited NADH/NADPH oxidase activity in WHHLMI rabbits. More importantly, the combined treatment with pitavastatin and valsartan significantly reduced plasma peroxynitrite concentrations compared with monotherapy.

Horiuchi *et al.* [109] have demonstrated that co-treatment with valsartan and fluvastatin significantly inhibits neointimal formation induced by cuff placement around the femoral artery. They also postulated that the cholesterol-independent inhibition of AT_1 receptor-mediated signaling by statins demonstrates a novel regulating mechanism that may contribute to the beneficial effects of these drugs beyond lowering of plasma cholesterol, especially together with an ARB. In addition, Li *et al.* [110] have shown that treatment with even a low dose of valsartan, together with a low dose of fluvastatin_that did not influence BP or plasma cholesterol, effectively attenuated atherosclerotic lesion size and lipid deposition with a decrease in oxidative stress and vascular inflammation in apolipoprotein E null mice treated with a high-cholesterol diet for 10 weeks. We also showed that the combined treatment with statin and ARB dramatically reduced plaque area accompanied with marked suppression of the production of vascular peroyxnitrite [111]. Dysfunction of eNOS was also shown to accelerate atherosclerotic lesion formation in mice [112], whereas overexpression of eNOS in mice with hypercholesterolemia resulted in increased eNOS-derived O_2^- production and promotion of atherogenesis [113]. In fact, we demonstrated that co-treatment with L-NAME significantly abolished pitavastatin-valsartan-induced plaque suppression [111].

PPARγ Agonist

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear super family. Emerging evidence indicates that the PPAR signaling pathway plays critical roles in the regulation of a variety of biological processes within the cardiovascular system [114]. Treatment with PPARγ agonist improves endothelial function in patients with type 2 diabetes [115]. We showed that pioglitazone markedly improved NO bioavailability in the Ang II-infused rabbit model. In addition, we made an observation that pioglitazone inhibited gp91phox expression, and attenuated toxic peroxynitrite ($ONOO^-$) formation [116].

Some ARBs, such as telmisartan, are selective activators of peroxisome proliferator activated receptor γ (PPARγ). Benson *et al.* [117] reported the novel observation that telmisartan may directly stimulate the PPARγ. Although this property has since also been reported for irbesartan [118] and a losartan metabolite [119], there is no doubt that telmisartan by 1 order of magnitude is the most powerful stimulator of PPARγactivity among the ARBs [117]. We showed that besides a class effect of ARBs, telmisartan additionally improves endothelial dysfunction and reduces atherosclerotic change through its PPARγ-mediated effects in WHHL rabbits [120].

CONCLUSIONS

Accumulating data suggest a close association of endothelial dysfunction and possibly EPC levels and function with cardiovascular events, suggesting that alterations in endothelial and EPC function plays an important pathophysiological role and promote development and complications of cardiovascular disease. Novel strategies to improve endothelial function may therefore have the potential to improve prognosis in vascular disease. Although ACE inhibitors and ARBs have provided an excellent starting point for therapies targeting the RAS, clinical trial evidence indicates that there is significant scope for testing whether increased and more comprehensive RAS suppression could yield additional benefits. One may therefore appropriately ask whether the introduction of another class of renin-angiotensin-aldosterone system (RAAS) blockers, such as aldosterone antagonists and renin inhibitors, will fulfill a clinical need. The catheter-type NO sensor is a potentially useful for investigating the relationship between increased NO bioavailability and reduced atherosclerosis. Importantly, the NO sensor may be applied to clinically evaluate endothelial function, i.e. reduced endothelium-derived NO bioavailability in patients with cardiovascular diseases. Future studies are needed to ascertain in clinical setting whether combined treatment (e.g. ARBs and renin inhibitors) could improve NO bioavail-

ability (tested with the NO sensor) and induce plaque regression and/or stabilization (tested using intravascular ultrasound [IVUS] and optical coherence tomography [OCT]) more effectively than monotherapy.

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