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Review

β-Adrenergic receptors and nitric oxide generation in the cardiovascular system

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Abstract. Nitric oxide plays a crucial role in cardiovascular homeostasis, with important vasodilatory, anti-thrombotic and anti-atherogenic properties. β-Adrenergic receptors (βARs), present on a wide variety of cardiovascular cells, including vascular endothelial cells, platelets, cardiac myocytes and leukocytes, have long been established as key players in maintaining cardiovascular homeostatic control. During the last few years a wealth of evidence has emerged which directly links stimulation of these cardiovascular βARs to nitric oxide (NO) generation, suggesting a new and important mechanism of adrenergic control of cardiovascular function. This review explores the cardiovascular cell systems in which this coupling of βARs and NO occurs, the intracellular signalling and regulatory mechanisms involved and the abnormalities in βAR-NO oxide coupling found in cardiovascular disease states.

Keywords. β-adrenergic receptors, nitric oxide, cardiovascular system, endothelium, platelets, cardiac myocytes.

Introduction

Since the discovery in 1987 that the endothelium-derived relaxing factor (EDRF) described by Furchgott and Zawadski [1] was nitric oxide (NO), it has come to be recognised as one of the most important molecules in mammalian physiology. NO acts as a signalling molecule in the cardiovascular and nervous systems, and plays a role in immune defence and wound healing as well as having a myriad of other actions. In the cardiovascular system, NO is generated mainly from vascular endothelial cells and to a lesser extent platelets, and its main functions are to cause vasodilatation, to inhibit platelet and leukocyte adhesion and platelet aggregation, and to inhibit vascular smooth muscle cell (VSMC) proliferation.

The existence of two subtypes of adrenergic receptors, the α-adrenoceptors (αARs) and the β-adrenoceptors (βARs), was first demonstrated in 1948 by Ahlquist [2], and since then they have been extensively studied and characterised. They are receptors for the endogenous catecholamines epinephrine and norepinephrine and are found widely distributed in the cardiovascular system. This review will focus on the βARs, of which there are three subtypes, and their roles in the maintenance of vascular tone, regulation of platelet aggregation and control of heart rate and contractility, focussing on the role of NO in these β-adrenergic functions.

Recent evidence suggests that these two important cell-signalling systems are interconnected and that, in many instances, stimulation of βARs is directly linked to NO production. This has been particularly demonstrated in both vascular endothelial cells and platelets, thus forming an important link between two significant pathways in vascular biology. In this review, the coupling mechanisms of βARs to NO generation will be explored in detail, and

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their importance in cardiovascular physiology will be examined. Furthermore, abnormalities in βAR-NO coupling that occur in cardiovascular pathophysiology will also be discussed.

NO

Furchgott and Zawadski [1] first reported that the acetylcholine (ACh)-mediated relaxation of vascular smooth muscle required the presence of intact endothelium. They proposed that a vasorelaxant substance was produced by the vascular endothelium, and they termed this substance EDRF. It was later demonstrated that EDRF was, in fact, the gas NO [3–5]. In mammalian cells, NO is synthesised from the amino acid L-arginine by oxidation of one of the terminal guanidine nitrogen atoms [6] to produce L-citrulline and NO. This reaction is catalysed by the NO synthase (NOS) family of enzymes, three main isoforms of which have been identified to date:

Type 1 (NOS 1) was first identified in both central and peripheral neuronal tissue, although it is also found outside the nervous system [7]. It is constitutively expressed, and its activation depends on elevation of intracellular levels of Ca^{2+}, which promotes binding of calmodulin [8].

Type 2 (NOS 2) is an inducible form of the enzyme, which acts independently of intracellular Ca^{2+} levels [8, 9]. It still requires calmodulin for its activation, but it binds even in the presence of low concentrations of Ca^{2+}. It is found in VSMCs [10], macrophages [9] and to a small extent, platelets [11], and usually requires cytokines or lipopolysaccharide for activation [7].

Type 3 (NOS 3) was first purified and cloned from vascular endothelial cells [12, 13] and is also found in cardiac myocytes [14] and platelets [11, 15]. Like NOS 1, it is constitutively expressed, and its activation is dependent upon the binding of calmodulin via an increase in Ca^{2+} [16]. However, NOS 3 can also be activated independently of Ca^{2+} elevation, by phosphorylation of various serine residues by a number of protein kinases [17, 18].

NO exerts many of its effects through its target molecule, soluble guanylyl cyclase (sGC), which it activates by binding to the heme moiety of the enzyme [19]. The activated sGC then converts guanosine triphosphate (GTP) to cyclic 3′,5′guanosine monophosphate (cGMP), the principal intracellular second messenger molecule for NO. Through this mechanism NO regulates vascular tone by relaxation of VSMCs, and is anti-atherogenic and antithrombotic by virtue of its inhibitory actions on VSMC proliferation [20, 21], platelet aggregation [22], and platelet [23] and leukocyte [24] adhesion to the vascular endothelium.

β-Adrenoceptors

In 1967, Lands et al. [25], found evidence for the existence of two types of βAR – β_1 and β_2 – by measuring the relative potency of sympathomimetic amines in different tissues. In 1989, a third βAR subtype – the β_3 adrenoceptor – was isolated and cloned [26]. Pharmacological evidence initially suggested a fourth type of βAR [27–29] but it has not been cloned, and recent evidence suggests that the putative β_4AR is probably a low-affinity state of the β_1AR [30, 31]. Only three βAR genes have, in fact, been identified at the genomic level. In the cardiovascular system, β_1ARs are found predominantly in the heart, where they increase heart rate (positive chronotropy) and force of contraction (positive inotropy), as well as increasing the rate of relaxation (positive lusitropy). β_2ARs are located on VSMCs, where they cause vasodilatation, and on platelets, where they can inhibit platelet aggregation. They are also found on vascular endothelial cells, and to a lesser degree in myocardium, where the effects of β_2AR stimulation are similar to those of β_1AR stimulation. There is some evidence for the existence of β_3AR in human heart [32], which may inhibit myocardial contractility through an NO-cGMP-mediated mechanism [33–35], although the unequivocal confirmation and physiological relevance of this remains somewhat elusive at present.

Signalling pathways of βAR-mediated NO generation

Several mechanisms of βAR signalling have been described, in a variety of cell types and species. Alongside the classical pathway involving G_s proteins, adenylyl cyclase (AC), cyclic 3′,5′ adenosine monophosphate (cAMP) and protein kinase A (PKA), described in early studies of VSMCs, βARs have shown to exert their effects through a number of other intracellular signalling pathways, such as the mitogen-activated protein kinase kinase (MEK)-p42/p44 mitogen-activated protein kinase (p42/44 MAPK, also known as ERK1/2) pathway, G_i protein coupling and the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) pathway. These signalling pathways, together with the evidence for their involvement in βAR-NO coupling, are discussed below and summarised in Figure 1.

cAMP-PKA signalling

All βARs are G-protein-coupled receptors which mediate their effects though the well-described actions of either the G-protein α-subunit or the $\beta\gamma$-subunit complex. The classical signal transduction mechanism for βARs involves coupling of the $G_{\alpha s}$ protein to AC, which becomes activated and catalyses the conversion of adenosine triphosphate (ATP) to cAMP. cAMP then activates PKA by

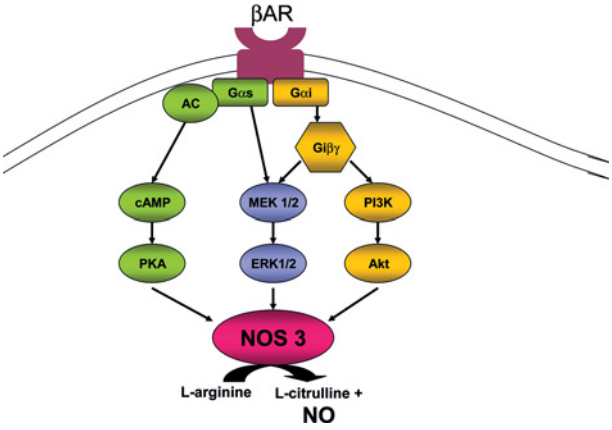

Figure 1. Potential intracellular pathways of βAR-mediated regulation of NO in cardiovascular cells.

binding to its regulatory subunit, causing it to dissociate from the catalytic subunit, thereby rendering the enzyme active. PKA is a serine/threonine protein kinase that targets a number of intracellular proteins, eliciting a series of specific cellular responses. For example, on VSMCs β_2ARs mediate vasodilatation via an elevation of cAMP, which activates PKA, which in turn phosphorylates myosin light-chain kinase, lowering its affinity for calmodulin and resulting in smooth muscle cell relaxation [36]. In cardiac myocytes, following β_1AR or β_2AR stimulation, PKA phosphorylates L-type calcium channels, promoting calcium entry into the cells, thus increasing cardiac contractility [37, 38].

Evidence for the involvement of the cAMP/PKA signalling pathway in βAR-mediated NO generation comes mostly from endothelial cell studies. Work from our own laboratory [39] demonstrated that in isolated human umbilical vein, β_2AR-mediated vasorelaxation is largely NO-dependent. The NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) attenuated the relaxation response to βAR stimulation or to the cAMP analogue dibutyryl cAMP, indicating that the NO-dependent component of the βAR relaxation response is mediated largely through elevation of cAMP. Parallel studies in cultured human umbilical vein endothelial cells (HUVECs) confirmed the presence of functional β_2ARs, which, when stimulated, raise intracellular levels of cAMP. We also demonstrated that stimulation of β_2ARs or receptor-independent elevation of intracellular cAMP in HUVECs increased NOS activity [39]. Furthermore, β_2AR-mediated NOS activation in HUVECs is associated with an increase in NOS 3 serine phosphorylation levels [40]. Protein kinase modification of NOS 3 by PKA is likely to be a key mechanism of βAR-mediated NOS activation in endothelial cells. Indeed, there is now abundant evidence of protein phosphorylation by PKA regulating NOS 3 activity in endothelial cells. For example, human NOS 3 is activated by phosphorylation of serine^{633} and serine^{1177} residues by PKA [18]. In bovine aortic endothelial cells, it has been reported that NOS 3 activation occurs in response to serine^{1177} phosphorylation and threonine^{495} dephosphorylation, these effects also being mediated by PKA [41]. It was recently shown that in HUVECs pretreated with the PKA inhibitor H89, βAR-mediated serine phosphorylation of NOS 3 is inhibited [42]. It is likely, therefore, that the increase in NOS 3 activity in response to β_2AR stimulation in HUVECs occurs in large part through elevation of cAMP with subsequent activation of PKA and serine phosphorylation of NOS 3. Studies in rat aorta have shown that β_2AR-mediated relaxation of this tissue has a significant NO-dependent component, in contrast to some reports [43–45]. In agreement with the accepted concept, βAR-mediated NO-independent relaxation, mediated through βAR on VSMCs, occurred principally through the cAMP/PKA signalling pathway. However, the NO-dependent component was partially inhibited by H89, indicating that, in this tissue, β_2AR-mediated NO generation is only partly mediated by activation of PKA [46]. Furthermore, Western-blotting studies showed that albuterol (a selective β_2AR agonist) increased NOS 3 serine phosphorylation, in agreement with previous findings in HUVECs [40], and that this increase was partially attenuated by H-89, demonstrating that in rat aorta, β_2AR-mediated NO generation occurs in part through NOS 3 phosphorylation by PKA [46]; the PKA-independent component of β_2AR-mediated NOS stimulation occurred through stimulation of the PI3K/Akt pathway, as discussed below.

The MEK/ERK pathway

No direct evidence exists for the MEK/ERK pathway being involved in βAR-NO coupling, but separate studies have shown first that activation of this pathway can occur in response to βAR activation, and, second, that it can play a role in NO production. Sexl et al. [47] have demonstrated that activation of the β_2AR on HUVECs stimulates cell proliferation and that this occurs via $G_{\alpha s}$ activation of MEK1/2, and subsequent phosphorylation of ERK1/2. More recently, it has been shown that adenosine, via the A_{2a} receptor, induces NO production in HUVECs and that this is mediated via the MEK1/2/ERK1/2 pathway [48]. There is also evidence that, in HUVECs, endothelin-induced NOS 3 regulation is tyrosine kinase-dependent [49], which is of relevance since ERK1/2 is a tyrosine kinase; furthermore, βAR-mediated PKA signalling can activate MAPK through switching of the coupling of βARs from a G_s protein to a G_i protein as a consequence of PKA-mediated β_2AR phosphorylation [50]. PKA itself has also been shown to activate the MAPK cascade through direct stimulation of Rap 1 [51]. Therefore, it is possible that, in some tissues or species, βAR-

mediated NO generation may also occur through MEK/ERK signalling.

PI3K/Akt signalling

In 1999, two groups independently showed that activation of NOS in endothelial cells, for example in response to vascular endothelial cell growth factor or shear stress, could occur by activation of the PI3K/Akt pathway, leading to phosphorylation of NOS 3 at serine$^{1177/1179}$ [17, 52]. Furthermore, this occurred independently of an increase in intracellular calcium levels. As β_2AR-mediated NOS activation in HUVECs also occurs independent of calcium [39], it seems likely that PI3K signalling could be one mechanism through which this occurs. Indeed, inhibition of Akt significantly attenuates NOS 3 serine phosphorylation in HUVECs [42], which has been shown to occur following β_2AR stimulation [40]. Similarly, in rat aorta, βAR-mediated NO-dependent vasodilatation, and serine phosphorylation of NOS 3, are both significantly attenuated both by wortmannin and by a selective Akt inhibitor, suggesting that the PI3K/Akt pathway can mediate β-adrenergic NOS activation in this tissue [46]. In recent years, it has become known that β_2ARs can couple not only to G_s protein, as has long been established, but also to G_i protein [53–55]. This may provide a mechanism whereby β_2ARs can stimulate Akt, since $\beta\gamma$ subunits derived from G_i, following its activation, can stimulate PI3K, which in turn activates Akt. This has been demonstrated in cat atrial myocytes [56], although whether this mechanism occurs in endothelial cells has yet to be determined.

βARs, NO and the vascular endothelium

It has long been known that vascular endothelial cells express βARs [57–61], but for many years the function of these receptors was unclear. Over the last 20 years or so, however, evidence has accumulated that they contribute to vasorelaxation through stimulation of endothelial NO biosynthesis. The original observation that endothelial βARs were important in relaxation of blood vessels came from Rubanyi and Vanhoutte [62], who demonstrated that removal of the endothelium reduced the relaxation of canine coronary arteries caused by βAR agonists. Since then many studies have reproduced these findings or shown similar effects with NO inhibition, in a variety of vascular tissues (*in vitro* and *in vivo*) from many different species, including rat [63–67], dog [68], pig [69], mouse [70], rabbit [71] and humans [39, 72, 73]. The extent of the contribution of NO to the βAR-mediated relaxation response differs between vascular beds. In some vessels βAR-mediated NO production may outweigh any direct vasorelaxant effect of βARs located on VSMCs [39, 71], whilst in others the contribution of NO is negligible [74, 75]. In some cases the issue is still unresolved, with different groups describing opposite effects in the same tissues. For instance, in the rat aorta, Gray and Marshall [64] reported that βAR-mediated relaxation was totally dependent on intact endothelium, whilst other investigators have shown it to be only partially dependent on the endothelium [43, 46, 76–78] or even to be endothelium-independent [44, 45]. Possible explanations for these inconsistencies may relate to differences in the degree of, and agent used for, pre-constriction between studies [45]; to differences in the ages of animals used [78]; or to incomplete removal of the endothelium in some studies [64].

Until recently, the mechanisms by which endothelial βARs may be coupled to NO biosynthesis remained unclear, although it was suggested that cAMP played a role in the βAR-mediated NO response [64, 69]. As discussed above, it has now been shown that at least two signalling pathways are involved. The first evidence for a direct, functional coupling of βARs and NOS in human endothelium that is mediated largely through cAMP elevation and is Ca^{2+}-independent came from our own work in human umbilical vein [39]. A further study [71] demonstrated that a similar endothelial βAR-cAMP-NO pathway regulates vasodilatation in the rabbit femoral artery *in vivo*. In HUVECs, βAR stimulation is associated with increased serine phosphorylation of NOS 3, and as discussed above, this appears to be mediated by both the cAMP/PKA and the PI3K/Akt pathways [40, 42]. Similarly, in rat aorta, β_2AR-mediated NO generation is mediated by both the cAMP/PKA and PI3K/Akt signalling systems [46].

Physiological significance of endothelial βARs

Previously it was thought that βARs located on VSMCs were predominantly responsible for β-adrenergic regulation of vascular tone. Since the discovery of the endothelium as an important metabolic tissue in its own right, which releases many vasoactive substances, the importance of endothelial cell βARs has become more evident, such that it is now recognised that they play an important role in the sympathoadrenal regulation of vascular tone. In addition, NO generated by endothelial βAR stimulation will also have anti-thrombotic and anti-atherogenic effects which are important in maintaining normal physiological function, via inhibition of platelet aggregation, VSMC proliferation and platelet and monocyte adhesion to the vascular endothelium. Furthermore, some disease states such as atherosclerosis and heart failure are associated with both endothelial dysfunction and impaired βAR function [79–81], as will be discussed later. Therefore, endothelial βAR-derived NO may be of great physiological and pathophysiological relevance in humans, and a

greater understanding of the signalling mechanisms involved may open up new possibilities for therapeutic intervention.

βARs, NO and platelets

βARs are present on human platelets and have been characterised to be of the β_2AR subtype, as determined by radioligand binding [82–84]. These receptors have not been extensively studied, as the predominant effects on platelets of the endogenous catecholamines epinephrine and norepinephrine are mediated through α_2ARs, which elicit platelet activation and aggregation [85, 86]. Although epinephrine and norepinephrine can act on both α_2ARs and β_2ARs, the overall effect of these catecholamines on platelets is pro-aggregatory, and this is related to both the relative selectivity of norepinephrine for α_2ARs versus β_2ARs and the greater numbers of α_2ARs as compared with β_2ARs present on platelets. It has been reported that, on average, each human platelet expresses 258 α_2ARs and 66 β_2ARs [87]. Platelet βARs are coupled to AC through G_s protein [88] and, when stimulated, cause an increase in intracellular cAMP concentration [82, 87] and subsequent activation of PKA [89], which in turn causes inhibition of aggregation [90]. Little data exist on the effect of βARs on platelet adhesion, and until recently, no link had been shown between βAR stimulation and NO biosynthesis in platelets even though, as discussed above, the relationship is now well established in vascular endothelium.

Platelets express both NOS 3 and NOS 2, although NOS 3 is predominant by far [11]. NO, generated by both platelets themselves and by the vascular endothelium, inhibits platelet adhesion to vascular endothelium [91] and platelet aggregation [22] through a cGMP-mediated mechanism. Indeed, NO generation by platelets may act as a negative feedback mechanism to regulate platelet aggregation following a pro-aggregatory stimulus [92]. Furthermore, platelet-derived NO plays an important role in the regulation of platelet recruitment [93]. Thus, platelet-derived NO may have important anti-thrombotic and anti-atherogenic roles *in vivo*.

The first evidence of coupling between β_2AR and NO biosynthesis in platelets came from our laboratory [94]. We found that stimulation of platelet β_2ARs resulted in an increase in NOS 3 activation, and this response was mediated via stimulation of AC and increased cAMP; furthermore, it occurred with no measurable change in intracellular Ca^{2+} levels, as previously seen in endothelial cells. However, further signalling pathways have not yet been investigated, and it remains to be determined whether PKA, tyrosine kinases (including MEK/ERK), Akt or possibly other kinase cascades (acting on platelet NOS 3) have any role to play in platelet βAR-mediated NO generation. In functional studies [94], isoproterenol inhibited U46619-induced platelet aggregation through β_2AR, but NOS inhibition had no effect on the isoproterenol anti-aggregatory response. On the other hand, isoproterenol decreased platelet adhesion to HUVEC monolayers, and this effect was abolished by NOS inhibition. It was concluded, therefore, that platelet β_2ARs, through activation of the L-arginine/NO system, may contribute to regulation of platelet adhesion to the vessel wall but not to platelet aggregation.

These data are further supported by a more recent study [95], which demonstrated that, at physiological concentrations, the catecholamines epinephrine and norepinephrine increase intraplatelet levels of cGMP as a consequence of βAR-mediated NO production. This indicates that platelet cGMP, as well as cAMP, plays a role in adrenergic regulation of platelet function physiologically. In a separate study, the same group demonstrated that adenosine increases platelet cGMP through an NO-dependent mechanism and that this plays a role in the anti-aggegating effect of adenosine [96]. As adenosine is similarly coupled to AC via G_s protein and induces its anti-aggregatory effect through cAMP elevation, it appears that cAMP/PKA may be a major mediator of NOS activation in platelets.

Physiological significance of platelet βARs

The mechanisms of βAR-mediated NO production in platelets have not been as extensively studied as those in endothelial cells, but nevertheless the functional significance of this pathway is clear. Blockade of NO biosynthesis does not appear to have an effect on βAR-mediated inhibition of platelet aggregation, although it does have a major effect on βAR-mediated inhibition of platelet adhesion to the endothelium, as discussed above. This suggests that, in platelets, βAR-stimulated NOS activation may have differential effects on the function or expression of glycoprotein Ib, which mediates adhesion, as compared with glycoprotein IIb/IIIa, which is involved in platelet aggregation. However, this has yet to be investigated. Nevertheless, βAR-mediated NO production from both endothelium and platelets may play a role in regulating platelet adhesion and maintaining the anti-thrombotic properties of the vessel wall. Furthermore, as discussed above, platelet-derived NO has been shown to have an important role in regulating platelet recruitment [93] and haemostasis [97], and may act as a negative feedback system to regulate platelet aggregation following a pro-aggregatory stimulus [92]. Although circulating catecholamines have a greater affinity for platelet αAR as compared with βAR, the βAR may offset the pro-aggregatory effects of αAR stimulation in response to endogenous catecholamines. *In vivo*, circulating plasma concentrations of epinephrine and norepinephrine range be-

tween 0.1 to 0.5 nmol/l and 0.3 to 3nmol/l respectively. However, in times of severe stress, for example, during myocardial infarction, circulating levels of these catecholamines can reach considerably higher levels (≥10 nM) [98]. Such high concentrations may contribute to the platelet aggregation seen in unstable angina and myocardial infarction through α_2AR stimulation [99, 100], and at those concentrations, significant activation of β_2ARs would also be expected to occur, thus offsetting the pro-aggregatory effects of these catecholamines.

βARs, NO and the heart

Norepinephrine, released from sympathetic adrenergic nerves, and epinephrine, released into the circulation by the adrenal glands, are important regulators of cardiac contractility, exerting their effects through adrenergic receptors (predominantly βAR) located in the heart. The human heart expresses both β_1AR and β_2AR [101] and, more recently, there has been some evidence of β_3AR messenger RNA (mRNA) expression in human heart tissue [33, 34]. In human heart β_1ARs are known to predominate, increasing force and frequency of contraction as well as increasing rate of relaxation, actions which are mediated through the well-described cAMP-PKA pathway [38, 102]. However, more recently, modulation of cardiac function by NO and cGMP has been described and suggested as an important mechanism of cardiac control. All three isoforms of NOS are expressed within various cell types of the heart [101, 103]. NOS 1 has been identified in cholinergic, sympathetic and non-adrenergic, non-cholinergic nerve terminals of the guinea pig heart [104]. NOS 2 is found in virtually all myocardial cell types following stimulation with inflammatory cytokines [105]. NOS 3 is constitutively expressed in endothelial cells from the endocardium and cardiac vessels [106, 107] as well as in cardiac myocytes [35, 106, 108].

Research into the effects of acetylcholine in the heart led to the discovery that the NO/cGMP system is an important second messenger signalling pathway of cardiac muscarinic cholinergic receptors [109]. Subsequently it was discovered that βARs were also coupled to NO biosynthesis. Balligand et al. [14] suggested that an NO-mediated mechanism was responsible for limiting the positive inotropic response to βAR agonists in rat ventricular myocytes. Further work by Sterin-Borda et al. [110] described how the isoproterenol-mediated positive inotropic response in isolated rat atria is accompanied by a calcium-dependent stimulation of NOS and increased cGMP levels. Inhibition of NOS and sGC enhanced the isoproterenol response, supporting the hypothesis of a parallel negative inotropic regulatory pathway induced by βAR stimulation.

Much of the early work on the establishment of NO-mediated βAR regulation in the heart has previously been reviewed in detail by Balligand [101], and mechanisms which result in the activation of NOS in cardiomyocytes have been reviewed by Bloch et al. [103]. Therefore, these will not be reviewed in detail here. However, in the last 4 years, several significant developments have furthered our understanding of the signalling mechanisms of βARs, and their coupling to NO within the heart, and these will be examined.

It is now well established that the different subtypes of βARs in the heart, mainly β_1AR and β_2AR, activate different signalling pathways due to differences in coupling pathways. Under normal physiological conditions, β_1AR stimulation leads to positive inotropy, lusitropy and chronotropy predominantly through G_s protein-cAMP-PKA signalling. β_2ARs couple to both G_s and G_i proteins. Coupling to G_s activates the cAMP-PKA pathway, mediating similar effects to β_1ARs. Coupling to the G_i protein mediates a negative inotropic response, through the $\beta\gamma$ subunit of G_i giving rise to PI3K/Akt activation, resulting in cardioprotective effects. This has recently reviewed by Zheng et al. [111].

The coupling of β_2ARs to G_i and activation of the PI3K/Akt pathway is also the mechanism by which stimulation of these receptors is believed to generate NO in the heart. In cat atrial myocytes [56], stimulation of β_2ARs was found to cause activation of the PI3K/Akt pathway, via a G_i-protein-coupled mechanism. Further work by the same group [112] showed that, in addition, NO produced by the β_2AR-Akt pathway acts locally via S-nitrosylation to inhibit β_2AR-G_s-cAMP signalling, offsetting normal muscarinic receptor-mediated inhibition of βAR-mediated inotropy.

Some years ago it was reported in functional studies that β_3ARs were present in the human heart and they could give rise to negative inotropic effects through G_i protein coupling and an NO-cGMP mediated mechanism [33, 35]. However, these studies used high levels of reverse transcription-polymerase chain reaction (RT-PCR) amplification and the rodent-selective β_3AR agonist BRL37344; thus the findings are difficult to extrapolate to humans. A study in mice demonstrated that βAR-stimulated inotropy was increased in both β_3AR gene-deficient (β_3–/–) and NOS 3 gene-deficient (NOS3–/–) mice [113]. Furthermore, isoproterenol increased myocardial cGMP in wild-type, but not β_3–/– mice, suggesting that, at least in this species, cardiac β_3AR-mediated NO generation may act as a negative feedback system to regulate βAR-stimulated positive inotropy. The first direct evidence of β_3AR-NOS 3 coupling came from a study by Pott et al. [114] who examined the effects of the β_3AR agonist BRL37344 in human atria. Further work by the same group [115] demonstrated that β_3AR-NOS 3 coupling is mediated by activation of the PI3K/Akt pathway, and fur-

thermore, NOS 3 activity is modulated by phosphorylation at the serine[1177] and serine[114] residues. Mechanisms of NOS 3 activation were also different between human atrial and ventricular tissue, being translocation-dependent in right atrium but not in left ventricle. Again, however, the physiological implications of this are not entirely clear, first because BRL37344 is rodent-selective, and second because this compound is not entirely selective for β_3AR, having actions also at β_1AR and β_2AR.

βARs, NO and other cardiovascular cells

There is comparatively little evidence of a link between βARs and NO in other cardiovascular cell types known to express NOS and βAR, such as macrophages and leukocytes. However, there is some evidence to suggest that βARs play a role in inhibiting NOS 2-induced NO generation in macrophages, as a mechanism of regulating the immune response, especially in inflammatory conditions where NO can contribute to the pathology. For example, Hasko et al. [116] showed that isoproterenol inhibits lipopolysaccharide-stimulated NO release from a macrophage cell line. Another study [117] confirmed and extended these results by demonstrating that epinephrine inhibits lipopolysaccharide-induced NO production (via NOS 2 activation) in murine macrophages through β_1AR and β_2AR. It has also been reported that the sympathetic nervous system, through βAR stimulation, reduces NO production from alveolar macrophages in a rat model of stress [118].

βAR-mediated NO generation in pathophysiology

Under normal physiological conditions NO, generated by the vascular endothelium, platelets and cardiac myocytes, is a key player in cardiovascular homeostasis. As discussed above, the recently discovered mechanisms of NO biosynthesis through stimulation of βARs in different cardiovascular cell types contribute importantly to the physiological regulation of vascular tone, platelet function and cardiac contractility. However, impairment of the L-arginine/NO pathway in either endothelium or platelets (and sometimes both) is a common feature of aging and many cardiovascular disorders. Whether such impairment is pathogenetic, or is a result of the vascular disease in these conditions is, however, uncertain. Such dysfunction may profoundly affect β-adrenergic responses in the cardiovascular system, and this may be of great pathophysiological relevance, as a lack of biologically active NO in such conditions may predispose to platelet adhesion to the vascular wall and subsequent thrombus formation, as well as to vasoconstriction. Here, abnormalities in βAR-NO coupling in aging and disease states are examined.

Type 2 diabetes mellitus

Both macrovascular and microvascular disease are important complications in type 2 diabetes mellitus. Much of the excess morbidity and mortality of the condition relates to atheromatous disease and its thrombotic complications, in which both vascular endothelium and platelets play an important role. Reduced NO production by the endothelium is well-documented in animal models of diabetes [119], and impairment of endothelium-dependent vasodilatation has been demonstrated in type 2 diabetic patients [120–122]. Patients with atherosclerosis, an important and major complication of type 2 diabetes, have a reduced expression of NOS 3 and suppressed NO production by the vascular endothelium [123]. Furthermore, patients with type 2 diabetes have reduced basal and insulin-stimulated NOS activity in skeletal muscle, which may contribute to the insulin-resistance of such patients [124]. Diabetes is also associated with impairment of platelet function, such as hypersensitivity of platelets to aggregating agents, enhanced thromboxane A_2 production and altered Ca^{2+} signalling. Platelets from type 2 diabetic subjects have been reported to have a reduced sensitivity to prostacyclin [125] and NO [126], and basal NOS 3 activity has been reported to be significantly lower in both type 1 and type 2 diabetic patients compared with healthy control subjects [127, 128].

Two studies have examined βAR-NO coupling in type 2 diabetes, one in endothelium and one in platelets. In 1999, Chowienczyk et al. [129] reported that albuterol-mediated vasodilatation, as measured by the digital volume pulse, is mediated in part through the L-arginine/NO pathway and that this is impaired in patients with type 2 diabetes. This finding was the first evidence of impaired βAR-NO coupling in this disease. More recently, work from our laboratory demonstrated that this impairment also occurs in platelets [130]. Platelets from patients with type 2 diabetes exhibited reduced basal NOS activity as compared with platelets from healthy control subjects, in agreement with previous findings [127, 128]. Furthermore, βAR- and AC-stimulated NOS activity and cGMP levels were also significantly reduced in these patients and were negatively correlated with both plasma glucose and HbA_{1c} levels. This indicates that poor glycaemic control is an important factor in the impairment of βAR-mediated NO generation seen in platelets from type 2 diabetic patients. Although changes in NOS 3 expression could not account for the impaired response seen in this study, it is possible that alterations in phosphorylation of NOS 3 or the availability of co-factors are responsible. Indeed, in a study investigating vascular dysfunction in internal mammary arteries from diabetic patients, both reduced endothelium-dependent vessel relaxation and reduced NOS 3 expression were demonstrated, together with attenuated threonine[308]-phosphorylation of Akt and decreased levels of serine[1177]-phosphorylated NOS 3 [131].

This result suggests impaired PI3K-mediated signalling in diabetic vessels, and it could account for the impaired βAR-NO coupling seen in endothelium, and possibly also platelets, in type 2 diabetes.

Hypertension

Essential hypertension is a multifactorial disease in which the exact pathogenetic mechanisms are still unclear. The importance of the endothelium in regulating basal vascular tone and arterial blood pressure is well established, and indeed, impaired endothelial-dependent vasodilatation and reduced NO production is well-documented in hypertension in both animal [132–134] and human studies [135–137]. However, this is not a universal finding, as several studies report no such evidence of impaired endothelial dysfunction in hypertension [138–141]. These conflicting results indicate that not all individuals with essential hypertension have impaired endothelial function, and where it is shown, it may be related to genetic and lifestyle differences or coexistence of other cardiovascular risk factors, for example hypercholesterolaemia, obesity, insulin resistance or smoking, all of which have been shown to be associated with endothelial dysfunction. There is also some evidence that it may be a consequence, rather than a cause, of the disease. For example, studies show that a transient increase in blood pressure results in impaired endothelium-dependent vasodilatation [142, 143].

In terms of βAR-mediated NO generation in hypertension, little work has been done and the results are still conflicting. Cockcroft et al. [138] investigated forearm blood flow by venous occlusion plethysmography and found no difference in isoproterenol-mediated vasodilatation between hypertensive patients and normotensive controls. In the same year, Arribas et al. [144] investigated endothelial βAR-mediated vasodilatation in isolated rat aorta and found impairment of the response in spontaneously hypertensive rats.

Aging

Cardiovascular function deteriorates with advancing age. Remodelling of the vascular wall, including intima and medial thickening, increased arterial stiffness, increased oxidative stress, impaired endothelial function and a reduction of cardiac output, peak heart rate and stroke volume all occur as part of the ageing process. Many studies have shown reduced NO-dependent vasodilatation in an elderly population [145–149]. βAR function also declines with increasing age (reviewed by Schutzer and Mader [150]), with physiological and molecular changes all playing a role, including reduced Akt-dependent phosphorylation of NOS 3 [151], which, as discussed above, is one signalling pathway involved in βAR-NO coupling. Therefore, although it has not been investigated in great detail, βAR-mediated NO production is also likely to be reduced as a consequence of ageing. This is supported by the findings of Arribas et al. [144], who demonstrated reduced βAR-mediated NO-dependent vasodilatation in isolated aorta from aged rats, and those of Goubareva et al. [152], who found that albuterol-stimulated NOS 3 activity is reduced in platelets from older healthy men as compared with platelets taken from younger men.

NO and βAR blockers

All the above evidence of βAR-mediated NO production has been concerned with stimulation of βARs by endogenous catecholamines or other βAR agonists. β-Receptor antagonists (β-blockers), might be expected to give rise to a reduction in NO biosynthesis. However, several β-blockers, including some which are used clinically, have been shown to have actions other than βAR blockade, such as agonist or inverse agonist properties. In this way, these agents may activate intracellular signalling mechanisms that may also regulate NO generation. For example, the β-blocker CGP 12177 has been shown to stimulate cAMP production in CHO cells expressing the human β_2AR [153]. Several other β-blockers have also been shown to have weak β_2AR agonist actions by stimulating cAMP [154, 155], or inverse agonist activity (through decreased cAMP accumulation) [155, 156]. Furthermore, several β-blockers which have β_2AR agonist activity at the level of cAMP accumulation or cAMP response element gene transcription do so via activation of the MEK/ERK pathway [157], which may also stimulate NO generation, as discussed above.

Some β-blockers have been shown to have vasodilatory properties that are NO-dependent. For example, carteolol has been shown to potentiate dilatation of rat mesenteric arteries, an effect blocked by inhibition of NOS [158]. Nebivolol was designed as a highly cardioselective β_1AR antagonist and is licensed for treatment of hypertension in Europe and the United Kingdom. It has no intrinsic sympathomimetic or αAR blocking activity at therapeutic doses [159], but it is distinct from many other β-blockers in that, in addition to its β_1AR antagonist activity, it possesses NO-mediated vasodilatory properties.

In 1991 it was demonstrated that nebivolol induced endothelium-dependent relaxation of canine coronary arteries [160]. The first evidence that this vasodilatory effect was mediated by NO in man came from a study by Bowman et al. [161], who infused nebivolol and atenolol into dorsal hand veins of healthy men. Nebivolol caused vasorelaxation of veins, which were preconstricted with phenylephrine or prostaglandin $F_{2\alpha}$, and the effect was antagonised by L-NMMA. Since then, several studies have confirmed the hypothesis that the vasodilator effect of

nebivolol is mediated by activation of the L-arginine/NO pathway [162–164]. For this reason, nebivolol is often considered to be useful for the management of essential hypertension where there is endothelial dysfunction [165–168]. Additionally, the NO-producing capacity of nebivolol is useful in reducing arterial stiffness, an important independent predictor of mortality in hypertensive patients. It has been shown that NO is an important regulator of arterial distensibility [169, 170], and β-blockers do not show improvement in arterial compliance in comparison with other anti-hypertensive agents [171]. In contrast to this, nebivolol has been shown to reduce pulse wave velocity (a measure of arterial distensibility) in sheep *in vivo*, due to release of NO [172], further demonstrating its usefulness in treating patients with hypertension. More recently it has been shown to provide cardioprotection for patients with heart failure (SENIORS trial) [173], although whether this is through NO generation is still unclear.

The signalling pathway(s) through which nebivolol activates the L-arginine/NO cascade is still unclear, and there are several hypotheses. It has been observed that nebivolol works through β_2AR-stimulated NO production [163–165, 174], and suggested signalling pathways include phospholipase C, phospholipase A_2 and cAMP, via a calcium-independent mechanism [175], or by activation of inositol phosphate [164]. A recent study in mouse renal arteries reports that nebivolol has a vasodilatory effect via stimulation of the endothelial β_2AR, elevated Ca^{2+}, increased NO production and activation of Ca^{2+}-activated K^+ channels [176]. However, nebivolol itself lacks direct β_2AR agonist activity, so it may be a metabolite of the drug that has this effect. Indeed, nebivolol has a greater potency as a vasodilator when administered by mouth than intra-arterially [177], suggesting that metabolism in the liver or elsewhere converts the drug to an active metabolite. Furthermore, it has been shown that *in vivo* metabolism in the mouse or *in vitro* metabolism by mouse hepatic microsomes leads to formation of a product that elicits endothelial β_2AR activation and NO biosynthesis [176]. In contrast, other studies provide evidence for β_3AR involvement in nebivolol-induced vasorelaxation in rat aorta [178], and human and rodent coronary arteries [179]. This latter study further suggests that nebivolol, in addition to its vasodilatory properties, has a pro-angiogenic effect in mouse aorta *in vitro*, and that these effects are mediated through stimulation of β_3AR, with an accompanying rise in cytosolic Ca^{2+}, and dephosphorylation of NOS 3 on threonine^{495} in endothelial cells [179]. In the rat, the NO vasodilator effect of nebivolol on renal vasculature and aorta appears to occur through a 5-hydroxytryptamine $(5\text{-HT})_{1A}$ receptor-mediated mechanism [180]. However, a recent study [181] suggests that the vasodilator effect of nebivolol in guinea pig heart is not mediated through β_2AR, β_3AR or 5-HT_{1A} receptors. This may be due to species differences. In a recent study in patients with essential hypertension, the antioxidant effects of nebivolol were compared with those of atenolol. Patients treated with nebivolol for 4 weeks showed an improvement in markers of oxidative stress (plasma hydroperoxides, low-density lipoprotein oxidation and isoprostanes) as compared with the atenolol group. In addition, there was a reduction of reactive oxygen species generation in endothelial cells exposed to plasma from nebivolol-, but not atenolol-treated, patients, suggesting that nebivolol may also increase NO by reducing its scavenging by reactive oxygen species [182]. Further work is required to elucidate the precise mechanism whereby this drug elicits NO biosynthesis in man.

Concluding remarks

Much has been learnt about β-adrenergic control of cardiovascular homeostasis in the last century, and the more recent discovery of NO as a biological signalling molecule has created an important new target for therapeutic intervention, particularly within the cardiovascular system. Over the last decade, the link between βAR stimulation and NO generation has been explored in detail, and the different regulatory signalling cascades involved are starting to emerge. Much work is needed to fully elucidate the importance of this new mechanism of adrenergic control in cardiovascular physiology and pathophysiology, but is clear that the discovery of βAR-NO coupling has revolutionized our understanding of βAR-mediated homeostatic control, and provides new possibilities in the therapy of cardiovascular disease states associated with dysfunction of βAR-mediated NO generation.

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