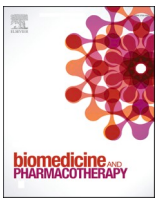
Biomedicine & Pharmacotherapy 168 (2023) 115804

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha



Metoprolol disrupts inflammatory response of human cardiomyocytes via β-arrestin2 biased agonism and NF-κB signaling modulation

Fabrizio Ricci [a,b,1], Andrea Di Credico [c,1], Giulia Gaggi [c], Giovanni Iannetti [a], Barbara Ghinassi [c], Sabina Gallina [a], Brian Olshansky [d], Angela Di Baldassarre [c,*]

[a] Department of Neuroscience, Imaging and Clinical Sciences, G.d'Annunzio University of Chieti-Pescara, 66100 Chieti, Italy and University Cardiology Division, Heart Department, SS Annunziata University Hospital, Chieti, Italy
[b] Department of Clinical Sciences, Lund University, 214 28 Malmö, Sweden
[c] Department of Medicine and Aging Sciences, and Reprogramming and Cell Differentiation Lab, Center for Advanced Studies and Technology (CAST), G. D'Annunzio University of Chieti-Pescara, 66100 Chieti, Italy
[d] University of Iowa, Iowa City, IA, USA

ARTICLE INFO

Keywords:
Metoprolol
Biased agonism
β-arrestin
Cardiomyocyte
Inflammation

ABSTRACT

Aims: Recent evidence supports non-class cardioprotective effects of metoprolol against neutrophil-mediated ischemia-reperfusion injury during exacerbated inflammation. Whether metoprolol exerts direct anti-inflammatory effect on cardiomyocytes is unknown. Accordingly, we aimed to investigate the direct anti-inflammatory effects of metoprolol in a cellular model of human induced pluripotent stem cell-derived cardiomyocytes (hiCMs) and to explore the role of β-arrestin2 (β-ARR2) biased agonism signaling pathway.

Methods and results: hiCMs were treated with TNF-α for 24 h, followed by 4-hour treatment with metoprolol or esmolol. Electrical response of hiCMs to β1-selective blockade was assessed by microelectrode arrays technology. The effect on inflammatory and adhesion molecule expression was evaluated in wild-type and β-ARR2 silenced hiCMs. To silence β-ARR2 expression, hiCMs were transfected with a specific small interfering RNA targeting β-ARR2 mRNA and preventing its translation.

TNF-α stimulation boosted the expression of IκB, NF-κB, IL1β, IL6, and VCAM1 in hiCMs. TNF-α-treated hiCMs showed similar physiological responses to metoprolol and esmolol, with no difference in field potential duration and beat period recorded. Adding metoprolol significantly decreased inflammatory response patterns in wild-type hiCMs by dampening TNF-α induced expression of NF-κB, IL1β, and IL6, but not in β-ARR2-knockout hiCMs. A similar response was not observed in presence of β1-selective blockade with esmolol.

Conclusions: Metoprolol exerts a non-class direct anti-inflammatory effect on hi-CMs. β1-selective blockade with metoprolol disrupts inflammatory responses induced by TNF-α and induces significant inhibition of NF-κB signaling cascade via β-ARR2 biased agonism. If confirmed at clinical level, metoprolol could be tested and repurposed to treat cardiac inflammatory disorders.

1. Introduction

Beta-adrenergic receptor blockers (β-blockers) are commonly prescribed drugs for patients with cardiovascular disorders, such as ischemic heart disease, heart failure, and arrhythmias [1]. Considering β-blocker trials, there is a consistently favorable relationship between the use of β-blockade post-myocardial infarction or in the setting of heart failure with reduced ejection fraction and clinical outcomes [2]. While clinical guidelines imply that all β-blockers have a consistent mechanism of action and a uniform class effect, preclinical research suggests a more

Abbreviations: β-ARR, β-arrestin; β-ARR1, β-arrestin 1; β-ARR2, β-arrestin 2; βAR, β-adrenergic receptors; β1AR, β1-adrenergic receptor; CTRL, control; DAPI, 4',6-diamidino-2-phenylindole; hiCMs, human induced pluripotent stem cell-derived cardiomyocytes; IL-1β, interleukin-1β; IL-6, interleukin-6; IKBα, NF-κB inhibitor alpha; NF-κB, nuclear factor kappa B; qPCR, quantitative real time PCR; siRNA, small interfering RNA; NF-α, tumor necrosis factor alpha; VCAM1, vascular cell adhesion molecule 1.

* Corresponding author at: Department of Medicine and Aging Sciences, "G. D'Annunzio" University of Chieti-Pescara, Via dei Vestini, 31, 66100 Chieti, Italy.
E-mail address: a.dibaldassarre@unich.it (A. Di Baldassarre).

[1] Equally contributed as first authors

https://doi.org/10.1016/j.biopha.2023.115804
Received 12 July 2023; Received in revised form 14 October 2023; Accepted 26 October 2023
Available online 31 October 2023

diverse range of mechanisms contributing to their beneficial cardiac profile. These benefits encompass not just heart rate modulation but also under-recognized neurohormonal, metabolic, and immunomodulatory effects that contribute to the overall efficacy of these drugs in treating cardiac conditions [3,4]. β-adrenergic receptors (βAR) are the primary mediators of adrenergic activation in the heart. While it is generally accepted that the signaling of different βARs leads to substantially different effects, β1AR is by far the predominant subtype in human adult cardiac myocytes, representing up to 80 % of total βAR density [5].

Recent in vitro and in vivo evidence supports the non-class, cardioprotective, and anti-inflammatory effects of metoprolol in several species, including humans, which are entirely mediated by β1AR, with no involvement of β2AR [6]. Indeed, metoprolol has been shown to reduce myocardial infarct size by stunning neutrophil migration, inhibiting neutrophil-platelet interactions during myocardial ischemia-reperfusion and halting exacerbated inflammation [7,8]. A similar disruptive in vivo effect on neutrophil dynamics is not shared by other β-blockers, such as propranolol or atenolol, which prompts questions about the clinical relevance of this mechanism. This differential effect of metoprolol compared against other drugs of the same family has been hypothesized to occur through a biased agonism signaling pathway involving β-arrestin (β-ARR) activation [9]. This pathway would be initiated upon binding of metoprolol to β1AR inducing a conformational change in the receptor's intracellular domain and exposing phosphorylation targets involved in β-ARR signaling cascade [7]. Phosphorylation enhances the affinity of the β1AR for binding to the adapter proteins β-ARRs that are abundantly expressed in the heart and include the two ubiquitously expressed isoforms β-ARR1 and β-ARR2. Notably, a few studies point toward a beneficial role played by β-ARR2 signaling in protecting cardiac cells against excessive inflammation after myocardial infarction, especially when β1AR has engaged [10]. Furthermore, by interacting with IκBα inhibitory proteins [11], β-ARRs have been shown to critically regulate signaling of NF-κB, a ubiquitously expressed transcription factor that regulates genes involved in immunity, inflammation, and cancer [12].

Beyond the cardioprotective properties mediated by targeting the hematopoietic compartment, whether metoprolol exerts a direct anti-inflammatory effect on human cardiomyocytes is unknown, especially in the setting of increased levels of pro-inflammatory cytokine TNFα. This molecule is associated with common cardiovascular conditions such as lymphocytic myocarditis [13], which lacks specific evidence-based treatments, and heart failure [14]. Accordingly, we aimed to investigate the cardioprotective effects of two selective β1-blockers approved for clinical use, metoprolol and esmolol, and to explore the role of β-ARR2 biased agonism to critically regulate the inflammatory response of the cardiomyocyte in a cellular model of human induced pluripotent stem cell-derived cardiomyocytes (hiCMs) exposed to exacerbated inflammation via TNF-α stimulation.

2. Methods

2.1. Cell culture and treatment

hiCMs (Ncardia, Charleroi, Belgium), obtained from human iPSCs [15] were seeded on fibronectin-coated plates at 9.4×10^4 cell/cm^2 in cardiomyocyte culture medium (Ncardia). After 4 days, cells were treated with 20 ng/mL TNF-α (Sigma-Aldrich) for 24 h. Then, 1 μM metoprolol tartrate (Seloken® solution for injection, Recordati Group, Italy) or 3 μM esmolol hydrochloride (Brevibloc® solution for injection, Baxter Healthcare Ltd) was added to the cell culture for 4 h. The treatment was administered using 1 μM metoprolol, which represents standard plasma concentration and is also considered safe for cardiac cell cultures. Metoprolol's therapeutic range varies between 25 and 100 mg, with one of most prescribed doses being 50 mg, resulting in maximal plasma levels of approximately 212 ng/mL, equivalent to ~1 μM. Such a concentration has been found to be non-toxic for cardiomyocytes when used within a range of 0.5–10 μM, while doses exceeding 10 μM have been shown to reduce cell viability [16]. Another study examining the pharmacokinetics and pharmacodynamics of esmolol demonstrated that an effective intravenous bolus of esmolol led to plasma concentrations of 1 μg/mL, which is equivalent to 3 μM [17].

2.2. ARRB2 silencing

hiCMs were transfected with 1 μM SMARTpool Accell siRNA anti-human ARRB2 (Dharmacon, Lafayette, CO, USA) or with 1 μM Non-targeting SMART pool siRNA (Dharmacon) as negative control for 72 h. At the end of incubation, hiCMs were treated with TNF-α followed by the administration of metoprolol or esmolol.

2.3. Immunofluorescence

Immunofluorescence was performed as previously described [18,19]. Briefly, cells were fixed in paraformaldehyde 4 % for 10 min and permeabilized with Triton 0.5 % for 15 min; after blocking with BSA 5 %, cells were stained with anti-β-ARR1 1:100 (Thermo Fisher Scientific, Waltham, Massachusetts), anti-β-ARR2 1:100 (Thermo Fisher Scientific), anti-NF-κB (p65 subunit) Alexa fluor 488 conjugated 1:50 (Abcam, Cambridge, UK), anti-cardiac Troponin T 1:100 (Abcam), anti-SERCA 1:100 (Thermo Fisher Scientific) overnight at 4 °C, followed, when required, by the incubation with the appropriate secondary antibody Alexa fluor 488 or 564 conjugated for 1 h at room temperature. Images were observed and acquired by EVOS M7000 (Thermo Fisher Scientific).

2.4. RNA extraction and reverse transcription

RNA extraction and reverse transcription were performed as previously described [20]. Briefly, the total RNA extracted using miRNEasy kit (QIAGEN, Germany) was retrotranscribed by the High-Capacity cDNA reverse transcription kit (Thermo Fisher Scientific), following manufacturer's procedure.

2.5. Quantitative real time PCR (qPCR)

qPCR analysis was performed using SYBR green (PowerUp SYBR Green Master mix, Thermo Fisher Scientific) as previously described [21]. The run method consisted of the following steps: 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min. Steps 2 and 3 were repeated for 40 cycles. The authenticity of the PCR products was verified by melt-curve analysis. Each gene expression value was normalized to 18 S. Fold changes were expressed in relation to the control condition, using the ΔΔCt method. The primers used are listed in Table 1.

2.6. Microelectrode arrays (MEA) technology

Microelectrode arrays (MEA), also known as multielectrode arrays, contain a grid of tightly spaced electrodes embedded in the culture surface of the well. For the analysis of cardiac electrical activity on MEA, hiCMs were seeded on 24 well MEA CytoView plate (M384-tMEA-24W, Axion Biosystem, Atlanta, GA, USA) precoated with 50 μg/mL of fibronectin (Sigma-Aldrich) at 20×10^4 cell/well. Cardiac electrical activity was recorded and analyzed by Maestro Edge MEA (Axion Biosystems) equipped with Axion Integrated Studio (AxIs) Navigator 3.2.3.1 software (Axion Biosystems) [22]. Spontaneous cardiac action potential propagating from cell to cell across the syncitium was recorded by MEA as an extracellular field potential. The time from the depolarization to repolarization is termed the field potential duration (FPD). FDP and beating rate were analyzed using the Cardiac Analysis tool. To show the cardiac cell activity waveform in response to each treatment, the FPD detection display was used. The analyzed endpoints for each treatment were: average FPD (i.e., the average of the time from the depolarization wave to the peak of the T-wave) and beat period (i.e. the time between

Table 1
Primer sequence for qPCR.

Gene Name	Primer sequence FW (5'→3')	Primer sequence RV (5'→3')	Reference
β-ARR1	ACATATGCCCTCCAGTGTCTTC	AGTCTGCAGGAAAGAGGTCATC	Primer Blast
β-ARR2	AGAAGTCGAGCCCTAACTGC	TGCGGTCCTTCAGGTAGTCA	Primer Blast
NF-κB	ATGTGGAGATCATTGAGCAGC	CCTGGTCCTGTGTAGCCATT	Primer Blast
IL-1β	AGCCATGGCAGAAGTACCTG	CCTGGAAGGAGCACTTCATCT	Primer Blast
IL-6	CCACCGGGAACGAAAGAGAA	GAGAAGGCAACTGGACCGAA	Primer Blast
IKBα	GAAGTGATCCGCCAGGTGAA	CTCACAGGCAAGGTGTAGGG	Primer Blast
VCAM1	TGGATAATGTTTGCAGCTTCTCA	CGTCACCTTCCCATTCAGTG	Primer Blast
18S	CATGGCCGTTCTTAGTTGGT	CGCTGAGCCAGTCAGTGTAG	[51]

β-ARR1, β-arrestin1; β-ARR2, β-arrestin2; NF-κB, nuclear factor kappa B; IL-1β, interleukin-1β; IL-6, interleukin-6; IKBα, NF-kappa-B inhibitor alpha; VCAM1, vascular cell adhesion molecule 1; 18S, 18S ribosomal RNA; FW, forward; RV, reverse

successive depolarization expressed in seconds). All data were imported in GraphPad PRISM 9.2 to perform statistical analysis.

3. Results

3.1. Metoprolol and esmolol elicited comparable physiological effects in hiCMs treated with TNF-α

We first examined the electrical response of hiCMs to pharmacological treatment. Cells were seeded on MEA plates (i.e., plates with microelectrodes embedded in the culture surface) and exposed first to TNF-α for 24 h and then to metoprolol or esmolol for 5 min (Fig. 1A); the electrical activity of untreated and treated hiCMs was monitored real-time and analyzed by measuring the average FPD and the beat period. The effects of the different treatments on the FPD waveform are reported in Fig. 1**B**. No differences in average FPD were found between control and cell treated with TNF-α alone, while, as expected, hiCMs treated with TNF-α + metoprolol and TNF-α + esmolol significantly increased the average FPD compared with control and TNF-α samples (Fig. 1C). The same pattern was observed in the analysis of the beat period, where TNF-α + metoprolol and TNF-α + esmolol treated cells demonstrated higher time intervals between successive depolarizations, indicating decreased hiCMs contraction rate (Fig. 1D).

3.2. Metoprolol inhibits the overexpression of NF-κB

To explore the molecular effects of metoprolol treatment, we exposed the hiCMs to 20 ng/mL TNF-α for 24 h; then a β1-selective blocker, metoprolol or esmolol, was added to the cell culture for 4 h.

We first analyzed NF-κB, an inducible transcription factor activated in response to TNF-α [23], and cardiac troponin T (cTnT), a sarcomeric protein that regulates CM contraction. On immunofluorescence analysis, TNF-α induced upregulation of NF-κB expression which, as expected, localized mainly at the cytoplasm level: studies of NF-κB trafficking evidenced, indeed, that its activation is associated with an early (30–60 min) nuclear translocation, followed by a return back into the cytoplasm compartment [24]. Exposure to metoprolol significantly reduced NF-κB overexpression in hiCMs. Indeed, quantification of immunofluorescence showed a nearly 50 % increase of NF-κB signal in the TNF-α sample compared with control, while subsequent treatment with metoprolol significantly weakened mean fluorescent intensity. A similar effect was not observed in presence of TNF-α + esmolol. Immunolabeling for cTnT, performed in parallel, depicted the typical sarcomeric striations [25], and no changes in cTnT expression or pattern of labeling could be observed for any sample (Fig. 2A-B, wild type).

Furthermore, to verify that TNF-α did not alter intracellular calcium homeostasis and hiCMs contractile function, we checked for the expression of the calcium pumps sarcoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a). SERCA2 expression levels were comparable in all samples, where it marked the perinuclear area and delineated the finely reticulated organization of the sarcoplasmic reticulum (Fig. 2C-D, wild type).

In the absence of exposure to TNF-α, metoprolol and esmolol did not affect the immunolabeling for NF-κB, cTnT or SERCA2 (Supplemental Fig. S1).

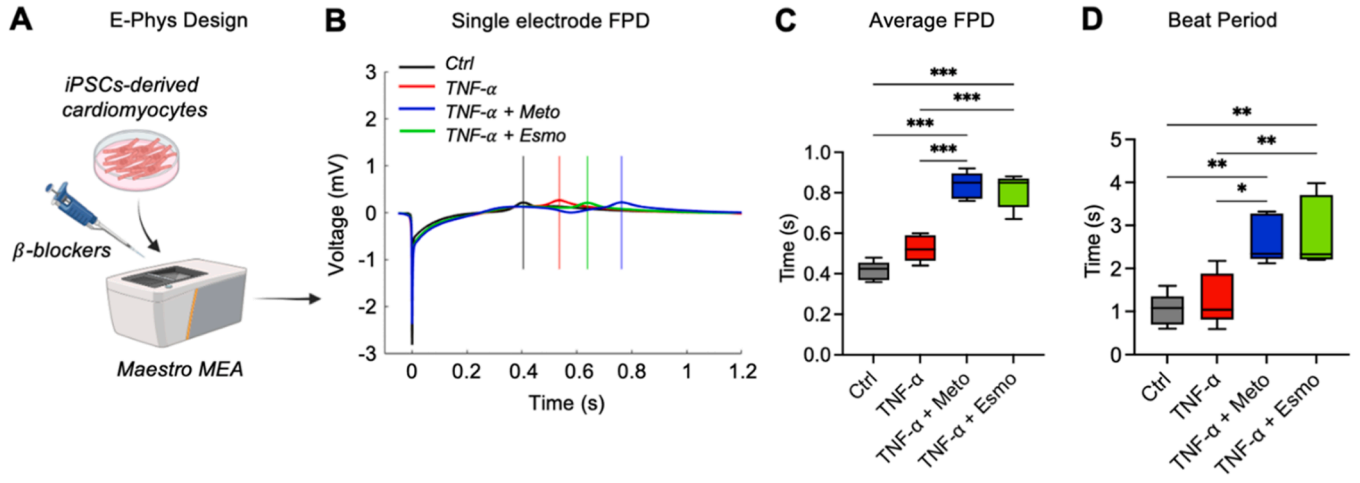


Fig. 1. Analysis of the electrical response of hiCMs to pharmacological testing. (A) Schematic illustration of the experimental design. Cells were seeded on MEA plate and treated with TNF-α 20 ng/mL for 24 h, followed by metoprolol or esmolol for 5 min. (B) Representative FPD waveform for each experimental condition; the figure highlights the effects of the different treatments on the time elapsing between cardiac cell depolarization and repolarization. (C) Average FPD and (D) the beat period expressed in seconds. Data are presented as mean ± standard deviation.

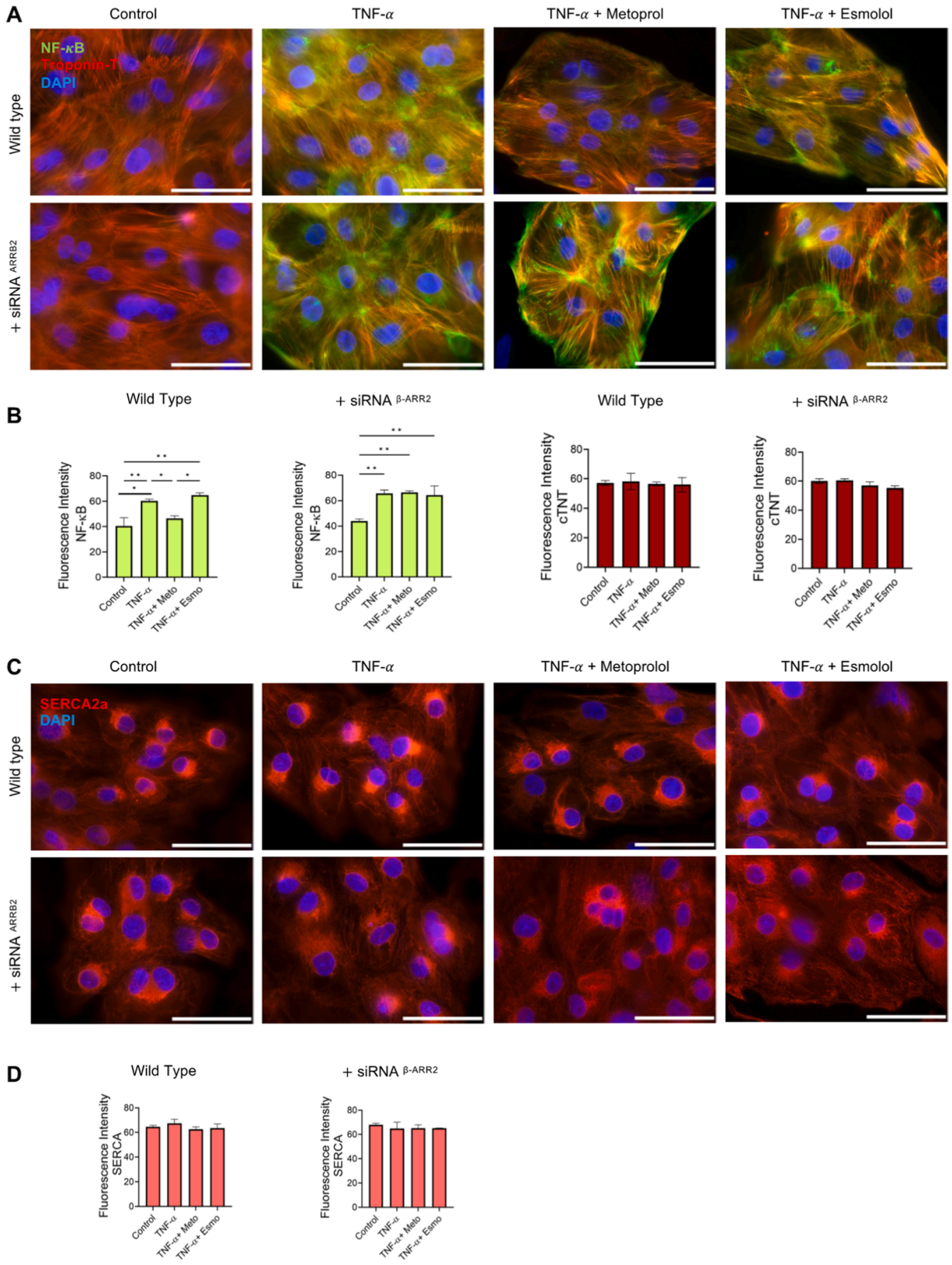


Fig. 2. Immunofluorescent analysis in wild type and in β-ARR2 silenced hiCMs treated with TNF-α and metoprolol or esmolol. (A) NF-κB (green fluorescence) and TnT (red fluorescence) expression and (B) their Fluorescent Intensity in wild-type and β-ARR2 silenced (+ siRNA $^{\beta\text{-ARR2}}$) hiCMs in the different experimental conditions, as indicated. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Pictures are representative of 5 different experiments. (C) SERCA2a (red fluorescence) expression and (D) its Fluorescent Intensity in wild-type and β-ARR2 silenced (+ siRNA$^{\beta\text{-ARR2}}$) hiCMs in the different experimental conditions, as indicated. Nuclei were counterstained with DAPI. Original Magnification 60x, scale bar 60 μm. Pictures are representative of 5 different experiments. Data are presented as mean ± SD ($n = 5$) *$p < 0.05$, **$p < 0.01$. Meto, metoprolol; Esmo, esmolol.

3.3. Metoprolol suppresses the expression of inflammatory and adhesion molecules

TNF-α exerts its pro-inflammatory effects by activating several signaling pathways, namely via IκB and NF-κB. Within unstimulated cells, NF-κB is complexed with inhibitory IκB proteins. The effect of TNF-α stimulation determines phosphorylation and degradation of IκB, with subsequent liberation of NF-κB and promotion of its nuclear translocation and transcriptional regulation of target genes such as inflammatory molecules [11]. We evaluated the response evoked by TNF-α on NF-κB transduction pathway. In particular, the gene expression of *IκB, NF-κB, IL1β, IL6,* and *VCAM1* was evaluated in TNF-α treated hiCMs, in presence or absence of metoprolol, with esmolol used again as control of the β1-selective blocker class (Fig. 3). As expected, *NF-κB, IL1β* and *IL6* expression was boosted by TNF-α stimulation; however, the addition of metoprolol to the culture media significantly decreased their

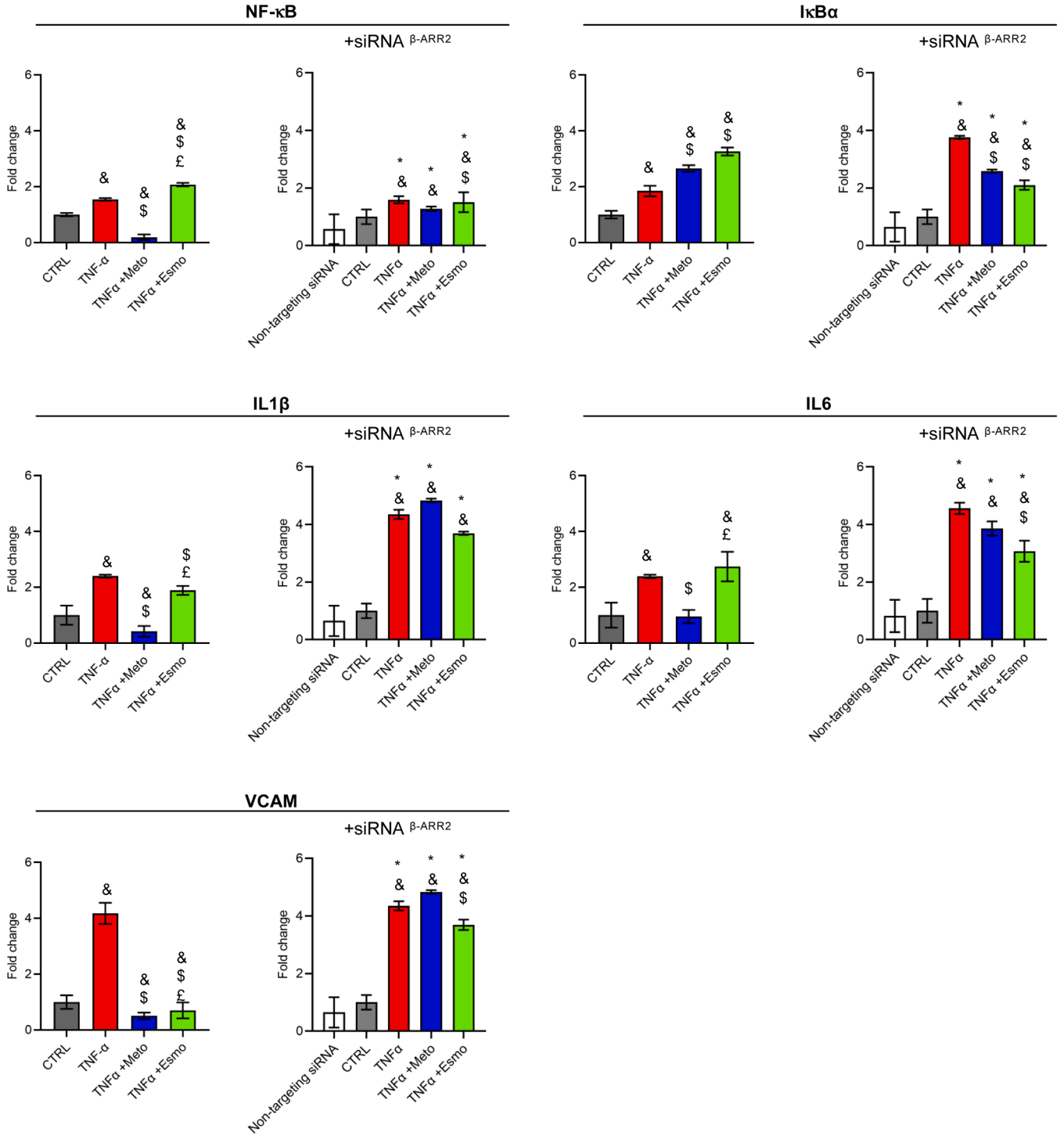


Fig. 3. Gene expression analysis in wild type and β-ARR2 silenced hiCMs treated with TNF-α and metoprolol or esmolol. Gene expression of NF-κB, IL1β, IL6 and VCAM1 in wild type and β-ARR2 silenced hiCMs was quantified by qPCR. Data are expressed as fold changes (mean ± SD) with respect to CTRL (for wild-type cells) or to non-targeting siRNA (for transfected hiCMs). 18 S was used as reference gene ($n = 3$, *$p < 0.05$ vs non-Targeting siRNA, $^{\&}p < 0.05$ vs CTRL, $^{\$}p < 0.05$ vs TNF-α, $^{£}p\ ^{<}0.05$ vs TNF-α + Metoprolol.

concentration at values comparable (*IL6*) or even lower (*NF-κB* and *IL1β*) than control. The attenuated inflammatory response observed with metoprolol was not reproduced by esmolol. This evidence supports an alternative mechanism other than general β1-blocker activity for the anti-inflammatory effect of metoprolol. A different trend was observed for IκBα transcript, whose expression, enhanced by TNF-α exposure, was further increased by β-blockade (Fig. 3).

We also tested mRNA levels of *VCAM*1, an adhesion molecule that participates in the cardiac inflammatory process, whose expression is upregulated by the activation of the TNF-α/IκB α/NF-κB signaling cascade [26]. *VCAM1* transcripts have almost tripled in presence of TNF-α, yet the induction was abolished after exposure to metoprolol; a similar response was observed in presence of esmolol. A summary of the effects evoked by metoprolol or esmolol alone on hiCMs gene expression of inflammatory and adhesion molecules is reported in Supplemental Fig. S2.

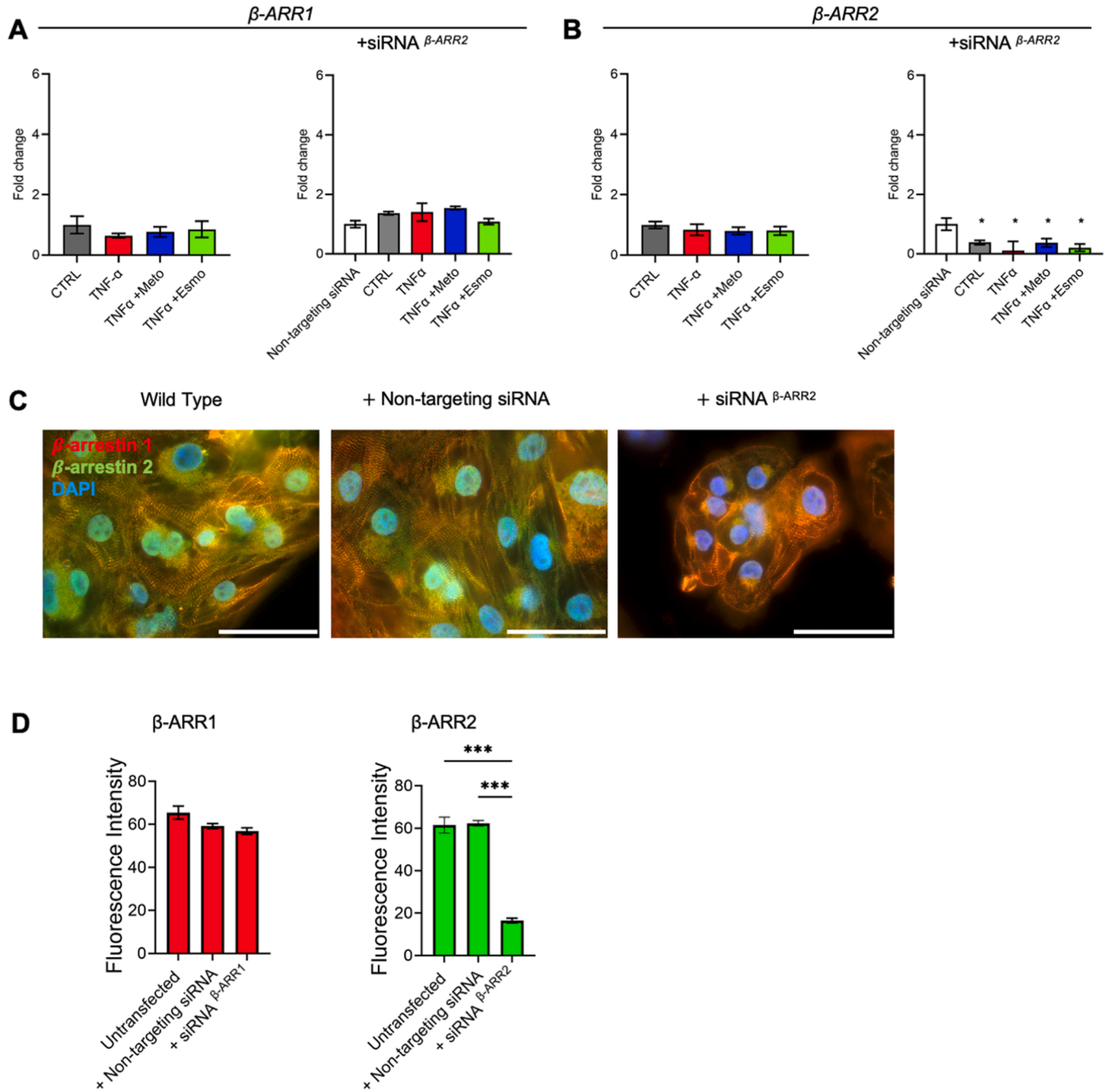


Fig. 4. Specificity and efficacy of β-ARR2 knockdown expression in hiCMs. hiCMs were transfected with β-ARR2 specific siRNA or with a non-targeting siRNA as control (CTRL). The specificity and efficacy of the β-ARR2 silencing were evaluated by qPCR and immunofluorescence. Gene expression of β-ARR1 (A) and β-ARR2 (B) in wild type and silenced hiCMs (+siRNA $^{β\text{-ARR2}}$). Data are expressed as fold changes (mean ± SD) with respect to CTRL (for wild-type cells) or to non-targeting siRNA (for transfected hiCMs). (C) β-ARR1 (red fluorescence) and β-ARR2 (green fluorescence) expression and (D) their fluorescent intensity quantification in wild type and in β-ARR2 silenced hiCMs (+siRNA $^{β\text{-ARR2}}$), as indicated. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Magnification 60x, scale bar 60 μm. Pictures are representative of 5 different experiments. Data are presented as mean ± SD ($n = 5$) ***$p < 0.001$.

3.4. β-ARR2 biased agonism mediates non-class anti-inflammatory properties of metoprolol

The binding of metoprolol to β1AR activates G-protein independent signaling via selective interaction with β-ARR2 [27]. Accordingly, all analyses were repeated on β-ARR2-knockout gene hiCMs, in order to test whether the anti-inflammatory effect of metoprolol was mediated by β-ARR2 signalling pathway.

To silence β-ARR2 expression, hiCMs were transfected with a specific siRNA targeting β-ARR2 mRNA and preventing its translation. After 72-hour transfection, the silencing specificity and efficacy were verified, and qPCR data demonstrated a significant drop of β-ARR2 transcript levels (gene silencing efficiency 80.2 ± 6.2 % relative to non-targeting siRNA), while β-ARR1 expression was unaffected (Fig. 4A-B). Expression of β-ARR2 protein significantly decreased in transfected cells, with β-ARR2 fluorescent intensity achieving nearly 25 % of untransfected control and non-targeting siRNA (Fig. 4C-D). The transfection of the non-targeting siRNA, which was performed as experimental control, did not affect β-ARR1, β-ARR2, TnT, NF-κB and SERCA immunostaining in comparison with the untransfected cells. Silenced hiCMs were then exposed to TNF-α, followed or not by β-blockade. As expected, TNF-α treatment induced a significant increase of NF-κB immunostaining in β-ARR2 silenced cells, but unlike wild-type cells, addition of metoprolol and esmolol was ineffective on NF-κB expression. No changes in TnT and SERCA staining were detected in both treated and untreated β-ARR2 silenced hiCMs (Fig. 2). Finally, we analyzed the effect of metoprolol on cardiac expression of inflammatory and adhesion molecules induced by TNF- α in the β-ARR2 gene knockout cells. The qPCR analysis evidenced that in silenced hiCMs *NF-κB, IL1β, IL6* and *VCAM1* transcripts increased by TNF-α exposure were not modulated by addition of metoprolol or esmolol to the culture media (Fig. 3), reinforcing the hypothesis that the attenuation of the hiCMs inflammatory response induced by metoprolol relies on β-ARR2 signaling pathway. On the other hand, in β-ARR2 silenced cells, *IκBα* mRNA levels significantly increased in response to TNF-α at levels higher than those measured in nontransfected hiCMs; this response was mitigated when cells were exposed to β-blockade (TNF-α + metoprolol and TNF-α + esmolol samples). The response of β-ARR2 gene knockout hiCMs to metoprolol or esmolol alone are reported in Supplemental Fig. S2.

4. Discussion

In a cellular model using hiCMs that were subjected to inflammatory conditions through stimulation via TNF-α, we observed a unique, pleiotropic anti-inflammatory effect of metoprolol. This non-class effect is mediated through β-ARR2 biased agonism, which serves as a key regulator of NF-κB activity. This specific regulation significantly influences downstream pro-inflammatory signaling mechanisms, thereby reducing the inflammatory response within the cellular model.

4.1. TNF-α and cardiac inflammation

TNF-α is an inflammatory molecule largely elevated in cytokine storm that plays a pivotal role in the progression of degenerative and inflammatory cardiac diseases. High levels of TNFα induce fibrosis, oxidative stress, and alterations in myocardial contraction and relaxation [14]. In addition to this direct negative influence on cardiac function, it may further worse clinical outcomes by establishing a dynamic crosstalk between sympathetic system and immune response [28,29]. In this study, we demonstrated that metoprolol could elicit a direct, non-class, anti-inflammatory response on hiCMs through β-ARR2 biased agonism reducing the inflammatory milieu via NF-κB modulation, potentially breaking the vicious circle between inflammation, oxidative stress, and cellular dysfunction.

4.2. Controlling for confounding effects

Prolonged exposures (>24–48 h) or high concentrations (20–100 ng/mL) of TNF-α induce adverse effects on the structural architecture of the cardiomyocyte, decreasing contractile activity, and altering response to β1AR stimulation [30,31]. Furthermore, TNF-α has been shown to affect diastolic function negatively [32] by down-regulating expression of SERCA2a, an intracellular protein responsible for sequestrating cytosolic calcium back into the sarcoplasmic reticulum during diastole, allowing for efficient uncoupling of actin-myosin and subsequent relaxation [33,34]. In order to control for possible confounding effects due to collateral functional dysregulations, we treated our hiCMS with 20 ng/mL TNF-α for 24 h [35]. This treatment condition triggers a robust inflammatory response and hiCMs production of inflammatory molecules, while preserving cellular viability and sparing sarcomeric architecture and function of calcium handling proteins[31, 36]. Accordingly, we did not observe post-treatment modifications in the expression of structural molecules (cTnT) and calcium handling proteins (SERCA2a). Notably, after 24-hour TNF-α treatment, there were no detectable changes of hiCMs electrical activity, with average FPD and beat period of treated cells comparable to control. On the other hand, acute hiCMs inflammatory reaction to TNF-α exposure was clearly demonstrated by a pronounced increase of NF-κB, IL1β, IL6, and VCAM1 transcripts. These data support TNF-α treatment was targeted to pro-inflammatory response, without altering sarcomeric structure and the calcium homeostasis. Overall, our experiments delivered a reliable model to evaluate the effect of metoprolol and esmolol on acute hi-CMs inflammation.

4.3. Metoprolol pleiotropic properties

Increasing evidence from animal and human studies suggest that *β*-blockers deliver direct anti-atherosclerotic effects. Particularly, metoprolol has been linked to a reduction in plaque thickness and decreased progression rate of carotid intima-media thickness [37]. Several potential mechanisms of metoprolol's action have been proposed, including reduced sympathetic activity, improved hemodynamics, and direct effects on the vascular endothelium. While confirming the atheroprotective effect of metoprolol, Ulleryd observed significant improvement of the inflammatory milieu with a reduction of serum inflammatory cytokines, e.g. TNF-α and CXCL1 [38]. Previous studies have also shown that metoprolol can reduce the expression of adhesion molecules in atherosclerotic lesions, inhibit the progression of atherosclerosis, and stabilize vulnerable plaques by diminishing inflammation and regulating the transition from low shear stress to physiological shear stress around the plaque [39]. Finally, metoprolol substantially reduces neutrophil-mediated inflammatory responses in both human and animal models of myocardial ischemia and reperfusion injury, resulting in an infarct-limiting effect not observed with atenolol or propranolol [6,7] . By proving that metoprolol can downregulate the synthesis of inflammatory and adhesion molecules in hiCMs under inflammatory conditions, our findings add valuable insights to the understanding of metoprolol's immunomodulatory effects and expand the current knowledge of metoprolol's therapeutic properties.

4.4. Comparative analysis: metoprolol vs esmolol

While β1AR stimulation is known to elicit an inflammatory response [40], in our experiment, the anti-inflammatory activity observed with metoprolol was not replicated with esmolol, another β1-selective blocker. The observation of the comparable electrical response of hiCMs to both metoprolol and esmolol rules out bioequivalence issues underlying the non-class anti-inflammatory effect of metoprolol, thus opening the search for alternative mechanisms to pure βAR antagonism.

4.5. Role of β-ARR2

While β-blockers inhibit the binding of agonists to βARs, some β-blockers may elicit cellular responses through G protein-independent, β-ARR-dependent biased agonism signaling pathways [5] In the human heart, β-ARR1 is the predominant subtype, followed by β-ARR2, which represents about 15–18 % of total β-ARR mass. It has been reported that metoprolol induces the interaction between β1-AR and β-ARR2, but not β-ARR1 [27]. In our experiment, we demonstrated that metoprolol may reduce the inflammatory response through an β-ARR2-dependent biased agonism pathway. Notably, β-ARR2 gene knockdown abolished the metoprolol effect on hiCM synthesis of inflammatory and adhesion molecules. Noticeably, after β-ARR2 silencing, the inflammatory response evoked by TNF-α in hiCMs was more intense, as evidenced by higher levels of NF-κB, IL1β, IL6 and VCAM1 transcripts.

4.5.1. Modulation of the IKK/I-κB/NF-κB signaling pathway

The IKK/I-κB/NF-κB signaling pathway is the most well-known mediator of TNF-α effect. In resting conditions, NF-κB is complexed with its inhibitor I-κB, but binding of TNF-α to its receptor determines the IκB degradation allowing NF-κB to act on the transcription of its target genes involving inflammatory molecules [11]. We observed that hiCMs response to TNF-α treatment increased the synthesis of NF-κB and I-κBα - the latter as compensatory response - but the addition of metoprolol downregulated NF-κB while further increasing I-κBα synthesis. This evidence suggests that the anti-inflammatory activity of metoprolol is at least partially due to its ability to upregulate the transcription of the NF-κB inhibitor I-κBα through a possible mechanism of biased agonism. It has previously reported, indeed, that after TNF-α stimulation βARR can bind and stabilize the NF-κB inhibitor I-κBα [12]. We have also observed that in TNF-α + esmolol sample I-κBα expression was higher than in TNF-α + metoprolol sample, but this induction was ineffective in reducing NF-κB and downstream inflammatory pathway. Clinical and preclinical studies on the anti-inflammatory effects of esmolol are few and limited to septic shock. Kimmoun demonstrated that esmolol could improve cardiac function by downregulating pro-inflammatory pathways, including the NF-κB signaling [41]. This apparent discrepancy with our findings can be probably explained by the use of a murine model of resuscitated septic shock, where the severe inflammatory burden is associated with critical systemic dysfunction.

4.5.2. Strengths and limitations

In this study, the use of human model of cardiomyocytes and state-of-the-art methodologies like microelectrode arrays and knock-out gene contributes to its strengths, providing rigorous and novel insights into the role of β-ARR2 in mediating metoprolol's anti-inflammatory effects. However, these findings are limited by the absence of animal models, which hinders the external validity and generalizability of the results. Additionally, the research focus on a single cell type and specific pathway does limit its broader applicability. Therefore, while the study lays foundation for better understanding of the immunomodulatory properties of metoprolol, further research involving animal models is crucial for comprehensive validation and applicability.

4.5.3. Implications and future directions

Overall, these findings support the hypothesis that β-ARR2 signaling pathway plays a pivotal role in mediating anti-inflammatory effects of metoprolol on hiCMs and confirm previous evidence of β-ARR2 mediating crosstalk between βAR and NF-κB signaling pathway. The exact role of β-ARR2 in regulating NF-κB activity is still far to be fully elucidated, but seems to be specific to the type of receptor engaged [42]. In the larger context of adrenergic receptors, stimulation of β2AR promotes β-arrestin2 stabilization of I-κBα in the cells induced by TNF-α, which leads to inhibition of NF-κB activation [43]. In several diseases, only a subset of β-blockers is therapeutically effective, with multiple signaling pathways and ligand bias explaining differences in effectiveness [44, 45]. Accordingly, it has been suggested to categorize βAR ligands based on multiple factors that comprehensively define their signaling profiles, emphasizing the importance of functional selectivity of ligand-receptor interactions triggering the activation of multiple signaling cascades and the bias of ligands to preferentially activate only certain cascades [46–50]. Further research is needed to understand better the role and relevance of biased agonism of metoprolol and other β-blockers in β-ARR pathways to modulate the inflammatory response of the cardiomyocyte. If confirmed at a clinical level, modulation of NF-κB signaling pathway with metoprolol could be tested and repurposed to treat cardiac inflammatory disorders.

5. Conclusions

Our study shows that metoprolol, a β1-selective blocker, exhibits non-class anti-inflammatory effects on hiCMs exposed to TNF-α-induced inflammation. These effects are mediated through β-ARR2 biased agonism, which plays a crucial role in regulating NF-κB activity and downstream pro-inflammatory signaling cascade. Our findings highlight the unique immunomodulatory properties of metoprolol and expand our understanding of its therapeutic potential beyond its well-known cardiovascular benefits. The role of β-ARR2 in mediating the anti-inflammatory effects of metoprolol underscores the importance of this signaling pathway in cardiac inflammation and the need for further research to elucidate the crosstalk between βARs and inflammatory pathways. The observed pleiotropic, direct anti-inflammatory effects of metoprolol on cardiomyocytes have important clinical implications. It suggests the viability of repurposing metoprolol as a potential pharmacological intervention for inflammatory cardiomyopathies. Future research should prioritize animal experiments and in vivo validations of these findings and further explore the potential of biased agonism as a therapeutic strategy for modulating the inflammatory response of the myocardium.

Funding

This work was supported by the European Union – Next Generation EU, under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 - M4C2, Investiment 1.5 - Call No. 3277 of 30.12.2021 - Italian Ministry of University, Award Number: ECS00000041, Project Title: "Innovation, digitalisation and sustainability for the diffused economy in Central Italy", Concession Degree No. 1057 of 23.06.2022 adopted by the Italian Ministry of University. CUP: D73C22000840006.

CRediT authorship contribution statement

Conception and design: FR, ADC, ADB: Analysis and interpretation of data. FR, ADB, GG, ADC, BG, SG, GI, BO: Drafting of the manuscript. FR, ADB, ADC, GG, GI: Critical manuscript revision for important intellectual content. BO, SG: All co-authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The individual data will be shared on reasonable request to the corresponding author.

Acknowledgements

The authors would like to express their sincere gratitude to Antonino

Scarinci, MD, for his invaluable clinical insights and inspiration throughout the research process.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115804.

References

[1] P. Joseph, K. Swedberg, D.P. Leong, S. Yusuf, The evolution of β-blockers in coronary artery disease and heart failure (Part 1/5), J. Am. Coll. Cardiol. 74 (2019) 672–682, https://doi.org/10.1016/j.jacc.2019.04.067.

[2] B. Olshansky, F. Ricci, A. Fedorowski, Importance of resting heart rate, S1050173822000731, Trends Cardiovasc. Med. (2022), https://doi.org/10.1016/j.tcm.2022.05.006.

[3] G.L. Ackland, S.T. Yao, A. Rudiger, A. Dyson, R. Stidwill, D. Poputnikov, M. Singer, A.V. Gourine, Cardioprotection, attenuated systemic inflammation, and survival benefit of B1-adrenoceptor blockade in severe sepsis in rats, Crit. Care Med. 38 (2010) 388–394, https://doi.org/10.1097/CCM.0b013e3181c03dfa.

[4] S. Coppola, S. Froio, D. Chiumello, β-Blockers in critically Ill patients: from physiology to clinical evidence, Crit. Care 19 (2015), 119, https://doi.org/10.1186/s13054-015-0803-2.

[5] A. Lymperopoulos, N. Cora, J. Maning, A.R. Brill, A. Sizova, Signaling and function of cardiac autonomic nervous system receptors: insights from the GPCR signalling universe, FEBS J. 288 (2021) 2645–2659, https://doi.org/10.1111/febs.15771.

[6] J. García-Prieto, R. Villena-Gutiérrez, M. Gómez, E. Bernardo, A. Pun-García, I. García-Lunar, G. Crainiciuc, R. Fernández-Jiménez, V. Sreeramkumar, R. Bourio-Martínez, et al., Neutrophil stunning by metoprolol reduces infarct size, Nat. Commun. 8 (2017), 14780, https://doi.org/10.1038/ncomms14780.

[7] A. Clemente-Moragón, M. Gómez, R. Villena-Gutiérrez, D.V. Lalama, J. García-Prieto, F. Martínez, F. Sánchez-Cabo, V. Fuster, E. Oliver, B. Ibáñez, Metoprolol exerts a non-class effect against ischaemia–reperfusion injury by abrogating exacerbated inflammation, Eur. Heart J. 41 (2020) 4425–4440, https://doi.org/10.1093/eurheartj/ehaa733.

[8] G. Heusch, P. Kleinbongard, Is metoprolol more cardioprotective than other beta-blockers? Eur. Heart J. 41 (2020) 4441–4443, https://doi.org/10.1093/eurheartj/ehaa764.

[9] M. Ippolito, J.L. Benovic, Biased agonism at β-adrenergic receptors, Cell. Signal. 80 (2021), 109905, https://doi.org/10.1016/j.cellsig.2020.109905.

[10] K. Watari, M. Nakaya, M. Nishida, K.-M. Kim, H. Kurose, β-Arrestin2 in infiltrated macrophages inhibits excessive inflammation after myocardial infarction, PLoS One 8 (2013), e68351, https://doi.org/10.1371/journal.pone.0068351.

[11] F. Christian, E. Smith, R. Carmody, The regulation of NF-κB subunits by phosphorylation, Cells 5 (2016) 12, https://doi.org/10.3390/cells5010012.

[12] D.S. Witherow, T.R. Garrison, W.E. Miller, R.J. Lefkowitz, β-Arrestin inhibits NF-κB activity by means of its interaction with the NF-κB inhibitor IκBα, Proc. Natl. Acad. Sci. 101 (2004) 8603–8607, https://doi.org/10.1073/pnas.0402851101.

[13] E. Ammirati, M. Frigerio, E.D. Adler, C. Basso, D.H. Birnie, M. Brambatti, M. G. Friedrich, K. Klingel, J. Lehtonen, J.J. Moslehi, et al., Management of acute myocarditis and chronic inflammatory cardiomyopathy: an expert consensus document, Circ. Heart Fail. 13 (2020), e007405, https://doi.org/10.1161/CIRCHEARTFAILURE.120.007405.

[14] S.M. Schumacher, S.V. Naga Prasad, Tumor necrosis factor-α in heart failure: an updated review, Curr. Cardiol. Rep. 20 (2018), 117, https://doi.org/10.1007/s11886-018-1067-7.

[15] A. Di Baldassarre, E. Cimetta, S. Bollini, G. Gaggi, B. Ghinassi, Human-induced pluripotent stem cell technology and cardiomyocyte generation: progress and clinical applications, Cells 7 (2018) 48, https://doi.org/10.3390/cells7060048.

[16] Q. Li, K. Huang, T. Ma, S. Lu, S. Tang, M. Wu, H. Yang, J. Zhong, Metoprolol protects against arginine vasopressin-induced cellular senescence in H9C2 cardiomyocytes by regulating the Sirt1/P53/P21 axis, Cardiovasc. Toxicol. 22 (2022) 99–107, https://doi.org/10.1007/s12012-021-09704-8.

[17] A.L. Sintetos, J. Hulse, E.L.C. Pritchett, Pharmacokinetics and pharmacodynamics of esmolol administered as an intravenous bolus, Clin. Pharmacol. Ther. 41 (1987) 112–117, https://doi.org/10.1038/clpt.1987.19.

[18] G. Gaggi, A. Di Credico, P. Izzicupo, F. Alviano, M. Di Mauro, A. Di Baldassarre, B. Ghinassi, Human mesenchymal stromal cells unveil an unexpected differentiation potential toward the dopaminergic neuronal lineage, Int. J. Mol. Sci. 21 (2020) 6589, https://doi.org/10.3390/ijms21186589.

[19] V. Di Giacomo, A. Matteucci, E. Stellacci, A. Battistini, A. Di Baldassarre, S. Capitani, E. Alfani, A.R. Migliaccio, L. Cocco, G. Migliaccio, Expression of signal transduction proteins during the differentiation of primary human erythroblasts, J. Cell. Physiol. 202 (2005) 831–838, https://doi.org/10.1002/jcp.20179.

[20] G. Gaggi, A. Di Credico, S. Guarnieri, M.A. Mariggiò, A. Di Baldassarre, B. Ghinassi, Human mesenchymal amniotic fluid stem cells reveal an unexpected neuronal potential differentiating into functional spinal motor neurons, Front. Cell Dev. Biol. 10 (2022), 936990, https://doi.org/10.3389/fcell.2022.936990.

[21] E. Rubino, M. Cruciani, N. Tchitchek, A. Le Tortorec, A.D. Rolland, Ö. Veli, L. Vallet, G. Gaggi, F. Michel, N. Dejucq-Rainsford, et al., Human ubiquitin-specific peptidase 18 is regulated by microRNAs via the 3'Untranslated region, a sequence duplicated in long intergenic non-coding RNA genes residing in Chr22q11.21, Front. Genet. 11 (2021), 627007, https://doi.org/10.3389/fgene.2020.627007.

[22] A. Di Credico, G. Gaggi, P. Izzicupo, L. Ferri, L. Bonanni, G. Iannetti, A. Di Baldassarre, B. Ghinassi, Real-time monitoring of levetiracetam effect on the electrophysiology of an heterogenous human iPSC-derived neuronal cell culture using microelectrode array technology, Biosensors 11 (2021) 450, https://doi.org/10.3390/bios11110450.

[23] I. Cicha, K. Urschel, TNF-α in the cardiovascular system: from physiology to therapy, Int. J. Interferon Cytokine Mediat. Res. (2015) 9, https://doi.org/10.2147/IJICMR.S64894.

[24] Z. Korwek, K. Tudelska, P. Nałęcz-Jawecki, M. Czerkies, W. Prus, J. Markiewicz, M. Kochańczyk, T. Lipniacki, Importins promote high-frequency NF-κB oscillations increasing information channel capacity, Biol. Direct 11 (2016), 61, https://doi.org/10.1186/s13062-016-0164-z.

[25] A. Di Baldassarre, M.A. D'Amico, P. Izzicupo, G. Gaggi, S. Guarnieri, M. A. Mariggiò, I. Antonucci, B. Corneo, D. Sirabella, L. Stuppia, et al., Cardiomyocytes derived from human cardiopoietic amniotic fluids, Sci. Rep. 8 (2018), 12028, https://doi.org/10.1038/s41598-018-30537-z.

[26] M.F. Troncoso, J. Ortiz-Quintero, V. Garrido-Moreno, F. Sanhueza-Olivares, A. Guerrero-Moncayo, M. Chiong, P.F. Castro, L. García, L. Gabrielli, R. Corbalán, et al., VCAM-1 as a predictor biomarker in cardiovascular disease, Biochim. Biophys. Acta BBA - Mol. Basis Dis. 1867 (2021), 166170, https://doi.org/10.1016/j.bbadis.2021.166170.

[27] M. Nakaya, S. Chikura, K. Watari, N. Mizuno, K. Mochinaga, S. Mangmool, S. Koyanagi, S. Ohdo, Y. Sato, T. Ide, et al., Induction of cardiac fibrosis by β-blocker in G protein-independent and G protein-coupled receptor kinase 5/β-arrestin2-dependent signaling pathways, J. Biol. Chem. 287 (2012) 35669–35677, https://doi.org/10.1074/jbc.M112.357871.

[28] S.D. Prabhu, Cytokine-induced modulation of cardiac function, Circ. Res. 95 (2004) 1140–1153, https://doi.org/10.1161/01.RES.0000150734.79804.92.

[29] P. Izzicupo, B. Ghinassi, M.A. D'Amico, A. Di Blasio, M. Gesi, G. Napolitano, S. Gallina, A. Di Baldassarre, Effects of ACE I/D polymorphism and aerobic training on the immune–endocrine network and cardiovascular parameters of postmenopausal women, J. Clin. Endocrinol. Metab. 98 (2013) 4187–4194, https://doi.org/10.1210/jc.2013-2305.

[30] M.K. Chung, T.S. Gulick, R.E. Rotondo, G.F. Schreiner, L.G. Lange, Mechanism of cytokine inhibition of beta-adrenergic agonist stimulation of Cyclic AMP in rat cardiac myocytes. impairment of signal transduction, Circ. Res. 67 (1990) 753–763, https://doi.org/10.1161/01.RES.67.3.753.

[31] A. Saraf, A. Rampoldi, M. Chao, D. Li, L. Armand, H. Hwang, R. Liu, R. Jha, H. Fu, J.T. Maxwell, et al., Functional and Molecular Effects of TNF-α on Human iPSC-Derived Cardiomyocytes, Stem Cell Res 52 (2021), 102218, https://doi.org/10.1016/j.scr.2021.102218.

[32] C.-T. Tsai, C.-K. Wu, J.-K. Lee, S.-N. Chang, Y.-M. Kuo, Y.-C. Wang, L.-P. Lai, F.-T. Chiang, J.-J. Hwang, J.-L. Lin, TNF- down-regulates sarcoplasmic reticulum Ca2 + ATPase expression and leads to left ventricular diastolic dysfunction through binding of NF- B to promoter response element, Cardiovasc. Res. 105 (2015) 318–329, https://doi.org/10.1093/cvr/cvv008.

[33] T. Samuel, R. Rosenberry, S. Lee, Z. Pan, Correcting calcium dysregulation in chronic heart failure using SERCA2a gene therapy, Int. J. Mol. Sci. 19 (2018) 1086, https://doi.org/10.3390/ijms19041086.

[34] M. Periasamy, S. Huke, SERCA pump level is a critical determinant of Ca2+ Homeostasis and cardiac contractility, J. Mol. Cell. Cardiol. 33 (2001) 1053–1063, https://doi.org/10.1006/jmcc.2001.1366.

[35] G. Shanmugam, M. Narasimhan, R. Sakthivel, R. Kumar R, C. Davidson, S. Palaniappan, W.W. Claycomb, J.R. Hoidal, V.M. Darley-Usmar, N. S. Rajasekaran, A biphasic effect of TNF-α in regulation of the Keap1/Nrf2 pathway in cardiomyocytes, Redox Biol. 9 (2016) 77–89, https://doi.org/10.1016/j.redox.2016.06.004.

[36] K.T. Moe, K. Khairunnisa, N.O. Yin, J. Chin-Dusting, P. Wong, M.C. Wong, Tumor necrosis factor-α-induced nuclear factor-kappaB activation in human cardiomyocytes is mediated by NADPH oxidase, J. Physiol. Biochem. 70 (2014) 769–779, https://doi.org/10.1007/s13105-014-0345-0.

[37] J. Wikstrand, G. Berglund, B. Hedblad, J. Hulthe, Antiatherosclerotic effects of β-blockers, Am. J. Cardiol. 91 (2003) 25–29, https://doi.org/10.1016/S0002-9149(03)00431-4.

[38] M.A. Ulleryd, E. Bernberg, L.J. Yang, G.M.L. Bergström, M.E. Johansson, Metoprolol reduces proinflammatory cytokines and atherosclerosis in ApoE $^{-/-}$ mice, BioMed. Res. Int. 2014 (2014) 1–7, https://doi.org/10.1155/2014/548783.

[39] C. Liang, L. Xiaonan, C. Xiaojun, L. Changjiang, X. Xinsheng, J. Guihua, H. Xiaobo, Z. Yanen, S. Runyi, L. Huixia, et al., Effect of metoprolol on vulnerable plaque in rabbits by changing shear stress around plaque and reducing inflammation, Eur. J. Pharmacol. 613 (2009) 79–85, https://doi.org/10.1016/j.ejphar.2009.03.075.

[40] P. Matarrese, S. Maccari, R. Vona, L. Gambardella, T. Stati, G. Marano, Role of β-adrenergic receptors and estrogen in cardiac repair after myocardial infarction: an overview, Int. J. Mol. Sci. 22 (2021) 8957, https://doi.org/10.3390/ijms22168957.

[41] A. Kimmoun, H. Louis, N. Al Kattani, J. Delemazure, N. Dessales, C. Wei, P. Y. Marie, K. Issa, B. Levy, B1-Adrenergic inhibition improves cardiac and vascular function in experimental septic shock, Crit. Care Med. 43 (2015) e332–e340, https://doi.org/10.1097/CCM.0000000000001078.

[42] D. Sharma, N. Parameswaran, Multifaceted role of β-arrestins in inflammation and disease, Genes Immun. 16 (2015) 499–513, https://doi.org/10.1038/gene.2015.37.

[43] H. Gao, Y. Sun, Y. Wu, B. Luan, Y. Wang, B. Qu, G. Pei, Identification of β-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-κB pathways, Mol. Cell 14 (2004) 303–317, https://doi.org/10.1016/S1097-2765(04)00216-3.

[44] V.J. Thanawala, G.S. Forkuo, W. Stallaert, P. Leff, M. Bouvier, R. Bond, Ligand bias prevents class equality among beta-blockers, Curr. Opin. Pharmacol. 16 (2014) 50–57, https://doi.org/10.1016/j.coph.2014.03.002.
[45] J.W. Wisler, S.M. DeWire, E.J. Whalen, J.D. Violin, M.T. Drake, S. Ahn, S. K. Shenoy, R.J. Lefkowitz, A unique mechanism of β-blocker action: carvedilol stimulates β-arrestin signaling, Proc. Natl. Acad. Sci. 104 (2007) 16657–16662, https://doi.org/10.1073/pnas.0707936104.
[46] T. Littmann, M. Göttle, M.T. Reinartz, S. Kälble, I.W. Wainer, T. Ozawa, R. Seifert, Recruitment of β -arrestin 1 and 2 to the β_2 -adrenoceptor: analysis of 65 ligands, J. Pharmacol. Exp. Ther. 355 (2015) 183–190, https://doi.org/10.1124/jpet.115.227959.
[47] B.A. Evans, M. Sato, M. Sarwar, D.S. Hutchinson, R.J. Summers, Ligand-directed signalling at β-adrenoceptors: ligand-directed signalling at β-adrenoceptors, Br. J. Pharmacol. 159 (2010) 1022–1038, https://doi.org/10.1111/j.1476-5381.2009.00602.x.
[48] S. Reiner, M. Ambrosio, C. Hoffmann, M.J. Lohse, Differential signaling of the endogenous agonists at the B2-adrenergic receptor, J. Biol. Chem. 285 (2010) 36188–36198, https://doi.org/10.1074/jbc.M110.175604.
[49] E.T. van der Westhuizen, B. Breton, A. Christopoulos, M. Bouvier, Quantification of ligand bias for clinically relevant β_2 -adrenergic receptor ligands: implications for drug taxonomy, Mol. Pharmacol. 85 (2014) 492–509, https://doi.org/10.1124/mol.113.088880.
[50] R. Seifert, Functional selectivity of G-protein-coupled receptors: from recombinant systems to native human cells, Biochem. Pharmacol. 86 (2013) 853–861, https://doi.org/10.1016/j.bcp.2013.07.029.
[51] G. Gaggi, A. Di Credico, S. Guarnieri, M.A. Mariggiò, P. Ballerini, A. Di Baldassarre, B. Ghinassi, Human fetal membrane-mesenchymal stromal cells generate functional spinal motor neurons in vitro, iScience 25 (2022), 105197, https://doi.org/10.1016/j.isci.2022.105197.