Targeting β₃-Adrenergic Receptors in the Heart: Selective Agonism and β-Blockade

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Abstract: Cardiac diseases, such as heart failure, remain leading causes of morbidity and mortality worldwide, with myocardial infarction as the most common etiology. HF is characterized by β-adrenergic receptor (βAR) dysregulation that is primarily due to the upregulation of G protein–coupled receptor kinases that leads to overdesensitization of β₁ and β₂ARs, and this clinically manifests as a loss of inotropic reserve. Interestingly, the “minor” βAR isoform, the β₂AR, found in the heart, lacks G protein–coupled receptor kinases recognition sites, and is not subject to desensitization, and as a consequence of this, in human failing myocardium, the levels of this receptor remain unchanged or are even increased. In different preclinical studies, it has been shown that β₁ARs can activate different signaling pathways that can protect the heart. The clinical relevance of this is also supported by the effects of β-blockers which are well known for their proangiogenic and cardioprotective effects, and data are emerging showing that these are mediated, at least in part, by enhancement of β₁AR activity. In this regard, targeting of β₁ARs could represent a novel potential strategy to improve cardiac metabolism, function, and remodeling.

Key Words: β-adrenergic receptor, G proteins, heart failure, β-blockers

(J Cardiovasc Pharmacol™ 2017;69:71–78)

INTRODUCTION

G protein–coupled receptors (GPCRs) are nodal regulators of mammalian cell physiology because they transduce cell signals from diverse ligands such as neurohormones, sensory stimuli, and ions through heterotrimeric G proteins.¹ In the heart, they represent the major modulators of both function and morphology with β-adrenergic receptors (βARs) representing “the heads of the line”, and for this reason they are considered the most important molecular targets in the cardiovascular system.²–⁴ Currently, 3 βAR subtypes (β₁AR, β₂AR, and β₃AR) have been identified in the myocardium, with β₁ and β₂ARs the most expressed and studied.⁵–⁷ However, since its discovery in 1989,⁶ it soon seemed clear that β₃ARs, the isoform with minor expression, can influence cardiovascular physiology. In particular, β₃ARs seem to have multiple roles that go from regulation of metabolism,⁸⁹ vasodilation, and relaxation⁸ to cardiac contractility.⁹ Thus, this receptor is of high interest especially for new potential therapeutic approaches for heart disease.

In this review, we will discuss what is known about the cardiac role of β₁ARs and how not only their activation but also the blockade could be beneficial or not in cardiac physiology and in disease.

β₃-Adrenergic Receptor (β₃AR) Structure

The mammalian β₃AR sequence consists of about 400–408 amino acids in a protein that has the typical structure of all GPCRs.¹⁰ The β₃AR has 7 transmembrane domains (7-TMDs) with an extracellular N-terminal that is glycosylated, and an intracellular C-terminal domain.¹⁰ Further, the Cys361 residue in the fourth intracellular domain is palmitoylated, a feature that has been shown to be associated with G protein-coupling and adenyl cyclase stimulation following agonist stimulation of the receptor¹¹ (Fig. 1). As shown in Figure 2, the protein sequence alignment between different mammalian species demonstrates that most of the homology between the β₃AR amino acid sequences is concentrated in the 7-TMDs and in the membrane-proximal regions of the intracellular loops. Interestingly, when the β₁AR protein sequence is compared with other βAR (β₁ and β₂AR) isoforms, it is still possible to observe a high level of homology in the 7-TMD sequence, but a significant divergence is present both in the third intracellular loop and in the C-terminal domain (Fig. 3). This difference probably represents the major factor affecting the pharmacologic regulation of the receptors and their response to a ligand. In this regard, the C-terminus of both β₁ and β₂ARs is rich in serine and threonine residues, and is subjected to GPCR kinase (GRK)-mediated regulation through phosphorylation.¹⁰ Further, these receptors also harbor a consensus sequence for protein kinase A (PKA).¹⁰ Of note, the β₃AR lacks all of these sites, and is more resistant to agonist-induced desensitization/
downregulation. Finally, these sequence divergences also support differential and intracellular signaling (including G protein-coupling) between the 3 βAR isoforms, which may determine their relative roles in physiology and in the disease.

β3AR: In Search of Signaling and Function

The human β3AR was cloned in 1989,7 and the studies demonstrated that this new βAR isoform was mainly implicated in lipolysis and thermogenesis regulation in adipose tissues.8,12 However, over the last 2 decades, different reports have also clearly shown that β3ARs are present in the cardiovascular system, mainly in myocardium and endothelium, where they have a prominent role in modulating cardiac function and angiogenesis, respectively.13,14 In this context, determining the specific pathways associated with these effects represents a tough challenge and remains a largely unresolved question regarding β3AR function. This is due, at least in part, to the fact that the role of the cardiac β3AR has not been studied with the same intensity as the β1- and β2ARs. Moreover, all the studies concerning β3AR function have not been focused on similar cell types, and the agonist and the doses used are significantly different between most studies.14,15 Another important difference is represented by the experimental model used in key studies.15 In fact, it is known that in the mouse, the gene encoding for the β3AR undergoes alternative splicing and gives rise to β3aAR and β3bAR variants.16,17 These 2 β3AR isoforms are coupled to adenylyl cyclase stimulatory Gz (Gzs) or to the inhibitory Gz (Gzi) protein subunits (β3bAR), or exclusively to the Gzs protein (β3aAR).17,18 By contrast, in humans, although some reports have proposed that β3AR can activate Gzi signaling in CHO/K1 cells19 and in adipocytes,20 it is a general assumption that, at least in ventricular myocardium, β3ARs are mainly coupled with Gzi proteins.21,22

For these reasons, the β3AR leads to effects that are either comparable or opposite to those elicited by β1- and β2AR stimulation. In fact, stimulation of β3AR, through Gzs activation, increases the generation of cyclic AMP (cAMP) and the activation of the PKA, similar to β1- and β2ARs.4,23 In the myocardium, after catecholamines stimulation of βARs, PKA phosphorylates many Ca2+ handling proteins and some myofilament components leading to positive inotropic, lusitropic, and chronotropic effects.4,23 (Fig. 4). However, because β3ARs are also coupled with Gzi, they can act as a brake to prevent β1 and β2ARs overactivation, and this has been proposed as a mechanism in the heart21,22 (Fig. 4). Moreover, in the heart, the stimulation of β3ARs leads to increased endothelial nitric oxide (NO) synthase (eNOS)22,24 or neuronal (nNOS) activation,25,26 giving rise to NO generation and activation of soluble guanylate cyclase to produce cGMP and cGMP-dependent protein kinase G (PKG) activation25–27 (Fig. 4). PKG is a serine/threonine kinase that mediates many of the biological effects of NO/cGMP.28,29 In particular, PKG downstream of β3ARs can enhance myocytes relaxation but cause negative inotropy, possibly through the phosphorylation of troponin I and L-type Ca2+ channel26–29 (Fig. 4). Importantly, the β3AR/NO-cGMP/PKG signaling axis seems to be a robust cardioprotective mechanism that can be beneficial in failing myocardium.22,26,29 In fact, PKG activation downstream of cGMP has been proven to reduce Ca2+ oscillations which can cause ventricular arrhythmias, hypercontracture and sarcolemmal rupture as well as mitochondrial
permeability transition pore that is a cause of cell death.\textsuperscript{30–32} Of note, both these phenomena occur during reperfusion of ischemic myocardium and can lead to progression toward heart failure (HF).\textsuperscript{30–32}

Pertaining to specific species differences of the $\beta_3$AR, humans have a natural mutation. In fact, there is a nonsynonymous polymorphism at amino acid 64 where a tryptophan (W) can exist instead of arginine (Figs. 1, 2). This mutation makes the $\beta_3$AR less responsive to catecholamine stimulation,\textsuperscript{33} which has been associated with some pathophysiological conditions (ie, obesity)\textsuperscript{34,35} (Box 1). This important difference suggests that nonhuman $\beta_3$AR and its signaling may not fully mirror the human system.

FIGURE 2. Multiple alignment of mammalian $\beta_3$AR. Protein sequence alignment of mouse, dog, pig, human, and Macaca mulatta $\beta_3$AR. The TMD is highlighted in yellow; the tryptophan (W) in position 64 is in red; the cysteine (C) in position 361 is in green.
In line with the notion that β1AR activation in failing myocardium is not detrimental, studies strongly support the idea that overexpression or persistent activation of β1AR is cardioprotective and can attenuate pathological LV hypertrophy induced by continuous infusion of isoproterenol and angiotensin II, or by transaortic constriction, in mice.\textsuperscript{44,45} Importantly, as shown in these studies, the activation of NOS and subsequent NO generation represent the main mechanism responsible for β1AR-induced cardioprotection. In agreement with this mechanism of action, another study, using small (mice) and large (pigs) animal models of ischemia/reperfusion (I/R) injury, demonstrated that administration of selective β1AR agonist BRL 37344 positively affected infarct size (acutely) and LV function (chronically).\textsuperscript{46} Further, this study showed that β1AR/NO signaling decreased opening of the mitochondrial permeability transition pore, thus conferring protection to the cardiac cells against cell death.\textsuperscript{46} Finally, it is well established that the role of exercise in cardioprotection,\textsuperscript{47} and β1ARs, has been also shown to be a mediator of this effect,\textsuperscript{48} especially in a setting of cardiac I/R injury.\textsuperscript{48} Of note, during exercise, it seems that Gβδ acts as the key mediator of β1AR-induced protection that leads to the PKA/Akt/eNOS activation, thus suggesting that, in some conditions, the β1AR, similarly to the cardioprotective β1AR,\textsuperscript{49} is coupled with both Gβδ and Gαi in cardiomyocytes (Fig. 4). In this regard, this signaling pathway activation has a multiple protective role in the injured myocardium like the promotion of revascularization of the ischemic tissue. In fact, activation of Akt and eNOS, and the consequent secretion of NO, has been proven to directly stimulate endothelial cell function and promote the neoangiogenesis.\textsuperscript{50} Further, as discussed above, the eNOS-mediated generation of NO is also responsible for cGMP andPKG activation, thus directly conferring beneficial effects on the myocardium.\textsuperscript{51}

**β-Blockers and β3ARs**

As described above, β3ARs have emerged as novel potential targets for the treatment of certain cardiovascular diseases including HF. The clinical relevance of this is further supported by the successful effects obtained with β-blocker treatment in patients with HF as they can block the noxious effects of catecholamines and prevent further β1 and β2ARs downregulation.\textsuperscript{4} Importantly, as proposed by us and others, the use of β-blockers may influence the expression/activity of β3ARs.\textsuperscript{35,52,53} However, because there are some specific differences in β-blockers (β1AR selective or nonselective), the full extent of whether any molecular changes in β3ARs are significant contributors to the therapeutic mechanisms of βAR antagonism in HF still remains to be elucidated. Accordingly, answering these mechanistic questions is important and may lead to novel therapeutic advances in HF.

In 2007, it was first reported that a relationship between β-blockers and β3AR may exist.\textsuperscript{54} In particular, in an HF rat model induced by transaortic constriction, it has been shown that, although metoprolol (a selective β1-blocker) treatment did not affect the expression levels of β3AR which was increased after HF, the use of carvedilol (a nonselective β-blocker) resulted in a robust β3AR downregulation.\textsuperscript{54} Nevertheless, soon after this report, a number of studies proposed

**BOX 1. Pathologies Associated With β3AR-Trp64Arg Polymorphism**

- Obesity, susceptibility to diabetes
- Insulin resistance
- Hyperuricemia
- Polymorphism

The β3AR in Cardiac Disease

Accumulating evidence has revealed that the β3AR, present in endothelium and myocardium, may have specific beneficial effects in the cardiovascular system including cardioprotection.\textsuperscript{26} This becomes critically crucial in cardiac diseases such as HF, a syndrome characterized by decreased cardiac output, caused by deficits in contractility and/or relaxation. Importantly, after an injury such as ischemia, to preserve cardiac output, there is an increase in sympathetic activity and in catecholamine release to stimulate βAR-mediated inotropic capacity. However, chronic exposure of the heart to high levels of catecholamines can lead to further pathologic changes in the heart that can induce a progressive deterioration of cardiac function and structure. Of note, catecholamines directly stimulate βARs, and the sustained activation of these receptors correlate with left ventricular (LV) dysfunction and mortality.\textsuperscript{56} In this regard, GRK2, the principal GRK involved in βAR regulation within the cardiomyocytes, phosphorylates the receptors attenuating their increased responsiveness.\textsuperscript{4} This process, called desensitization, at early stage represents a protective mechanism, but in chronic stage can cause β1 and β2ARs dysregulation and signaling abnormalities (eg, downregulation and overdesensitization) and promote the progression of the disease.\textsuperscript{4} Importantly, as discussed above, β3ARs lack GRK recognition sites and are not subject to desensitization and downregulation, and in fact, their levels within human failing myocardium remain unchanged\textsuperscript{37} or become upregulated.\textsuperscript{38} Enhancement of β3ARs could represent either a protective mechanism against the detrimental effects of chronic βAR stimulation or a detrimental mechanism that may lead to further deterioration of HF. In this regard, the role of this receptor in the heart has been debated for years, and some reports have suggested that due to its cardiodepressant effect, sustained activation of β3ARs in HF could contribute to impaired cardiac function.\textsuperscript{39} Consistently, the antagonism of this receptor has been proposed as a potential strategy against HF development.\textsuperscript{39,40} However, by contrast with this hypothesis, it has been demonstrated that in the failing myocardium, β3ARs are able to inhibit, through activation of Na\textsuperscript{+}-K\textsuperscript{+} pump, the deleterious accumulation of Na\textsuperscript{+} in cardiac myocytes\textsuperscript{41,42} thus blocking cAMP generation,\textsuperscript{43} and consequently, reducing the activation of the cAMP-downstream oxidative pathways. Mechanistically, this effect decreases the glutathionylation of the β1 Na\textsuperscript{+}-K\textsuperscript{+} pump subunit and enhances the Na\textsuperscript{+}-K\textsuperscript{+} pump activity in presence of β3AR agonists.\textsuperscript{43}

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that β1-blockers and their subsequent beneficial effects in HF could induce enhancement of the β3-AR expression and activity. For example, Sharma et al.53 showed that metoprolol treatment was able to improve cardiac function in diabetic rats mainly through β3-AR upregulation and NO generation. Analogous, in a canine model of mitral regurgitation, we recently found that metoprolol can promote β3-AR upregulation and enhance its protective signaling (ie, nNOS/NO/cGMP).54 Moreover, correlative data have been recently shown for the potential of β3-AR agonistic activity of nebivolol, a highly selective β1-blocker.52,55 In particular, in a model of I/R injury, nebivolol administration activated cardiac

FIGURE 3. Multiple alignment of human βARs. Protein sequence alignment of human β1, β2, and β3AR. The TMD is highlighted in yellow.
FIGURE 4. Schematic representation of \(\beta_3\)AR signaling activation in cardiomyocytes. \(\beta_3\)ARs are coupled to both stimulatory G proteins (Gs) and inhibitory G proteins (Gi). Although the Gs pathway induces the generation of cAMP and cGMP which, in turn, activates the PKA and PKG, respectively, the activation of Gi signaling pathway is able to stimulate only the generation of cGMP and the activation of PKG. PKA has multiple roles in cardiomyocytes and is able to induce the phosphorylation of several key factors involved in the regulation of contractility, such as cardiac troponin I (cTnI) and phospholamban (PLN). The latter affects Ca\(^{2+}\) cycling to the contractile proteins. Furthermore, PKA can phosphorylate the L-type Ca\(^{2+}\) channel (LTCC) increasing the influx of extracellular Ca\(^{2+}\). Importantly, PKA can also activate protein kinase B (Akt) with the subsequent activation of the endothelial NO synthase (eNOS). Of note, eNOS activation increases the generation of NO which, in turn, activates the soluble guanylate cyclase to produce cGMP and PKG activation. Similar to PKA, PKG is able to phosphorylate PLN and cTnI. However, PKG can induce the inactivation of LTCC, thus reducing the extracellular Ca\(^{2+}\) influx. Moreover, cGMP can stimulate phosphodiesterase 2 (PDE2), reducing the cAMP levels and the activation of PKA. Of note, after the Gi signaling pathway activation, the \(\beta_3\)AR is able to give rise to NO through both eNOS and neuronal NOS (nNOS) located on the sarcoplasmic reticulum (SR).

\(\beta_3\)ARs leading to a significant reduction of infarct size.\(^{55}\) Similarly, Zhang et al\(^{52}\) demonstrated, in a setting of HF in mice, induced by left anterior descending artery ligation, that nebivolol was able to reduce cardiac fibrosis and apoptosis and improved cardiac function. Interestingly, this report showed also that after 4 weeks postmyocardial infarction, there was a significant reduction of cardiac \(\beta_3\)AR levels, and that nebivolol was able to restore the expression of this receptor.\(^{52}\)

Importantly, further to the direct effect in cardiomyocytes, \(\beta_3\)-blockers, such as nebivolol have also been reported to act on endothelium.\(^{56,57}\) Of note, enhancement of neangiogenesis in the failing heart is considered one of the mechanisms of protection activated by this class of drugs.\(^{58}\) In this context, nebivolol through \(\beta_3\)AR activation increases the generation of NO, a key mediator of endothelial function,\(^{50}\) enhancing endothelial proliferation and increasing vasodilation.\(^{56,57}\)

**Clinical Perspectives of \(\beta_3\)AR Receptor Targeting**

As described above, the \(\beta_3\)AR represents an emerging attractive target for pharmacological modulation in the injured heart that, for its compensatory effects, prevents the effects of excessive catecholamines stimulation on the heart.\(^{43,44}\) In fact, selective \(\beta_3\)AR agonism has been proven to confer protection in the failing heart through a specific cGMP/NO signaling pathway.\(^{56}\) A particular role for NO has been associated with the enhancement of endothelial cell proliferation,\(^{50}\) and within failing myocardium it can lead to a beneficial effect on cardiac function and remodeling.\(^{57,58}\) As described above, \(\beta_3\)ARs are expressed not only in cardiomyocytes but also in endothelial cells, thus supporting the idea that this receptor can also promote proangiogenic mechanisms.\(^{9,56,57}\) Moreover, in adipocytes, the \(\beta_3\)AR is implicated in metabolic regulation [fatty acid (FA) oxidation, lipolysis, and thermogenesis],\(^{59}\) that can also be a crucial mechanism to rescue the heart from failure. Importantly, this mechanism is particularly important in several pathological conditions that affect cardiac function, such as ischemia and pressure overload.\(^{60}\) In fact, in these conditions, the heart, in the presence of limited oxygen supply, suppresses glucose and FA oxidation with a shift in cardiac substrate metabolism from FA oxidation to glycolysis.\(^{61}\) Glycolysis without a concomitant increase in glucose oxidation results in an accumulation of different harmful catabolites such as lactate and protons that are the cause of intracellular acidosis and Na\(^+\) and Ca\(^{2+}\) overload.\(^{62}\) These effects strongly reduce the capacity of the heart to provide sufficient energy for contractile work because more energy is spent to restore ion homeostasis and lead to an increase in lipid accumulation within the cardiac cells with consequent lipotoxicity.\(^{61}\)

In this context, as recently demonstrated in adipocytes, nebivolol, through \(\beta_3\)AR activation, is able to improve adipocyte metabolism.\(^{63}\) Therefore, it is plausible that long-term \(\beta_3\)AR stimulation, through selective agonists and/or \(\beta\)-blockers (ie, nebivolol), can be used as a novel therapeutical approach also to improve metabolism within the failing myocardium.

Currently, 2 clinical trials, the *Beta 3 Agonist Treatment in Heart Failure* (Beat-HF; clinicaltrials.gov: NCT01876433), or in the prevention of HF development, the *Assessment of Efficacy of Mirabegron, a New Beta 3-adrenergic Receptor in the Prevention of Heart Failure* (Beta3_LVH; clinicaltrials.gov: NCT02599480), are trying to evaluate the effects of \(\beta_3\)AR agonism on HF progression and development.
In this context, patients will be treated with the selective β3AR agonist, mirabegron (also known as YM-178), a drug already approved in the United States, Japan, and Europe, for the treatment of overactive bladder. This drug, through the activation of β3AR, exerts a myorelaxant effect in the detrusor muscle thus improving the bladder filling. These trials have recently started, so no results are available yet. Anyway, in the meantime that these trials will give us the proof-of-concept of the beneficial role of β3ARs in human HF, the most important clinical evidence that we currently have regarding the role of this receptor remains the proved efficacy of β-blockers. The cardioprotective effect of βAR-blockade has been largely attributed, for decades, to antagonism of cardiac β1- and β2ARs and a resulting heart rate reduction. However, as discussed above in this review, there is emerging evidence that this class of drugs may also act through β1AR signaling pathway activation which is not blocked by the drugs currently used in clinical HF-therapy (Box 2). However, the main question that still remains unanswered is why not all patients respond favorably to these agents. For these reasons, further elucidation on specific mechanisms of action of these drugs is extremely interesting. In particular, it will be important to evaluate how different β-blockers can impact, in a positive or a negative manner, β3AR activity, and if this effect is a significant contributor of therapeutic mechanisms in HF.

BOX 2. β-blockers and β3AR Expression/Activity

1. Both metoprolol and nebivolol enhance β3AR expression and activity.
2. Carvedilol reduces the expression of β3AR.

CONCLUSIONS

The β3AR is a novel and intriguing receptor, with multiple functions within the cardiovascular system. In this review, we have discussed how many disparities have been generated around the signaling and the function of this receptor. However, the emerging concept in the literature is that β3AR is mostly protective for the cardiovascular system and its agonism with selective ligands or activation during β1- and β2AR blockade could represent a future therapeutic strategy to prevent development of HF. Anyway, as discussed above, the β3AR function in the heart is still poorly investigated, and for this reason, further investigations are required to clarify the causal mechanistic relationship between β3AR expression and cardiac dysfunction and protection. More importantly, since presently, only β1AR-blockers have been associated with improvement of the β3AR signaling pathway, it will be crucial to define the specific signaling pathways associated with β-blocker-dependent activation/inhibition of β3ARs because it can help in personalizing anti-HF therapy.

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