ADRENERGIC MECHANISM IN THE CONTROL OF ENDOTHELIAL FUNCTION

Daniela Sorriento[1]
Bruno Trimarco[1]
Guido Iaccarino[2]

[1] Department of Internal Medicine of Federico II University of Naples, Napoli, Italy
[2] School of Medicine of University of Salerno, Baronissi (SA), Italy

Addresses for Correspondence

Corresponding author
Daniela Sorriento, PhD
Università degli Studi di Napoli "Federico II"
Via Pansini 5, 80131 Napoli, Italia
Tel: 0817462220
Fax: 0817462256
Email: danisor@libero.it

Senior author
Guido Iaccarino, MD, PhD
Università di Salerno
Via Salvador Allende, 84081 Baronissi (SA), Italia
Tel: 089965021
E-mail: giaccarino@unisa.it

Abstract

There is considerable evidence that many disease are associated with endothelial dysfunction and reduced nitric oxide production such as hypertension, obesity, dyslipidemias, diabetes, heart failure, atherosclerosis. Notably these conditions are also characterized by alteration in the adrenergic tone. Whether these two mechanisms are just epiphenomenal each other or there is a functional link, it is still to be established. A starting ground to establish this issue is that vascular endothelium plays an important role in the function of cardiovascular system and that adrenergic receptors on endothelial cells contribute to the regulation of vasomotor tone. The aim of this excerpt is to review current knowledge on the physiology of endothelial adrenergic receptors to contribute to the basis for newer and better approaches to endothelial dysfunction in the setup of cardiovascular conditions.

Introduction

The endothelium controls several vascular functions, including vasculature tone and permeability, thrombosis, hemostasis and angiogenesis[1-4]. It is noteworthy that all these functions can be regulated by the activation of receptors and often the same receptor can activate multiple endothelial functions. The adrenergic system is the major regulator of cardiac and vascular function and of endothelial vasorelaxation by means of α and β adrenergic receptors activation. The adrenergic receptors (ARs) are part of a large family of G protein coupled receptors (GPCR) which mediate the functional effects of catecholamines like epinephrine and norepinephrine. The ARs family includes three β (β_1, β_2, β_3), three α_1 (α_{1A}, α_{1B}, α_{1D}) and three α_2 (α_{2A}, α_{2B}, α_{2C}) receptor subtypes. These receptors actively participate to the release of nitric oxide (NO) in order to regulate endothelial function[5]. NO plays a crucial role in endothelium homeostasis, with important vasodilatory, anti-thrombotic and anti-atherogenic properties. NO mediates most of the endothelial functions: it has been invoked as a mechanism in vasorelaxation, endothelium permeability and neoangiogenesis[3]. NO in the endothelium is constitutively produced by the endothelial NO synthase, eNOS[6]. This latter is then further activated through calcium levels [7] and phosphorylation of various serine residues by a number of protein kinases [8, 9]. Indeed, it has been demonstrated that NO is activated by means of the PI3K pathway in response to the stimulation of tyrosine kinase [10, 11].

The impaired ability of vascular endothelium to stimulate vasodilation is referred to as "Endothelial Dysfunction" and the major cause is the decreased bioavailability of NO in different conditions which can be due to various mechanisms: reduced eNOS expression, altered NO production and increased NO catabolism. Endothelial dysfunction plays a key role in the development of cardiovascular disease such as hypertension, type 2 diabetes and heart failure. The identification of the underlying pathogenic mechanisms will lead to the discovery of newer and more potent tools to

treat such diseases. On this issue, endothelial dysfunction has been associated to signal transduction abnormalities observed in hypertension. In particular, adrenergic vasorelaxation has been demonstrated to be impaired in hypertensive patients, probably due to the presence of increased desensitization and impaired signalling of βAR. Adrenergic receptors on endothelium have been longely not considered functional to the regulation of the vascular tone. On the contrary, it is possible to identify very specific roles for such receptors in several endothelial function. This review will summarize the effects of adrenergic receptors on endothelial functions, focusing on modulation of NO synthesis and angiogenesis.

α adrenergic receptors

αAR are GPCRs that couple to Gαq protein. The Gαq subunit is a primary activator of phospholipase C (PLC). Activation of PLC promotes the cleavage of the inositol substrate phosphatidyl-inositol 4,5 bisphosphate (PIP2) to yield diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG and IP3 promote the activation of a protein kinase C (PKC). α_1AR can also activate specific adenylate (adenylyl) cyclases (AC) leading to an increase in cAMP levels. The activation of specific PLCs and ACs requires a complex balance of signals from G-proteins, especially the Gα subunits, within specific cell contexts. DAG and cAMP are second messengers that affect a wide array of cell signaling pathways and responses.

1. α_1AR and Nitric oxide

Several reports [12, 13] have produced evidence for the functional presence of vasorelaxant α_1AR in the brachial and pulmonary arteries isolated from the rabbit and rat, respectively. According to these reports, the pharmacological stimulation of α_1AR located on endothelial cells, is able to generate NO, whereas the stimulation of α_2AR releases a relaxing prostanoid[12, 13]. Filippi demonstrated that nanomolar concentrations of phenylephrine, which are devoid of any contractile effect, induced a

slight endothelium-dependent vasorelaxation in the rat mesenteric vascular bed through the stimulation of α_{1D}AR, located on endothelial cells, which act through phospholipase C stimulation, followed by IP1 generation, and nitric-oxide synthase activation. Conversely, the increase in perfusion pressure induced by micromolar concentrations of phenylephrine is attributable to the stimulation of α_{1A}AR[14].

2. α_1AR and angiogenesis

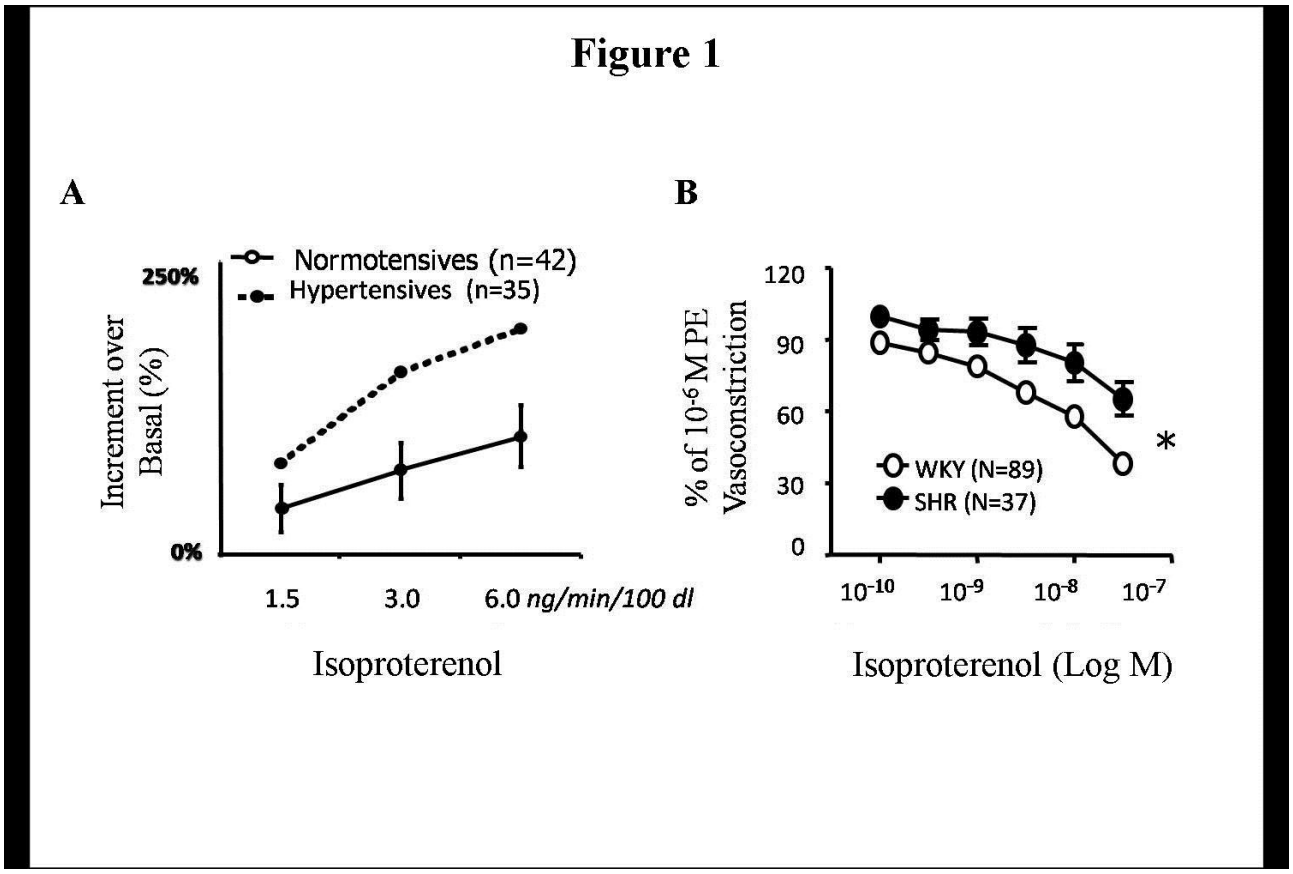
Neo-angiogenesis has long been known to be a highly ordered multistep molecular process under tight regulation by endothelial cells[15] and closely associated with endothelial cell proliferation and migration and to the capability of these cells to modulate the levels of VEGF, the most important cytokine system involved in the formation of new vessels[16]. A series of biological, chemical, hormonal effectors can interfere with this process. Several data support the notion that α_1-adrenergic receptor should also be ranked among these agents. Indeed, it has been demonstrated that the α_{1A}- and the α_{1B}-AR subtypes but not the α_{1D} subtype are expressed in cultured rat aorta endothelial cells. The activation of these α_1-AR in endothelial cells provide a negative regulation of angiogenesis[17]. Indeed, pharmacological antagonism of α_1-AR in endothelial cells from WKY rats by doxazosin enhanced, while stimulation of these adrenergic receptors with phenylephrine, inhibited endothelial mechanisms of angiogenesis such as cell proliferation and DNA synthesis, ERK and retinoblastoma protein (Rb) phosphorylation, cell migration and tubule formation[17]. A similar phenotype can be observed *in vivo*, since an increased α_1-adrenergic receptor density in the ischaemic hindlimb, compared to non-ischaemic hindlimb, suggested an enhanced α_1-adrenergic receptor tone in the ischaemic tissue. Treatment with doxazosin did not alter systemic blood pressure but enhanced neo-angiogenesis in the ischaemic hindlimb[17].

3. α_2ARand Nitric oxide

It has been demonstrated that α_2 adrenergic agonists cause endothelium dependent relaxation, that is reduced or abolished by inhibitors of L-arginine/NO pathway. It depends on the activation of α_2AR on endothelial cells which stimulates the release of NO, an action that would tend to attenuate vasoconstriction produced by the activation of post-junctional vascular α_1AR[18-20]. The α_2AR subtype that cause endothelium dependent relaxation belongs to the $\alpha_{2A/D}$ subtype, despite the prominent presence of α_{2C}AR (77% of α_{2C} versus 23% of $\alpha_{2A/D}$)[21]. It appears that this ratio may not be constant, since it varies within the vascular bed. Indeed, Bockman demonstrated that in the rat mesenteric artery the α_2AR is coupled to endothelium dependent NO-mediated relaxations and belongs to the $\alpha_{2A/D}$ subtype appearing in its α_{2D} version [22]. It has been demonstrated that endothelium dependent relaxation to α_2 adrenergic agonists is prevented by pertussis toxin [23-28], suggesting the involvement of G_i proteins in the signal transduction from the receptor to the activation of nitric oxide synthase [29, 30]. Indeed, α_2 adrenergic agonists cause activation of G_i proteins in endothelial cells and stimulate NO synthase activity [31, 32]. Contrary to what expected, cAMP is not involved in the signal transduction pathway for $\alpha_{2A/D}$AR mediated NO formation [22]. Indeed, the use of forskolin to oppose α_2 adrenergic receptor mediated inhibition of cAMP formation in endothelium did not affect the relaxant response to α_2AR agonists, suggesting that cAMP is not involved in the coupling of α_2AR to NO. There are physiological modulation of endothelium dependent relaxation to α_2 adrenergic agonists. Such relaxation is upregulated by chronic increase in blood flow [33] or exercise training [34]. Insulin enhances NO mediated vasorelaxation both in animal [25] and human [32] vasculature.

β-adrenergic receptors

βARs signal by coupling to the stimulatory G protein, Gs, leads to the activation of adenylyl cyclase and accumulation of the second messenger cAMP[35, 36]. However, recent studies indicate that under certain conditions βAR, and particularly β_2AR, can couple to Gi as well as to Gs [37-41]. It is now widely accepted that βAR exist on endothelial cells [10, 38, 40, 42] and contribute to the regulation of vasomotor tone. βAR are classically known to be present in the vascular smooth muscle cells (VSMC) where they cause vasodilation. The relative relevance of endothelial VSMC in adrenergic vasodilation is demonstrated by the observation that, in presence of intact endothelium, vasorelaxation to βAR agonist, isoproterenol (ISO), is sensitive to low doses of ISO (10^{-10}M-10^{-8}M). On the contrary, in absence of endothelium, the vasorelaxation is sensitive to higher doses of ISO (10^{-7}M-10^{-5}M). This appears to hold true through experimental models (rat or man) and vascular districts (*see Figure 1*).



1. *β_1 and β_2 adrenergic receptors*

It is now recognized that βAR located in the endothelium play an important role in the relaxant response to ISO, since the non selective β_1-and β_2-adrenergic receptor antagonist propranolol antagonized this relaxant effect[43, 44]. However, recent studies carried out in humans, in umbilical veins *in vitro*[10]or in the forearm *in vivo*[45], showed that vasorelaxation to ISO is abolished by the selective β_2AR antagonist ICI-118551 and remains unchanged in the presence of the β_1AR antagonist CGP-20712, indicating that, as in the vascular smooth muscle cells [46], the endothelial βAR are totally or at least predominantly of the β_2 subtype [10, 45].

β_2AR are seven transmembrane receptors coupled through G_s proteins to a cAMP dependent intracellular pathway[47]. It has been demonstrated that PKA posphorylation of the third intracellular loop of the β_2AR increases the affinity of the receptor for G_i protein[48, 49]. This switch leads to two consequences: first, it decreases the rate of cAMP generation, since G_i activation inhibits adenylyl cyclase activity. Second, it increases non cAMP dependent signaling through G_i, such as activation of the extracellular signal-regulated kinases ERK1/2 and PI_3K[50-54]. Gi coupled receptors have been shown to regulate non-receptor tyrosine kinases, such as SRC, which acts as an intermediate between G_i and other molecules like RAS and PI_3K [53, 55].

2. *β_2AR and Nitric oxide*

For years it has been given for granted that vascular β_2AR mediate adrenergic vasorelaxation through direct activation of vascular smooth muscle cells[56]. However, recent data challenge this vision, and show that β_2AR-dependent vasorelaxation is mediated at least in part, by endothelium through nitric oxide (NO) dependent processes[10]. We have recently demonstrated that the β_2AR are expressed on endothelial cells (EC) and their stimulation causes endothelial nitric oxide synthase (eNOS) activation[57]. In particular, β_2AR couple to eNOS and induce NO dependent vasodilation [57]. The mechanism of eNOS activation following β_2AR stimulation is known to be AKT dependent[58].

Indeed, the activity of eNOS is regulated by both a calcium/calmodulin dependent fashion[59]and AKT dependent eNOS phosphorylation in Ser 1177 [8, 60-63]. AKT is primarily activated in response to stimulation of transmembrane receptors with intrinsic tyrosine kinase activity or indirectly coupled to tyrosine kinases or to seven transmembrane G protein-coupled receptor[11, 61, 64]. Therefore AKT acts as integrator of different signal transduction pathways converging on eNOS, including endothelial β_2AR receptor[9, 58, 62, 63, 65].

3. β_2AR and angiogenesis

In the endothelium βARs control other important endothelial functions like angiogenesis, that is tightly associated to endothelial cell migration and proliferation [57, 65, 66]. We demonstrated that β_2AR stimulation with ISO and the overexpression of β_2AR increases endothelial cell proliferation. Moreover, β_2AR stimulation induces ERK phosphorylation and the MEKK inhibitor, U0126, inhibits β_2AR induced cell proliferation [66] suggesting that β_2AR dependent cell proliferation is dependent on ERK activation. We studied post-ischaemic angiogenesis in the hindlimb (HL) of β_2AR knock-out mice (β_2AR-/-) in vivo and explored possible molecular mechanisms in vitro. Angiogenesis was severely impaired in β_2AR-/- mice subjected to femoral artery resection, but was restored by gene therapy with ADβ_2AR. The proangiogenic responses to a variety of stimuli were impaired in β_2AR-/- EC *in vitro*[17]. Moreover, removal of β_2ARs impaired the activation of NFκB, a transcription factor that promotes angiogenesis; ISO did not induce NFκB activation in β_2AR(-/-) EC[17]. ADβ_2AR administration restored β_2AR membrane density and reinstated the NFκB response to ISO [17]. These results suggest that β_2ARs control angiogenesis through the tight regulation of nuclear transcriptional activity.

4.

5. α_1ARand β_2AR differently regulate neo-angiogenesis

α_1- and β_2-adrenergic receptors mediate opposite effects on neo-angiogenesis, comparable to their regulation of the vascular tone. In particular, the α_1-AR is inhibitory, whereas the β_2-AR is stimulant to neo-angiogenesis. Interestingly, in ischaemia, the α_1-AR are upregulated, thus causing a predominance of α_1-adrenergic receptor signalling over that of β_2-AR, which is downregulated. Furthermore, in conditions such as hypertension, where the α_1-AR tone is higher than that of the β_2-AR, there is also an impairment in neo-angiogenesis [66, 67]. It is interesting to note that in the ischaemic hindlimb, α_1-AR blockade resulted in a normalization of β_2-AR density together with improved neo-angiogenesis. α_1-AR upregulation, in particular, might be a regulatory mechanism aimed at preventing excessive angiogenesis. This upregulation might be triggered by ischaemia, through regulatory sequences within the gene promoter, which have been demonstrated for both the α_{1A}- and α_{1B}-adrenergic receptor[68, 69].

6. β_3 adrenergic receptors

In rat thoracic aorta, Trochu showed that β_3AR are mainly located on endothelial cells and act in conjunction with β_1AR and β_2AR to mediate relaxation through activation of NO synthase pathway and subsequent increase in tissue cyclic GMP content and is reduced by endothelium removal or in presence of L-NMMA [70]. This β_3AR mediated aorta relaxation seems to be independent of G_i proteins stimulation, since the blockage of G_i protein by PTX does not modify β_3AR agonists induced relaxation. On the contrary, selective potassium channels blockers of K (Ca), K (ATP) and K (v) decreased β_3AR agonists induced relaxation. So it appears that this effect results from the activation of several potassium channels, K (Ca), K (ATP) and K (v) [71].

Pathological implications

It was reported that noradrenaline-induced release of nitric oxide is enhanced in mineralcorticoid hypertension [72] indicating that α_2AR may play an important role in the regulation of vascular tone not only in physiological but also in pathological conditions. The implications of impaired βAR signalling in the pathophysiology of several cardiovascular disorders has been studied in animals and humans. Data from these studies indicate that changes in βAR function are induced by heart failure [73, 74] and hypertension [75, 76]. Moreover, alteration in βAR function were found also with physiological aging [77, 78], due to receptor downregulation and desensitization. Exercise restored the impaired signalling and βAR dependent vasorelaxation[79]. We and others have observed that impaired βAR signalling may account for dysfunctional βAR vasorelaxation in hypertension. In this condition, β_2AR overexpression in hypertensive rat carotids corrects impaired vasorelaxation to βAR stimulation to levels similar to those seen in normotensive rats[57]. We proved that impaired endothelium dependent vasorelaxation in spontaneously hypertensive rats (SHR) can be corrected by increasing the signal transduction pathways leading to nitric oxide synthase activation [80]. In particular, since eNOS is activated in response to phosphorylation by AKT and impaired AKT activity is involved in endothelial dysfunction, AKT overexpression should result in the correction of impaired phenotype. Indeed, insulin and ISO cause AKT membrane localization and this subcellular localization is impaired in SHR. AKT overexpression, through means of adenovirus mediated AKT gene transfer to the endothelium, increases the amount of AKT localized to the membrane and corrects impaired NO release and endothelium dependent vasodilation to agonists of both the GPCR and tyrosine kinase (TK) dependent pathways.

Conclusions

In the last years great advances have been made in the study of adrenergic receptors signaling and function in the endothelium also thanks to the development of new technologies. Indeed, genetic mouse models have significantly improved our understanding of the mechanisms of action of specific drugs *in vivo*. The ability to induce transgene expression at defined times or in defined tissues is an important goal as well as the ability to induce or repress the expression of endogenous genes in a developmental or tissue specific fashion. Indeed, deletion of the genes encoding for adrenergic receptor subtypeshas helped to identify the specific subtypes whichmediate *in vivo* effects of specific drugs. Thus, the combination of molecular biological, genetic, and pharmacological techniques greatly facilitates our understanding of adrenergic receptor function *in vivo*, and in turn leads to more effective and specific therapeutic treatment in humans. βARs, for instance, are already target of therapeutic intervention in many diseases: βAR stimulation in asthma and obesity or βAR blocking in hypertension and coronary insufficiency. In conclusion, giving the importance of endothelial function in most physiological and pathological conditions, it is clear that the increasing knowledge of adrenergic receptors function in the endothelium is helpful for future progresses in clinical application.

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Legend

Figure 1: βAR vasodilation is impaired in hypertension: A) In hypertensive patients, forearm vasodilation to ISO yielded an increase in forearm blood flow that was significantly lower to that observed in normotensive patients, at each dose of ISO. B) In hypertensive rats SHR, βAR-induced vasorelaxation to ISO in control-treated carotids was significantly impaired compared with that observed in normotensive WKY(* F= 5.756, $p< 0.01$, 2-way ANOVA).