ADRENERGIC MECHANISM IN THE CONTROL OF ENDOTHELIAL FUNCTION

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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 \(\alpha\) and \(\beta\) 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 \(\beta\) (\(\beta_1, \beta_2, \beta_3\)), three \(\alpha_1\) (\(\alpha_{1A}, \alpha_{1B}, \alpha_{1D}\)) and three \(\alpha_2\) (\(\alpha_{2A}, \alpha_{2B}, \alpha_{2C}\)) 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). α1AR 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. **α1AR and Nitric oxide**

Several reports have produced evidence for the functional presence of vasorelaxant α1AR in the brachial and pulmonary arteries isolated from the rabbit and rat, respectively. According to these reports, the pharmacological stimulation of α1AR located on endothelial cells, is able to generate NO, whereas the stimulation of α2AR releases a relaxing prostanoid. 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 $\alpha_{1D}$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 $\alpha_{1A}$AR.$^{14}$

2. $\alpha_{1}$AR 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 $\alpha_{1}$-adrenergic receptor should also be ranked among these agents. Indeed, it has been demonstrated that the $\alpha_{1A}$- and the $\alpha_{1B}$-AR subtypes but not the $\alpha_{1D}$ subtype are expressed in cultured rat aorta endothelial cells. The activation of these $\alpha_{1}$-AR in endothelial cells provide a negative regulation of angiogenesis.$^{17}$ Indeed, pharmacological antagonism of $\alpha_{1}$-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 $\alpha_{1}$-adrenergic receptor density in the ischaemic hindlimb, compared to non-ischaemic hindlimb, suggested an enhanced $\alpha_{1}$-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. $\alpha_2$AR and Nitric oxide

It has been demonstrated that $\alpha_2$ adrenergic agonists cause endothelium dependent relaxation, that is reduced or abolished by inhibitors of L-arginine/NO pathway. It depends on the activation of $\alpha_2$AR 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 $\alpha_1$AR\textsuperscript{18-20}. The $\alpha_2$AR subtype that cause endothelium dependent relaxation belongs to the $\alpha_2$A/D subtype, despite the prominent presence of $\alpha_2$CAR (77% of $\alpha_2$C versus 23% of $\alpha_2$A/D)\textsuperscript{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 $\alpha_2$AR is coupled to endothelium dependent NO-mediated relaxations and belongs to the $\alpha_2$A/D subtype appearing in its $\alpha_2$D version \textsuperscript{22}. It has been demonstrated that endothelium dependent relaxation to $\alpha_2$ adrenergic agonists is prevented by pertussis toxin \textsuperscript{23-28}, suggesting the involvement of $G_i$ proteins in the signal transduction from the receptor to the activation of nitric oxide synthase \textsuperscript{29, 30}. Indeed, $\alpha_2$ adrenergic agonists cause activation of $G_i$ proteins in endothelial cells and stimulate NO synthase activity \textsuperscript{31, 32}. Contrary to what expected, cAMP is not involved in the signal transduction pathway for $\alpha_2$A/DAR mediated NO formation \textsuperscript{22}. Indeed, the use of forskolin to oppose $\alpha_2$ adrenergic receptor mediated inhibition of cAMP formation in endothelium did not affect the relaxant response to $\alpha_2$AR agonists, suggesting that cAMP is not involved in the coupling of $\alpha_2$AR to NO. There are physiological modulation of endothelium dependent relaxation to $\alpha_2$ adrenergic agonists. Such relaxation is upregulated by chronic increase in blood flow \textsuperscript{33} or exercise training \textsuperscript{34}. Insulin enhances NO mediated vasorelaxation both in animal \textsuperscript{25} and human \textsuperscript{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\(_2\)AR, 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. $\beta_1$ and $\beta_2$ adrenergic receptors

It is now recognized that $\beta$AR located in the endothelium play an important role in the relaxant response to ISO, since the non selective $\beta_1$- and $\beta_2$-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 $\beta_2$AR antagonist ICI-118551 and remains unchanged in the presence of the $\beta_1$AR antagonist CGP-20712, indicating that, as in the vascular smooth muscle cells$^{46}$, the endothelial $\beta$AR are totally or at least predominantly of the $\beta_2$ subtype$^{10, 45}$.$

$\beta_2$AR are seven transmembrane receptors coupled through $G_s$ proteins to a cAMP dependent intracellular pathway$^{47}$. It has been demonstrated that PKA phosphorylation of the third intracellular loop of the $\beta_2$AR 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 adenyllyl cyclase activity. Second, it increases non cAMP dependent signaling through $G_i$, such as activation of the extracellular signal-regulated kinases ERK1/2 and PI3K$^{50-54}$. $G_i$ 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 PI3K$^{53, 55}$.

2. $\beta_2$AR and Nitric oxide

For years it has been given for granted that vascular $\beta_2$AR mediate adrenergic vasorelaxation through direct activation of vascular smooth muscle cells$^{56}$. However, recent data challenge this vision, and show that $\beta_2$AR-dependent vasorelaxation is mediated at least in part, by endothelium through nitric oxide (NO) dependent processes$^{10}$. We have recently demonstrated that the $\beta_2$AR are expressed on endothelial cells (EC) and their stimulation causes endothelial nitric oxide synthase (eNOS) activation$^{57}$. In particular, $\beta_2$AR couple to eNOS and induce NO dependent vasodilation$^{57}$. The mechanism of eNOS activation following $\beta_2$AR stimulation is known to be AKT dependent$^{58}$. 

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Indeed, the activity of eNOS is regulated by both a calcium/calmodulin dependent fashion\textsuperscript{59} and AKT dependent eNOS phosphorylation in Ser 1177 \textsuperscript{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\textsuperscript{11, 61, 64}. Therefore AKT acts as integrator of different signal transduction pathways converging on eNOS, including endothelial $\beta_2$AR receptor\textsuperscript{9, 58, 62, 63, 65}.

3. $\beta_2$AR and angiogenesis

In the endothelium $\beta$ARs control other important endothelial functions like angiogenesis, that is tightly associated to endothelial cell migration and proliferation \textsuperscript{57, 65, 66}. We demonstrated that $\beta_2$AR stimulation with ISO and the overexpression of $\beta_2$AR increases endothelial cell proliferation. Moreover, $\beta_2$AR stimulation induces ERK phosphorylation and the MEKK inhibitor, U0126, inhibits $\beta_2$AR induced cell proliferation \textsuperscript{66} suggesting that $\beta_2$AR dependent cell proliferation is dependent on ERK activation. We studied post-ischaemic angiogenesis in the hindlimb (HL) of $\beta_2$AR knock-out mice ($\beta_2$AR\textsuperscript{-/-}) in vivo and explored possible molecular mechanisms in vitro. Angiogenesis was severely impaired in $\beta_2$AR\textsuperscript{-/-} mice subjected to femoral artery resection, but was restored by gene therapy with AD$\beta_2$AR. The proangiogenic responses to a variety of stimuli were impaired in $\beta_2$AR\textsuperscript{-/-} EC \textit{in vitro}\textsuperscript{17}. Moreover, removal of $\beta_2$ARs impaired the activation of NF$\kappa$B, a transcription factor that promotes angiogenesis; ISO did not induce NF$\kappa$B activation in $\beta_2$AR\textsuperscript{-/-} EC\textsuperscript{17}. AD$\beta_2$AR administration restored $\beta_2$AR membrane density and reinstated the NF$\kappa$B response to ISO \textsuperscript{17}. These results suggest that $\beta_2$ARs control angiogenesis through the tight regulation of nuclear transcriptional activity.
5. \( \alpha_1 \text{ARand } \beta_2 \text{AR differently regulate neo-angiogenesis} \)

\( \alpha_1 \)- and \( \beta_2 \)-adrenergic receptors mediate opposite effects on neo-angiogenesis, comparable to their regulation of the vascular tone. In particular, the \( \alpha_1 \)-AR is inhibitory, whereas the \( \beta_2 \)-AR is stimulant to neo-angiogenesis. Interestingly, in ischaemia, the \( \alpha_1 \)-AR are upregulated, thus causing a predominance of \( \alpha_1 \)-adrenergic receptor signalling over that of \( \beta_2 \)-AR, which is downregulated. Furthermore, in conditions such as hypertension, where the \( \alpha_1 \)-AR tone is higher than that of the \( \beta_2 \)-AR, there is also an impairment in neo-angiogenesis \(^{66, 67}\). It is interesting to note that in the ischaemic hindlimb, \( \alpha_1 \)-AR blockade resulted in a normalization of \( \beta_2 \)-AR density together with improved neo-angiogenesis. \( \alpha_1 \)-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 \( \alpha_1A^- \) and \( \alpha_1B^- \)-adrenergic receptor \(^{68, 69}\).

6. \( \beta_3 \text{adrenergic receptors} \)

In rat thoracic aorta, Trochu showed that \( \beta_3 \)-AR are mainly located on endothelial cells and act in conjunction with \( \beta_1 \)-AR and \( \beta_2 \)-AR 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 \( \beta_3 \)-AR mediated aorta relaxation seems to be independent of \( G_i \) proteins stimulation, since the blockage of \( G_i \) protein by PTX does not modify \( \beta_3 \)-AR agonists induced relaxation. On the contrary, selective potassium channels blockers of K (Ca), K (ATP) and K (v) decreased \( \beta_3 \)-AR 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 mineralocorticoid hypertension indicating that $\alpha_2$AR may play an important role in the regulation of vascular tone not only in physiological but also in pathological conditions. The implications of impaired $\beta$AR signalling in the pathophysiology of several cardiovascular disorders has been studied in animals and humans. Data from these studies indicate that changes in $\beta$AR function are induced by heart failure and hypertension. Moreover, alteration in $\beta$AR function were found also with physiological aging, due to receptor downregulation and desensitization. Exercise restored the impaired signalling and $\beta$AR dependent vasorelaxation. We and others have observed that impaired $\beta$AR signalling may account for dysfunctional $\beta$AR vasorelaxation in hypertension. In this condition, $\beta_2$AR overexpression in hypertensive rat carotids corrects impaired vasorelaxation to $\beta$AR stimulation to levels similar to those seen in normotensive rats. 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. 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 subtypes has helped to identify the specific subtypes which mediate *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).