Heterodimerization with 5-HT_{2B}R Is Indispensable for β_2AR-mediated Cardioprotection

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***Running title*:** Heterodimer of β_2-AR and 5-HT_{2B}R Protects Heart

Subject Terms:
Cardiotoxicity
Cell Signaling/Signal Transduction
Ischemia
Myocardial Biology

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ABSTRACT

***Rationale*:** The β_2-adrenoceptor (β_2-AR), a prototypical G protein-coupled receptor (GPCR), couples to both G_s and G_i proteins. Stimulation of the β_2-AR is beneficial to humans and animals with heart failure presumably because it activates the downstream G_i-PI3K-Akt cell survival pathway. Cardiac β_2-AR signaling can be regulated by crosstalk or heterodimerization with other GPCRs, but the physiological and pathophysiological significance of this type of regulation has not been sufficiently demonstrated.

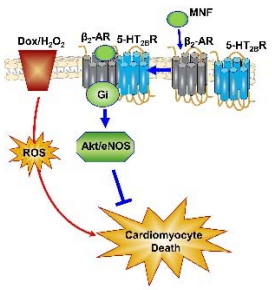
***Objective*:** Here, we aim to investigate the potential cardioprotective effect of β_2-adrenergic stimulation with a subtype-selective agonist, (*R,R'*)-4-methoxy-1-naphthylfenoterol (MNF), and to decipher the underlying mechanism with a particular emphasis on the role of heterodimerization of β_2-ARs with another GPCR, 5-hydroxytryptamine receptors 2B (5-HT_{2B}Rs).

***Methods and Results*:** Using pharmacological, genetic and biophysical protein-protein interaction approaches, we studied the cardioprotective effect of the β_2-agonist, MNF, and explored the underlying mechanism in both *in vivo* in mice and cultured rodent cardiomyocytes insulted with doxorubicin (Dox), hydrogen peroxide (H_2O_2) or ischemia/reperfusion. In Dox-treated mice, MNF reduced mortality and body weight loss, while improving cardiac function and cardiomyocyte viability. MNF also alleviated myocardial ischemia/reperfusion injury. In cultured rodent cardiomyocytes, MNF inhibited DNA damage and cell death caused by Dox, H_2O_2 or hypoxia/reoxygenation. Mechanistically, we found that MNF or another β_2-agonist zinterol markedly promoted heterodimerization of β_2-ARs with 5-HT_{2B}Rs. Upregulation of the heterodimerized 5-HT_{2B}Rs and β_2-ARs enhanced β_2-AR-stimulated G_i-Akt signaling and cardioprotection while knockdown or pharmacological inhibition of the 5-HT_{2B}R attenuated β_2-AR-stimulated G_i signaling and cardioprotection.

***Conclusions*:** These data demonstrate that the β_2-AR-stimulated cardioprotective G_i signaling depends on the heterodimerization of β_2-ARs and 5-HT_{2B}Rs.

Keywords:

β_2-adrenoceptor, 5-HT_{2B} receptor, GPCR, cardioprotection, heterodimerization, doxorubicin, ischemia/reperfusion injury, cardiac, cardiomyocyte.



Nonstandard Abbreviations and Acronyms:

5-HT	Serotonin
5-HT_{2B}R	5-HT_{2B} receptor
β_1-AR	β_1-adrenoceptor
β_2-AR	β_2-adrenoceptor
γH2AX	H2A histone family member X
Ci-GnRHR	*Ciona intestinalis* gonadotropin-releasing hormone receptor
CK	creatine kinase
Co-IP	co-immunoprecipitation
Dox	Doxorubicin
eNOS	endothelial nitric oxide synthase
FRET	Fluorescence Resonance Energy Transfer
GPCR	G protein-coupled receptor
GRAB	GPCR-activation-based
GRAB-Epi	GPCR-activation-based-epinephrine
Iso	Isoproterenol
LDH	lactate dehydrogenase
MNF	(*R,R'*)-4-methoxy-1-naphthylfenoterol
PI	propidium iodide

PTX	pertussis toxin
ROS	reactive oxygen species
siRNA	short interfering RNA

INTRODUCTION

G protein-coupled receptors (GPCRs) or seven transmembrane receptors bind to ligands and thereby transduce signals via coupling to cognate G proteins. The traditional model of GPCR signaling is based on activation of a GPCR in its monomeric state [1]. However, increasing evidence suggests that GPCRs can exist as dimers or oligomers, and that heterodimerization or heterooligomerization of GPCRs could produce receptor phenotypes with distinct signaling properties [2, 3]. Thus, a deeper understanding of the signaling of these GPCR complexes can provide insights into the role of GPCR heterodimerization in physiology and diseases.

β_2-Adrenoceptor (β_2-AR) is a prototypical GPCR capable of coupling to both G_s and G_i proteins [4-7]. In cardiomyocytes, stimulation of the β_2-AR-G_s-adenylyl cyclase-cAMP-PKA signaling cascade produces positive inotropism, whereas activation of the β_2-AR-G_i pathway, in addition to counteracting the G_s-mediated positive inotropic effect, turns on a strong cell survival signal mediated by the $G_{\beta\gamma}$-PI3K-Akt signaling pathway [8, 9]. Importantly, the β_2-agonists clenbuterol and fenoterol have been shown to produce benefits on both humans and animals with heart failure [10, 11].

Heart failure is an end-stage condition of a damaged heart caused by insulting stimuli including diseases, toxic substances and physical injuries. Hormones such as norepinephrine and angiotensin II are elevated in heart failure due to neuroendocrine adjustment to compensate for reduced circulation. These hormones in turn stimulate their corresponding GPCRs in cardiomyocytes and paradoxically cause hypertrophy and cell death [12]. For instance, prolonged catecholamine stimulation triggers cardiomyocyte apoptosis and pathological cardiac remodeling via activation of the cardiotoxic β_1-AR-Ca^{2+}/calmodulin kinase II signaling independent of PKA activation [13-15].

Opposing to cardiotoxic stimulation of the β_1-AR, stimulation of the β_2-AR is cardioprotective [9, 10]. The important role of the β_2-AR in the heart has been demonstrated in studies involving animals lacking functional β_2-ARs or human subjects carrying loss-of-function β_2-AR genotypes. In mice, deficiency of the β_2-AR results in increased mortality and cardiomyocyte apoptosis in response to catecholamine stimulation [16], or doxorubicin (Dox) treatment [17]. In humans, β_2-AR exhibits three common missense genetic variabilities: Arg16Gly, Gln27Glu and Thr164Ile with mean allele frequencies in the minor alleles of 0.476, 0.204 and 0.004, respectively [18]. Heart failure patients harboring a single Ile164 allele display a higher risk of death or heart transplantation as compared with patients homozygous for the wild-type Thr164 genotype

[19], and this correlates with blunted cardiac adrenergic response in these patients [20]. The importance of the β_2-AR-G_i signaling in cardioprotection has been further evidenced by our recent study in heart failure patients harboring the G_i-signaling-defective β_2-AR-Gly16 allele, displaying increased risks of the composite endpoint relative to patients homozygous for the major allele β_2-AR-Arg16 in an allele-dose-dependent manner [21]. Collectively, these findings suggest that the β_2-AR and particularly the β_2-AR-G_i pathway constitute an important native myocardial protection mechanism.

Increasing evidence indicates that β_2-ARs may exist as monomers, homodimers or heterodimers [22-26]. In particular, heterodimerization of β_2-ARs with other GPCRs, including β_1-ARs and β_3-ARs, occurs as suggested by fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer and co-immunoprecipitation (Co-IP) studies [22-26]. Heterodimerization can alter the pharmacological properties of a GPCR, such as ligand-binding affinity, receptor internalization, cAMP accumulation and ERK1/2 phosphorylation [1, 2, 22, 23, 25, 26]. However, the physiological and pathophysiological implications of this mode of signaling regulation are largely unexplored given the complexity of GPCR expression profiles in different tissues and cell types.

In the heart, the β-AR signaling crosstalks with various neurohormonal regulatory systems. For instance, peripheral serotonergic stimulation is closely linked with the β-adrenergic system in regulating cardiac morphology and function. Previous studies have shown that serotonin (5-hydroxytryptamine, 5-HT) enhances catecholamine-mediated β-AR signaling in cardiac cells [27]. The 5-HT receptor 2B (5-HT_{2B}R) is the most abundant 5-HT receptor subtypes expressed in adult human heart (Supplementary Figure I). Similar to β_2-adrenergic stimulation, activation of the 5-HT_{2B}R enhances survival of cardiomyocytes via activation of the PI3K-Akt cell survival pathway [28]. In contrast, deficiency of the 5-HT_{2B}R blunts multiple actions of β-adrenergic stimulation with isoproterenol (Iso) on cardiac physiology and pathology [29, 30]. Furthermore, the 5-HT_{2B}R is adaptively upregulated and contributes to the functional compensation of the heart in spontaneously hypertensive rats [31]. In addition, transgenic overexpression of the 5-HT_{2B}R in the heart also leads to compensated cardiac hypertrophy in mice [32], while deficiency of the receptor leads to dilated cardiomyopathy with reduced number and size of cardiomyocytes [33]. These previous studies have revealed a cardiac beneficial consequence of enhanced 5-HT_{2B}R signaling. Nevertheless, it is still controversial as to the exact function of the 5-HT_{2B}R in the heart [34, 35]. Regarding signaling of the 5-HT_{2B}R, it has been classified as a G_q protein-coupled receptor [36]. It is noteworthy that heterodimerization of the 5-HT_{2B}R with the angiotensin AT_1 receptor has been implicated in adrenergic stimulation-induced cardiac hypertrophy [30]. But it remains unknown whether the crosstalk between the 5-HT_{2B}R and β-AR signaling pathway is attributed to heterodimerization of the two sub-families of GPCRs.

In the present study, we seek to determine whether β_2-adrenergic stimulation by a highly selective agonist, (*R,R'*)-4-methoxy-1-naphthylfenoterol (MNF) [37], alleviates Dox- or oxidative stress-induced cardiotoxicity and, if so, to investigate whether stimulation of the β_2-AR *per se* is sufficient to induce cardioprotection. Here we show that while MNF, profoundly protects the heart against Dox-, hydrogen peroxide (H_2O_2)- or ischemia/reperfusion-induced cardiac damage, stimulation of the β_2-AR *per se* is insufficient to elicit the protective effect and, to our surprise, that heterodimerization with the 5-HT_{2B}R is

indispensable for β_2-AR-mediated cardioprotection.

METHODS

Detailed experimental procedures, animals, materials and statistical analysis are described in methods in Online Data Supplement, and all are available within the article and its Online Data Supplement files. Please see the Major Resources Table in the Supplemental Materials.

RESULTS

MNF alleviates Dox-induced mortality, cardiac dysfunction, and myocardial ischemia/reperfusion injury.

As illustrated in the schematic diagram, mice were subjected to Dox treatment (20 $mg \cdot kg^{-1}$, *i.p.*) and followed up for 23 days (Figure 1A). Dox treatment of mice caused a high mortality which peaked on the 7^{th} day (Figure 1B). On day 23, the survival rate of the Dox-treated mice was 20%, while that of the control mice was 100%. While MNF in drinking water (in $mg \cdot kg^{-1} \cdot d^{-1}$; 0.075 (low MNF) or 0.75 (high MNF)) had little effect on mouse basal survival (Supplementary Figure IIA), it overtly attenuated Dox-induced mortality in a dose-dependent manner (Figure 1B). Similarly, MNF treatment potently protected mice against Dox-elicited reduction in body weight, without altering their basal growth rate as compared with the control group (Figure 1C).

Next, we examined cardiac function and tissue damage in mice treated with Dox for 7 days. Pretreatment of mice with MNF or control vehicle was conducted three days prior to Dox, then on day 7 of MNF treatment echocardiography and sample collection were performed (Figure 1D). Dox impaired cardiac function as indicated by substantial dilation of the left ventricles and marked cardiac dysfunction indexed by reduction of ejection fraction (38.1% *vs.* 64.4% in the control) and fractional shortening (17.9% *vs.* 36.0% in the control) (Figure 1E). Co-administration with MNF significantly ameliorated these functional impairments (Figure 1E). The beneficial effect of MNF was also manifested by the prevention of body weight loss on day 7 (Figure 1F), as in Figure 1C. Furthermore, MNF markedly suppressed Dox-induced cardiomyocyte apoptosis, as evidenced by reduced TUNEL-positive cells (Figure 1G). In addition, MNF reduced serum lactate dehydrogenase (LDH) elevation caused by Dox (Figure 1H), indicating that MNF protects the heart against Dox-induced cardiac cell injury and cell death.

We also studied the effect of MNF on myocardial ischemia/reperfusion injury in mice. MNF or control vehicle was given via drinking water for 3 days before induction of myocardial ischemia and reperfusion. The animals were allowed to recover and were sacrificed after 24 h for sample collection (Figure 1I). We found that treatment with MNF reduced infarct sizes (Figure 1J), serum LDH (Figure 1K) and creatine kinase (CK) activities (Figure 1L) and TUNEL-positive cells (Figure 1M), indicating a reduction in myocardial injury and cell death. Collectively, these data suggest that treatment with MNF protects mice against Dox-induced cardiotoxicity and death, as well as myocardial ischemia/reperfusion injury.

MNF attenuates H_2O_2-, Dox- or hypoxia/reoxygenation-induced cardiomyocyte death.

Previous studies have shown that H_2O_2 is one of the reactive oxygen species (ROS) involved in Dox-induced oxidative stress [38]. H_2O_2 produces oxidative damage to membranes and other cellular structures and ultimately leads to cell death. We found that MNF dose-dependently attenuated H_2O_2-induced cell death, as illustrated by LDH release and the number of propidium iodide (PI) staining-positive cells, in cultured adult mouse cardiomyocytes (Figure 2A). Similarly, in cultured adult rat ventricular myocytes, MNF at 0.1 μM fully abolished H_2O_2-induced cardiomyocyte necrosis indexed by PI-positive staining (Figure 2B) and LDH release, as well as apoptosis evidenced by activation of caspase 3/7 (Figure 2C).

Various mechanisms participate in Dox-induced cardiomyocyte death, including oxidative stress and DNA damage. Dox is cytotoxic presumably because it intercalates between stacked DNA base pairs to inhibit topoisomerase II and subsequently induces DNA double-strand breaks [39, 40]. Cell death (apoptosis and necrosis) could occur when repair mechanisms fail. We next determined whether the beneficial effect of MNF is mediated by reducing DNA damage. Dox increased the level of H2A histone family member X (γH2AX), a marker for DNA double-strand break [41], by 76.8%, which was fully abolished by co-treatment with MNF in mouse myocardium (Figure 2D). We found that MNF not only ameliorated Dox-induced ROS in cardiomyocytes (Supplementary Figure III) but also suppressed apoptosis (evidenced by caspase 3/7 activity) and necrosis (indexed by LDH release) in cultured cardiomyocytes subjected to Dox (Figure 2E) or hypoxia/reoxygenation treatment (Figure 2F). Taken together, these results indicate that MNF attenuates Dox- or oxidative stress-induced cardiomyocyte death.

Post-treatment with MNF attenuates H_2O_2- or Dox-induced cardiomyopathy and cardiomyocyte death in vitro and in vivo.

To investigate whether the cardiac deleterious effects of Dox or H_2O_2 could be reversed by post-treatment with a β_2-agonist, MNF was administered after cardiomyocytes or mice were treated with Dox or H_2O_2. Post-treatment with MNF significantly attenuated Dox- or H_2O_2-induced LDH release and caspase 3/7 activity in cultured rat cardiomyocytes (Figures 3A and 3B). In the *in vivo* study, mice induced to develop cardiomyopathy (by 20 $mg \cdot kg^{-1}$ of Dox, *i.p.*) were given access to drinking water with or without MNF (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$) one day after the pretreatment of Dox followed by cardiac function and tissue damage characterization (Figure 3C). We found that MNF partially restored cardiac function (Figure 3D) and reduced myocardial injury and apoptosis (Figures 3E and 3F) in Dox-treated mice. These results suggest that post-treatment with MNF also produces a cardioprotective effect.

Stimulation of β_2-AR by agonists promotes heterodimerization of β_2-AR and 5-HT_{2B}R.

Because activation of either the β_2-AR or the 5-HT_{2B}R promotes cardiomyocyte survival via the PI3K-Akt pathway [28] and because the cardiac 5-HT system interacts with the β-AR signaling, we hypothesized that the β_2-AR and the 5-HT_{2B}R may have functional crosstalk in the heart particularly in cell survival and

cell death regulation. To determine whether β_2-ARs and 5-HT_{2B}Rs physically interact with one another, we conducted a FRET assay. Indeed, co-expression of HA-β_2-AR and FLAG-5-HT_{2B}R in HEK293 cells led to significant FRET signals (Figure 4A). Importantly, the presence of MNF drastically increased the FRET signals, suggesting an enhancement of the interaction of the two receptors (Figure 4A). In addition, Co-IP assays revealed that the two receptors had physical interaction which was profoundly potentiated by MNF (Figure 4B) or another β_2-agonist zinterol (Figure 4C). These results provided multiple lines of evidence that β_2-agonists enhance heterodimerization of β_2-ARs and 5-HT_{2B}Rs.

MNF selectively activates β_2-AR but not 5-HT_{2B}R.

The selectivity of MNF to the β_2-AR was demonstrated in the present study using the genetically encoded GPCR-activation-based-epinephrine (GRAB-Epi) sensor [42]. In response to MNF, GRAB-Epi sensors exhibited large fluorescence increases, suggesting stabilization of the β_2-AR backbone into its active conformation (Figure 4D). Relative to the non-selective β-AR agonist Iso, MNF acted as a partial agonist with an EC_{50} value of 50 nmol/L that was comparable to the value of Iso (32 nmol/L). In contrast, MNF showed little activity for the GRAB-5-HT_{2B}R sensor as compared to 5-HT (Figure 4D). Functionally, induction of the $[Ca^{2+}]_i$ flux response – an index for activation of the G_q-phospholipase C signaling pathway – by MNF was not detected up to a concentration of 10 □mol/L in a stable CHO-K1 cell-line overexpressing 5-HT_{2B}R, while 5-HT induced a robust increase with an EC_{50} value of 2.3 nmol/L (Figure 4E). Furthermore, MNF was much less potent (IC_{50} = 9.7 μmol/L) than the 5-HT_{2B}R antagonist SB-206553 (IC_{50} = 44.5 nmol/L) in inhibiting the $[Ca^{2+}]_i$ flux response induced by 5-HT (Figure 4E). These results define MNF as an agonist of the β_2-AR rather than an agonist for both of the heterodimerized receptors.

β_2-Agonist promotes heterodimerization of β_2-ARs and 5-HT_{2B}Rs under oxidative stress conditions.

Heterodimerization of the receptors was also examined under stress conditions by Co-IP. In HEK293 cells overexpressing both β_2-ARs and 5-HT_{2B}Rs, treatment with H_2O_2 enhanced heterodimerization, which could be further increased by MNF (Figure 4F). Similarly, hypoxia/reoxygenation (under the same experimental conditions as in Figure 2F) increased heterodimerization of β_2-ARs and 5-HT_{2B}Rs in rat cardiomyocytes, and the presence of MNF further enhanced the heterodimerization (Figure 4G). Therefore, it is evidenced that β_2-adrenergic stimulation promoted heterodimerization of 5-HT_{2B}Rs and β_2-ARs under oxidative stress conditions in cardiomyocytes.

β_2-AR is necessary for MNF-induced cardioprotection.

To determine whether the cardioprotective effect of MNF depends on the β_2-AR, we examined the effects of MNF on wild-type (WT) and β_2-AR KO mice subjected to Dox treatment. MNF was able to decrease Dox-induced mortality in WT mice but this effect was not statistically significant in β_2-AR KO mice (Figure 5A, Supplementary Figure IIB). MNF also failed to restored Dox-induced reductions in ejection fraction and fractional shortening in β_2-AR KO mice (Figure 5B). Furthermore, Dox-induced increase in serum LDH levels was not counteracted by MNF in β_2-AR KO mice (Figure 5C). Lastly, the

protective effect of MNF on β_2-AR KO mice against Dox-induced myocardial apoptosis was not statistically significant, as assayed by TUNEL staining (Figure 5D and 5E). In adult rat cardiomyocytes, pretreatment with propranolol, a nonselective β-AR antagonist, abolished the effects of MNF in attenuating H_2O_2-induced LDH leakage and cell death (Figure 5F). These data show that the cardioprotective effect of MNF depends on the β_2-AR.

5-HT_{2B}R is required for β_2-agonist-induced cardioprotection.

We investigated the potential involvement of the 5-HT_{2B}R in the cardioprotective effect of β_2-agonists. Since homozygous knockout of the 5-HT_{2B}R causes cardiomyopathy in the developing hearts and is largely lethal to mouse pups [33, 36], normally developed heterozygous 5-HT_{2B}R knockout (5-$HT_{2B}R^{+/-}$) mice were used to investigate the involvement of the 5-HT_{2B}R in MNF-mediated cardioprotection (Supplementary Figure IVA). Importantly, MNF treatment did not significantly improve animal survival, cardiac function (indexed by ejection fraction, fractional shortening) and myocyte death (assessed by serum LDH level) in Dox-treated 5-$HT_{2B}R^{+/-}$ mice (Figures 6A to 6C, Supplementary Figure IIC). Similarly, MNF protected adult WT mouse cardiomyocytes against H_2O_2-elicited injury, as manifested by the reductions in LDH release and PI-positive cells, but had no significant effect on cardiomyocytes isolated from 5-$HT_{2B}R^{+/-}$ mice (Figure 6D). Furthermore, in adult rat cardiomyocytes, inhibition of the 5-HT_{2B}R with SB-206553 (1 μmol/L) largely blocked MNF-induced protective effects on cell viability and LDH release (Figure 6E). This was validated by the fact that siRNA-mediated knockdown of 5-HT_{2B}R in rat cardiomyocytes also attenuated MNF-induced protection against H_2O_2 (Figure 6F, Supplementary Figure IVB). The same was true for the cardioprotective effect of zinterol. Knockdown of 5-HT_{2B}R attenuated the protective effect of zinterol in rat cardiomyocytes insulted by H_2O_2 (Figure 6G). These data indicate that the cardioprotective effect of β_2-agonists requires the presence of the 5-HT_{2B}R.

β_2-AR-stimulated cardioprotective G_i-Akt signaling is 5-HT_{2B}R-dependent.

We and others have previously shown that β_2-AR-stimulated cardioprotection is mediated by the receptor coupling to G_i proteins [8, 9, 43, 44]. We have also shown that MNF, like most β_2-agonists, activates both the β_2-AR-coupled G_s and G_i signaling pathways [45]. Here, we determined whether the β_2-AR-G_i signaling plays a role in MNF-elicited cardioprotection by treating rat cardiomyocytes with pertussis toxin (PTX), a disruptor of G_i signaling. As a positive control, zinterol-induced protective effects on cardiomyocytes, as evidenced by the reduction of H_2O_2-induced LDH release and cell death, were blocked by PTX treatment (Figure 7A). Similarly, the ability of MNF to effectively attenuate H_2O_2-induced LDH leakage and cell death was largely abolished in the presence of PTX (Figure 7A).

It has been shown that the serine/threonine kinase Akt is activated in response to stimulation of the β_2-AR-coupled G_i signaling [8, 9], and that β_2-AR-mediated endothelial nitric oxide synthase (eNOS) activation is Akt-dependent [46]. Both Akt and eNOS are important signaling molecules downstream of the β_2-AR-G_i cardioprotective pathway [5, 46-48]. Here, we found that treatment of rat cardiomyocytes with MNF significantly increased the phosphorylation of Akt at Ser473 and eNOS at Ser1177, a response that was

fully blocked by pretreatment with ICI-118551, a selective β_2-AR antagonist, or PTX (Figure 7B). Similarly, MNF increased Akt and eNOS phosphorylation in cardiomyocytes isolated from WT mice, but it had no statistically significant effect on cardiomyocytes isolated from β_2-AR KO mice (Figure 7C), suggesting that, in the absence of the β_2-AR, a β_2-agonist cannot activate a G_i signaling via the 5-HT_{2B}R. Notably, inhibition of the 5-HT_{2B}R with SB-206553, a selective 5-HT_{2B}R antagonist, also abolished MNF-induced increase in Akt and eNOS phosphorylation (Figure 7D), indicating that the 5-HT_{2B}R is required for the β_2-AR-stimulated G_i signaling. This conclusion was substantiated by the fact that siRNA-mediated knockdown of 5-HT_{2B}R also effectively attenuated the protective effects of MNF on Dox-induced apoptosis and necrosis, similar to PTX treatment (Figure 7E). Meanwhile, knockdown of 5-HT_{2B}R impaired MNF-elicited cardioprotection with the loss of its G_i-sensitive component (Figure 7E). In line with it cardiodeleterious effects, Dox itself reduced Akt and eNOS phosphorylation in mouse hearts, and MNF substantially restored it (Figure 7F). This effect is reminiscence of the cardioprotective effect of MNF *in vivo* (Figure 1). Together, these results indicate that MNF induces a 5-HT_{2B}R-dependent β_2-AR-G_i-Akt-eNOS signaling to protect cardiomyocytes from Dox- or oxidative stress-induced injury.

Heterodimerization of 5-HT_{2B}Rs and β_2-ARs enhances β_2-AR-stimulated G_i coupling and cardioprotection.

Next, we sought to determine whether heterodimerization of β_2-ARs and 5-HT_{2B}Rs promotes G_i protein coupling, and if so, the consequence of the heterodimerization, or lack thereof, on β_2-agonist-induced cardioprotection. First, Co-IP was performed on cardiomyocytes overexpressing Myc-5-HT_{2B}R and one of the FLAG-β_2-AR variants – WT or Gly16 – to detect the presence of the 5-HT_{2B}R/β_2-AR heterodimers. We have previously shown that the β_2-AR-Gly16 variant is G_i-signaling-defective [21]. In the absence of MNF, the extents of heterodimerization were the same for the Myc-5-HT_{2B}R/FLAG-β_2-AR-WT pair and the Myc-5-HT_{2B}R/FLAG-β_2-AR-Gly16 pair (Figure 8A). Interestingly, heterodimerization of FLAG-β_2-AR-WT and Myc-5-HT_{2B}R, but not that of FLAG-β_2-AR-Gly16 and Myc-5-HT_{2B}R, was substantially enhanced by MNF (Figure 8A), underscoring that the β_2-AR-stimulated G_i coupling is required for ligand-enhanced heterodimerization between the 5-HT_{2B}R and the β_2-AR.

To delineate the molecular basis of the β_2-AR-stimulated G_i coupling, we conducted a Co-IP study to detect physical interactions among β_2-ARs, 5-HT_{2B}Rs and $G_{i\alpha3}$ in cardiomyocytes. We detected receptor-$G_{i\alpha3}$ interactions in cardiomyocytes overexpressing Myc-5-HT_{2B}R and/or FLAG-β_2-AR (Figure 8B). Importantly, MNF was able to enhance the Co-IP signals in cells overexpressing both Myc-5-HT_{2B}R and FLAG-β_2-AR-WT (Figure 8B, left panel). The Co-IP signals were more intense in cells overexpressing both receptors as compared to cells overexpressing just one of the receptors. Notably, overexpression of the G_i-signaling-defective FLAG-β_2-AR-Gly16 variant blocked the effect of MNF (Figure 8B, right panel). These results not only indicate that the 5-HT_{2B}R, the β_2-AR and the G_i protein can form a complex upon β_2-adrenergic stimulation, but also suggest that the β_2-AR-stimulated G_i coupling is enhanced by the co-presence of the β_2-AR and the 5-HT_{2B}R.

In the functional study, MNF induced more protection against Dox in cardiomyocytes overexpressing both Myc-5-HT_{2B}R and FLAG-β_2-AR-WT as compared to the cells expressing native 5-HT_{2B}R and β_2-AR

and overexpressed β-galactosidase (Figure 8C). Most importantly, overexpressing FLAG-β_2-AR-Gly16, even with Myc-5-HT_{2B}R, abolished MNF-induced cardioprotection, although individual overexpression of Myc-5-HT_{2B}R alone showed a trend towards increased cardioprotection. Together, the data of Figure 8A and Figure 8C indicate that MNF-enhanced heterodimerization of β_2-ARs and 5-HT_{2B}Rs potentiates β_2-AR-stimulated cardioprotection.

Finally, overexpression of both Myc-5-HT_{2B}R and FLAG-β_2-AR also restored β_2-AR-stimulated Akt and eNOS phosphorylation in cardiomyocytes treated with Dox (Figure 8D), suggesting that the 5-HT_{2B}R/β_2-AR heterodimer is sufficient to mediate the β_2-AR-stimulated G_i signaling. The findings together highlight that the 5-HT_{2B}R/β_2-AR heterodimer is the receptor species transducing the β_2-AR-stimulated cardioprotective G_i signaling (Figure 8E).

DISCUSSION

The cardioprotective effect of β_2-adrenergic stimulation has been demonstrated in numerous studies involving various insults such as hypoxia, ischemia/reperfusion, H_2O_2, and chemotherapeutic agents [8-10, 16, 17, 43]. The major finding of the current study is that a β_2-agonist promotes 5-HT_{2B}Rs and β_2-ARs to form heterodimers and subsequently induces a 5-HT_{2B}R-dependent cardioprotective β_2-AR-G_i signaling. Specifically, we provide multiple lines of evidence to demonstrate that the selective β_2-agonist MNF protected cardiomyocytes against Dox-, H_2O_2 or ischemia/reperfusion-induced damages both *in vivo* and *in vitro* (Figures 1 to 3). FRET and Co-IP assays reveal that β_2-ARs and 5-HT_{2B}Rs formed heterodimers; and β_2-agonists, such as MNF and zinterol, enhanced their interaction (Figure 4). In adult rat ventricular myocytes, MNF also increased the phosphorylation of Akt, an important cell survival mediator downstream of the β_2-AR-G_i signaling pathway. Importantly, the effects of MNF on G_i-Akt-eNOS signaling and cardioprotection were not only blocked by PTX but also by knockdown or pharmacological inhibition of the 5-HT_{2B}R (Figures 6 and 7). In genetic knockout or knockdown models, the cardioprotective effect of MNF or zinterol was lost (Figures 5 and 6), suggesting that both the β_2-AR and the 5-HT_{2B}R are necessary but insufficient alone to mediated a β_2-agonist-induced cardioprotective effect. Sufficiency for the transduction of a β_2-AR-stimulated cardioprotective G_i signaling is fulfilled by the presence of the 5-HT_{2B}R/β_2-AR heterodimer (Figure 8).

The 5-HT_{2B}R was previously regarded as a contributor of cardiac hypertrophy and fibrosis [29, 30]. This perception is largely based on observations that pharmacological blocking or genetic manipulation to attenuate the 5-HT_{2B}R blunts catecholamine- or angiotensin II-induced cardiac hypertrophy and cardiac cell death [29, 30, 34, 35]. Yet there is limited progress in development of 5-HT_{2B}R antagonists for the treatment of cardiac disorders [49, 50]. Surprisingly, results from the current study show that the β_2-agonist-induced cardioprotection is dependent on the 5-HT_{2B}R. This apparent contradiction can be rationalized if the 5-HT_{2B}R regulates the cardioprotective β_2-AR-G_i signaling not via downstream signaling crosstalk but by direct receptor-receptor interaction. This is supported by the findings that MNF fails to activate 5-HT_{2B}R, but promotes 5-HT_{2B}Rs and β_2-ARs to form heterodimers (Figure 4). The fact that stimulation of the β_2-AR

by MNF enhances formation of the 5-HT_{2B}R/β_2-AR heterodimers which promote G_i protein coupling, G_i-Akt-eNOS signaling and G_i-mediated cardioprotection (Figures 7E and 8) indicates that the heterodimerized receptors are responsible for the transduction of the cardioprotective G_i signaling (Figure 8E). The heterodimerization may involve recruitment of the 5-HT_{2B}R by the activated β_2-AR followed by change of the coupling preference of the β_2-AR from G_s to G_i proteins. In this regards, Rashid and colleagues have reported that the heterodimer of the G_s-coupled dopamine D_1 receptor and the G_i-coupled dopamine D_2 receptor activates a $G_{q/11}$-signaling in response to the dual full D_1 agonist and partial D_2 agonist SKF83959 [51], suggesting that heterodimerization is capable of altering G protein coupling preference. Moreover, Saikai and colleagues have pharmacologically characterized the heterodimers of the ascidian, *Ciona intestinalis*, gonadotropin-releasing hormone receptors (Ci-GnRHRs) among four GnRHR subtypes (Ci-GnRHR1 to 4) and found that the mainly G_s-coupled R2 (monomer/homodimer), when heterodimerized with the R4, which is devoid of any ligand-binding or signaling activities, produces an R2-R4 heterodimer with an enhanced ability to couple to G_i proteins [52]. Our data show that the MNF-induced cardioprotection as well as β_2-AR-G_i signaling was abolished by ablation of the 5-HT_{2B}R or inhibition of the receptor with SB-206553 (Figures 6 and 7), yet MNF was largely inactive on the 5-HT_{2B}R (Figures 4D and 4E). We hypothesize that agonist binding on the β_2-AR causes a conformational change on the receptor favoring its interaction with the 5-HT_{2B}R which further enhances its G_i signaling. Nevertheless, the exact mechanism underlying the heterodimerization of the β_2-AR with the 5-HT_{2B}R merits future investigation.

In adult human or mouse hearts, the averaged expression level of the 5-HT_{2B}R is about 10 times less than that of the β_2-AR at the transcription level, although individual expression levels vary widely in humans (Supplementary Figures I and V). As our finding suggests, the β_2-AR-stimulated cardioprotective G_i signaling depends on the co-existence of the 5-HT_{2B}R. Therefore, genetic variation that alters cardiac 5-HT_{2B}R expression might dictate clinical outcomes in patients suffering from heart failure, given the salutary effect of the β_2-AR-G_i signaling on the failing heart [21].

As another important beneficial action, MNF exhibits an anticancer effect via activating the β_2-AR-coupled G_s signaling in a broad range of cancer cell lines [53-55]. It has been well-documented that Dox is an antineoplastic agent of the anthracycline class widely used in the treatment of breast cancer, Kaposi's sarcoma, lymphoma and acute lymphoblastic leukemia [56, 57], but elicits severe irreversible cardiotoxicity in 30%-40% of patients - producing massive myocyte loss, cardiomyopathy and heart failure [56, 57]. Cardiotoxicity is the most important factor limiting the use of Dox in cancer chemotherapy and the amelioration of this problem would optimize its use in clinical oncology. Since MNF possesses both antineoplastic [53-55, 58] and cardioprotective properties, it merits future investigation to determine whether a combination of MNF with Dox or other chemotherapeutic agents not only enhances tumor suppression but also reduces their cardiotoxicity.

We conclude that a β_2-agonist produces a cardioprotective effect via promoting heterodimerization of β_2-ARs and 5-HT_{2B}Rs with subsequent activation of a pro-survival β_2-AR-G_i-Akt-eNOS signaling. Heterodimerization with the 5-HT_{2B}R is indispensable for β_2-AR-mediated cardioprotection. Nevertheless, one should exercise caution in the interpretation of the current data particularly those derived from genetic

knockout animals as global rather than cardiac-specific homozygous or heterozygous knockout mice were used. Molecular changes and drug actions on non-myocytes could affect outcomes of cell injury and dysfunction in Dox-treated hearts. In addition, the current Dox treatment regimen is known to trigger a broad range of toxicity in multiple organs. Thus, the complication of extra-cardiac confounding factors in the phenotypic outcomes should be considered.

ACKNOWLEDGEMENTS

We thank L. Jin, N. Li, W.Q. Zhang, P. Xie, X.T. Sun and W. Zheng for their excellent technical support. We would also like to thank Dr. Yi Rao for providing the 5-HT_{2B}R KO mice.

SOURCES OF FUNDING

This work was supported by the National Key R&D Program of China (2018YFA0800501, 2018YFA0800701, and 2018YFA0507603), the National Natural Science Foundation of China (31671177, 81630008, 81790621, 81872752 and 31521062) and the Beijing Natural Science Foundation (5182010).

DISCLOSURES

Irving W. Wainer and Rui-Ping Xiao are co-inventors on the issued patents reporting the synthesis and use of 4'-methoxy-1-naphthylfenoterol. They have transferred all of the rights to these patents to the U.S. Government, which has sole rights to their use and licensing.

SUPPLEMENTAL MATERIALS

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FIGURE LEGENDS

Figure 1. MNF reduces mortality, alleviates Dox-induced cardiac dysfunction and ameliorates myocardial ischemia/reperfusion injury. (A) An experimental scheme of a survival analysis of the effect of (*R,R'*)-4-methoxy-1-naphthylfenoterol (MNF) on a mouse model of acute doxorubicin (Dox)-induced cardiomyopathy (only that of the 'insult with treatment' arms are shown). Male C57BL/6J mice were randomized into groups and were given a high dose of MNF (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$, p.o.), a low dose of MNF (0.075 $mg \cdot kg^{-1} \cdot d^{-1}$, p.o.), or drinking water (vehicle) daily. On the third day after the onset of oral treatment (day 0), mice in each group were subcategorized and each subgroup of mice received either Dox (20 $mg \cdot kg^{-1}$ in saline, i.p.) or vehicle administration. (B) Kaplan-Meier survival curves. n_b indicates the number of animals at the beginning of the study. n_e indicates the number of living animals at the end of the study. n_b = 30 for Dox group, 20 for Dox+MNF groups and 10 for Control group. Dox+MNF groups were compared with the Dox only group by log-rank test. *P* values were adjusted for 2 tests. (C) Changes in body weight with time. Averaging of the body weight data was performed daily. Comparison of the datasets was performed using repeated measures analysis (mixed-effect model) with Dunnett's post-hoc test (*vs* Dox groups). Adjusted *p* values of Dox+MNF (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$) groups (*vs* their respective Dox groups) are shown. (D) An experimental scheme for the determination of the effects of MNF on a mouse model of acute Dox-induced cardiomyopathy. C57BL/6J mice were pretreated with MNF (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$, p.o.) or vehicle followed by Dox treatment (20 $mg \cdot kg^{-1}$, i.p.) on the third day as described in the legend of Fig. 1A. On day 7 after Dox treatment, the dimension and function of the mouse hearts were measured by echocardiography (ECHO). The mice were weighed and then sacrificed. Myocardial injury was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and serum lactate dehydrogenase (LDH) assay as described in Methods. Representative M-mode echocardiograms, averaged data for cardiac contractile function - ejection fraction and fractional shortening (E), body weights of the mice (F), representative photomicrographs (scale bar, 50 μm) and averaged data for TUNEL staining of cardiac sections (G) and average serum LDH activities rescaled as folds of the vehicle control (H) from mice 7 d after vehicle or Dox injection (20 $mg \cdot kg^{-1}$, i.p.) with or without treatment with MNF (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$). Arrows indicate TUNEL-positive cells. *n* = 9 for Control group and 8 for other groups in E, *n* = 10 for Control and MNF groups and 9 for other groups in F, *n* = 8 in G and H. Comparisons of the datasets were performed using two-way ANOVA with Bonferroni-corrected post-hoc *t* test for F and Kruskal-Wallis with post-hoc Mann-Whitney test (Dox group *vs* Dox+MNF group) for E, G and H. (I) An experimental scheme for the determination of the effects of MNF on a mouse model of myocardial ischemia/reperfusion. C57BL/6J mice were pretreated with MNF (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$, p.o.) or vehicle. Temporary ligation of the

left anterior descending coronary artery was performed on the third day of treatment to induce 30 min of myocardial ischemia followed by reperfusion. The mice were sacrificed after 24 h. Infarct sizes and areas at risk (AAR) on the left ventricles (LV) were determined by dual Evans blue–triphenyltetrazolium chloride (TTC) staining. Myocardial injury was assessed by serum LDH assay, serum creatine kinase (CK) assay and TUNEL staining. Representative images of Evans blue-TTC-stained heart slides with infarct zones in white and AAR in both red and white (upper panels, scale bar, 1 mm) and averaged data of infarct sizes (expressed as a percentage of AAR) and AAR (expressed as a percentage of LV area) (lower panels) (J), averaged data of serum LDH activities (K) and serum CK activities (L) rescaled as folds of the vehicle controls, and percentages of TUNEL-positive cells in cardiac sections (M) are presented. n = 8 for the Vehicle groups and n = 9 for the MNF groups in J. n = 8 for MNF groups and n = 9 for Vehicle groups in K and L. n = 5 for M. P values were determined using Mann-Whitney U test in J-M.

Figure 2. Protective effects of MNF in cardiomyocytes subjected to H_2O_2, Dox or hypoxia/reoxygenation treatment. (A-C) Cardiomyocytes from adult mice (A) or adult rats (B and C) were pretreated with MNF at the indicated concentrations for 1 h and then co-incubated in the presence or absence of 10 μmol/L (A) or 15 μmol/L (B and C) of H_2O_2 for 15-20 h. LDH activities released into the culture media of mouse cardiomyocytes (n = 9 mice in A) and rat cardiomyocytes (n = 10 rats for MNF group and 16 for other groups in C) were measured. Propidium iodide (PI) staining was performed to detect necrotic cardiomyocytes. Representative microscopic images of the cardiomyocytes taken under bright field (left) and fluorescent field (right) are shown in B (top panels). PI-positive cells are indicated by arrows. Scale bar, 100 μm. Percentages of PI-positive cells in cultured mouse cardiomyocytes (n = 5 mice in A) and rat cardiomyocytes (n = 11 rats in B bottom panel) and caspase 3/7 activities in cultured rat cardiomyocytes (n = 5 rats in C) were determined. (D) Western blot analysis of heart lysates for the DNA damage marker γH2AX in mice treated with or without MNF (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$, p.o.) subjected to Dox (20 $mg \cdot kg^{-1}$, i.p.) or vehicle. Representative blots of γH2AX and GAPDH (loading control) using specific antibodies (top panel). γH2AX-to-GAPDH ratios (bottom panel, rescaled as folds of the vehicle control, n = 5 mice). (E) LDH released into the culture media (n = 10 rats) and averaged caspase 3/7 activities (n = 10 rats for MNF group and 14 for other groups) in isolated adult rat cardiomyocytes in the presence or absence of MNF (0.1 μmol/L) with or without Dox (15 μmol/L overnight). (F) LDH released into the culture media (n = 6 rats) and averaged caspase 3/7 activities (n = 6 rats) in adult rat cardiomyocytes subjected to 12 h of hypoxia (95% N_2 and 5% CO_2) followed by 3.5 h of reoxygenation or 15.5 h of normoxia treatment in the presence or absence of MNF (0.1 μmol/L). Data for LDH release and caspase 3/7 activity were rescaled as folds of the vehicle control (A, C, E and F). Comparisons were carried out using Kruskal-Wallis with Dunn's post-hoc test for A and C (upper panel) or Mann-Whitney test (Dox group *vs* Dox+MNF group) for E (upper panel), Welch's ANOVA with Tamhane's T2 post-hoc test for B, E (bottom panel) and F (bottom panel), and two-way ANOVA with Bonferroni-corrected post-hoc t test for C (bottom panel), D and F (upper panel).

Figure 3. MNF reverses H_2O_2- or Dox-induced cardiomyocyte death and cardiac dysfunction. (A and B) Cardiomyocytes from adult rats were treated with H_2O_2 (15 μmol/L) (A) or Dox (15 μmol/L) (B). MNF (0.1 μmol/L) or vehicle was added after 1 h and the cells were cultured for 15-20 h. Released LDH (rescaled as folds of the vehicle control, n = 6 rats for A and B) and caspase 3/7 activities (rescaled as folds of the vehicle control, n = 5 rats for A and 6 for B) were then measured. (C) An experimental scheme for the determination of the reversing effects of MNF on a mouse model of acute Dox-induced cardiomyopathy. C57BL/6J mice were treated with Dox (20 $mg \cdot kg^{-1}$, i.p.) or vehicle. On the following day, the survived mice were randomized into groups and treated with MNF (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$, p.o.) or vehicle for 6 days. Cardiac function of the mice was then assessed by ECHO. Myocardial injury was assessed by TUNEL staining and serum LDH assay. (D-F) Averaged data of ejection fraction and fractional shortening (D), serum LDH activities rescaled as folds of the control (E) and TUNEL-positive cells in cardiac sections (F) are presented. n_b = 15, n_e = 8 for the Dox groups, n_b = 11, n_e = 8 for the Dox+MNF groups, n = 5 for other groups in D and E, n = 4 mice for Control and MNF groups and 5 for other groups in F. Data were analyzed using Welch's ANOVA with Tamhane's T2 post-hoc test for A (upper panel), two-way ANOVA with Bonferroni-corrected post-hoc t test for A (bottom panel), B (upper panel), D (right panel) and E and Kruskal-Wallis with post-hoc Mann-Whitney test (Dox group *vs* Dox+MNF group) for B (bottom panel), D (left panel) and F.

Figure 4. MNF enhances heterodimerization of β_2-AR and 5-HT_{2B}R. (A) HEK293 cells were transfected with either HA-β_2-AR plasmids or FLAG-5-HT_{2B}R plasmids, or both, and incubated with or without MNF (0.1 μmol/L) or zinterol (Zint, 1 μmol/L). The transfected and drug-treated cells were subjected to a FRET assay. The degrees of interaction between HA-β_2-AR and FLAG-5-HT_{2B}R in HEK293 cells with different treatments are presented in terms of Delta F % (n = 6 for FLAG group and n = 15 for other groups). (B and C) Lysates from the cells in panel A were immunoprecipitated with anti-HA or anti-FLAG antibody followed by immunoblotting with an antibody of the reciprocal species. Some cell lysates (30 μL) were saved for positive controls and loading controls in immunodetection (n = 4). (D) Activation of the β_2-AR by MNF and the β-agonist isoproterenol (Iso) (left panel) and activation of the 5-HT_{2B}R by MNF and 5-HT (right panel) were measured using the genetically encoded GPCR-activation-based (GRAB) sensor assay. The change in fluorescent signal ($\Delta F/F_0$) indicates receptor responsiveness (n = 3). (E) $[Ca^{2+}]_i$ flux responses of CHO-K1/5-HT_{2B}R cells upon stimulation with 5-HT and MNF were measured by FLIPR. Data are expressed as percentages of the maximum effect of 5-HT (n = 3) (left panel). The antagonist mode of the assay was conducted by administering 5-HT after pretreatment of the CHO-K1/5-HT_{2B}R cells with the

compound SB-206553 or MNF. Data are expressed as percentage inhibition of the $[Ca^{2+}]_i$ flux response of 5-HT in the absence of the compound (n = 3) (right panel). (F and G) Co-IP assays to detect interactions of β_2-ARs and 5-HT_{2B}Rs were performed as described above using the corresponding antibodies against the epitope-tags on HEK293 cells transfected with either HA-β_2-AR plasmids or FLAG-5-HT_{2B}R plasmids, or both (F), or on adult rat cardiomyocytes infected with FLAG-β_2-AR adenoviruses or Myc-5-HT_{2B}R adenoviruses, or both (G) after the cells were subjected to H_2O_2 (15 μmol/L, 15-20 h) or vehicle treatment (F), or 12 h of hypoxia followed by 3.5 h of reoxygenation (H/R) or 15.5 h of normoxia treatment (G) in the presence or absence of MNF (0.1 μmol/L) (n = 6 for F and G). Data were rescaled as folds of the vehicle control (B, C and F) or the normoxia control (G). Fitting of the data points into the four-parameter logistic model and calculation of the EC_{50} or IC_{50} values were performed with GraphPad Prism (version 8.0.1) (D and E). Comparisons of the datasets were carried out using Mann-Whitney U test for B and C, Welch's ANOVA with Tamhane's T2 post-hoc test for A, F (right panel) and G and one-way ANOVA with Tukey's post-hoc test for F (left panel).

Figure 5. The cardioprotective effect of MNF is β_2-AR-dependent. (A-D) FVB mice (WT) and β_2-AR KO mice were pretreated with MNF (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$, p.o.) or vehicle followed by Dox treatment (20 $mg \cdot kg^{-1}$, i.p.) on the third day as described in the legend of Fig. 1. (A) Kaplan-Meier survival curves. n = 33 for the WT Dox group, and 20 for other groups. Dox+MNF group was compared with Dox group in each genotype by log-rank test. P values were adjusted for 2 tests. (B) Representative M-mode echocardiograms and cardiac contractile function - ejection fraction and fractional shortening. (C) Averaged serum LDH activities rescaled as folds of the vehicle control. (D) Representative photomicrographs and averaged data for TUNEL staining of cardiac sections. Arrows indicate TUNEL-positive cells. Scale bar, 50 μm. n_b = 15 for the Dox only groups, 13 for the Dox+MNF groups and 10 for other groups in B, C and E. n_e = 10 mice for B, C and E. (F) Adult rat cardiomyocytes were pretreated with or without propranolol (Prop, 1 μmol/L, 15 min, n = 7 for upper panel and 6 for bottom panel). MNF (0.1 μmol/L) or its vehicle was co-incubated in the presence or absence of 15 μmol/L of H_2O_2 for 20 h. LDH release (rescaled as folds of the untreated control) and the percentages of PI-positive cells were determined. Comparisons were carried out using two-way ANOVA with Bonferroni-corrected post-hoc t test for B, Kruskal-Wallis with post-hoc Mann-Whitney test (Dox group *vs* Dox+MNF group) for E, Welch's ANOVA with Tamhane's T2 post-hoc test for C and one-way ANOVA with Tukey's post-hoc test for F.

Figure 6. 5-HT_{2B}R is required for β_2-agonist-induced cardioprotection. (A-C) C57BL/6J mice (WT) and heterozygous 5-HT_{2B}R KO mice (+/-) were pretreated with MNF (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$, p.o.) or vehicle followed by Dox treatment (20 $mg \cdot kg^{-1}$, i.p.) on the third day as described in the legend of Fig. 1. (A) Kaplan-Meier survival curves. n = 30 for the WT Dox group, 20 for the WT Dox+MNF group and 10 for other groups. Dox+MNF group was compared with Dox group in each genotype by log-rank test. P values were adjusted for 2 tests. (B) Cardiac contractile function - ejection fraction and fractional shortening. (C) Averaged serum LDH activities rescaled as folds of the control. n_b = 8 for the Dox only groups and the Dox+MNF groups and 5 for other groups in B and C. n_e = 5 mice for B and C. Cardiomyocytes from adult mice (D) or adult rats (E-G) were pretreated with MNF (0.1 μmol/L) or Zint (1 μmol/L) for 1 h and then co-incubated in the presence or absence of 10 μmol/L (D) or 15 μmol/L (E-G) of H_2O_2 for 15-20 h. SB-206553 (1 μmol/L) was added 1 h before MNF or vehicle treatment (E). Cells were transfected with 5-HT_{2B}R siRNA or scrambled siRNA for 24 h before MNF, Zint or vehicle treatment (F and G). LDH activities released into the culture media (rescaled as folds of the respective control) of mouse cardiomyocytes (D) and rat cardiomyocytes (E-G) were measured. Percentages of PI-positive cells in cultured mouse cardiomyocytes (D) and rat cardiomyocytes (E and F) were determined. Averaged caspase 3/7 activities (rescaled as folds of the untreated scrambled siRNA control) in rat cardiomyocytes were measured (G). n = 6 mice for D and F (upper panel) and n = 5 rats for E, G and F (bottom panel). Comparisons were carried out using Kruskal-Wallis with post-hoc Mann-Whitney test (Dox group *vs* Dox+MNF group) for B and C. H_2O_2 groups in D-G were compared using two-way ANOVA with Bonferroni-corrected post-hoc t test while groups without H_2O_2 were only appearing as reference groups without analyzing.

Figure 7. MNF induces a 5-HT_{2B}R-dependent cardioprotective β_2-AR-G_i-Akt signaling. (A) Adult rat cardiomyocytes were pretreated with or without pertussis toxin (PTX, 0.75 μg/mL, 3 h). MNF (0.1 μmol/L) or its vehicle (Veh) was co-incubated in the presence or absence of 15 μmol/L of H_2O_2 for 20 h. Zint (1 μmol/L) was used as a positive control for β_2-AR-G_i activation. LDH release (n = 9) and the percentages of PI-positive cells (n = 8) were determined. The MNF and H_2O_2 groups or the Zint and H_2O_2 groups were compared with the H_2O_2 alone groups. The PTX-pretreated MNF and H_2O_2 groups or Zint and H_2O_2 groups were compared with the corresponding groups without PTX. (B) Isolated adult rat cardiomyocytes were pretreated with PTX (0.75 μg/mL, >3 h) or ICI-118551 (ICI, 1 μmol/L, 15 min), and then incubated with

MNF (0.1 μmol/L) or Veh at 37 °C for 1 h. Cells were lysed for western blotting determination of phosphorylated Akt (p-Akt), phosphorylated eNOS (p-eNOS), Akt, eNOS and GAPDH. Representative images of the western blots (upper panel), averaged data of Akt phosphorylation (middle panel, $n = 5$) and averaged data of eNOS phosphorylation (bottom panel, $n = 8$) are presented. (C) Cardiomyocytes from adult WT or β_2-AR KO mice were incubated with MNF (0.1 μmol/L) or Veh at 37 °C for 1 h. Cells were lysed for western blotting (middle panel, $n = 5$; bottom panel, $n = 4$). (D) Isolated adult rat cardiomyocytes were pretreated with SB-206553 (1 μmol/L, 1 h), and then incubated with MNF (0.1 μmol/L) or Veh at 37 °C for 1 h. Cells were lysed for western blotting ($n = 6$). (E) LDH released into the culture media ($n = 7$) and averaged caspase 3/7 activities ($n = 6$) (rescaled as folds of the untreated scrambled siRNA control) were determined in adult rat cardiomyocytes treated by different combinations of MNF (0.1 μmol/L), Dox (15 μmol/L overnight) and PTX (0.75 μg/mL) following a 24 h-transfection with scrambled siRNA or 5-HT_{2B}R siRNA. (F) C57BL/6J mice were injected with Dox (20 $mg \cdot kg^{-1}$, i.p.) or Veh. On the next day, MNF (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$, p.o.) or Veh in drinking water was administered to the mice for 6 days as described in the legend of Fig. 3C. The mice were sacrificed 7 days after Dox treatment. Lysates prepared from left ventricular tissues were subjected to western blotting ($n = 6$). Data were rescaled as folds of the Veh control (A, B, D and F) or the WT Veh control (C). *P* values were determined using Welch's ANOVA with Tamhane's T2 post-hoc test in A (right panel), two-way ANOVA with Bonferroni-corrected post-hoc *t* test in F and Kruskal-Wallis with Bonferroni-corrected post-hoc Mann-Whitney test comparing H_2O_2 group to H_2O_2+MNF group, H_2O_2 group to H_2O_2+Zint group, H_2O_2+MNF group to H_2O_2+MNF+PTX group and H_2O_2+Zint group to H_2O_2+Zint+PTX group in A (left panel), Vehicle group, MNF+ICI group or MNF+PTX group to MNF group in B, WT+MNF group and β_2-AR KO+MNF group to their respective control group in C, and Veh+MNF group and SB-206553+MNF group to their respective control group in D. Dox groups in E were compared using two-way ANOVA with Bonferroni-corrected post-hoc *t* test while groups without Dox were only appearing as reference groups without analyzing.

Figure 8. The MNF-induced 5-HT_{2B}R/β_2-AR heterodimer is sufficient to mediate G_i-Akt signaling and cardioprotection. Adult rat cardiomyocytes were infected with different combinations of β-galactosidase (β-gal), Myc-5-HT_{2B}R, FLAG-β_2-AR (-WT) and FLAG-β_2-AR-Gly16 adenoviruses for 24 h followed by agonist-stimulation (0.1 μmol/L of MNF or Veh). (A) Co-IP assays to detect heterodimerization of β_2-ARs and 5-HT_{2B}Rs were performed on the cardiomyocytes as described in the legend of Fig. 4 ($n = 4$). (B) To detect interactions of FLAG-β_2-AR and/or Myc-5-HT_{2B}R with $G_{i\alpha3}$ in the cardiomyocytes, agonist-stimulation was performed in the presence of the crosslinker dithiobis(succinimidyl propionate), which was subsequently inactivated with an excess amount of Tris. Co-IP was performed on lysates from these cells using the corresponding antibody against the epitope-tag followed by immunoblotting detection of $G_{i\alpha3}$ ($n = 6$). (C) The adenovirus- and agonist-treated cardiomyocytes were challenged with Dox (15 μmol/L). LDH released into culture media and averaged caspase 3/7 activities (rescaled as folds of the unchallenged β-gal control) were determined after overnight cell culture ($n = 6$ for upper panel and $n = 7$ for bottom panel). (D) Adult rat cardiomyocytes overexpressed with β-gal or both FLAG-β_2-AR and Myc-5-HT_{2B}R were treated with Dox (15 μmol/L) or Veh overnight. Subsets of these cells were further treated with MNF (0.1 μmol/L) or Veh at 37 °C for 1 h. Cells were lysed for western blotting ($n = 8$ for middle panel and $n = 6$ for bottom

panel). Data were rescaled as folds of the Veh control (A and B) or the untreated β-gal control (D). *P* values were determined using one-way ANOVA with Tukey's post-hoc test in B and D (bottom panel), Welch's ANOVA with Tamhane's T2 post-hoc test in C (upper panel) and Kruskal-Wallis with Bonferroni-corrected post-hoc Mann-Whitney test comparing β_2-AR-WT+MNF group and β_2-AR-Gly16+MNF group to their respective control group in A, β-gal+Dox+Vehicle group, 5-HT_{2B}R+β_2-AR-WT+Dox+MNF group or 5-HT_{2B}R+β_2-AR-Gly16+Dox+MNF group to β-gal+Dox+MNF group in C (bottom panel), and β-gal+Dox group to β-gal+Dox+MNF group, 5-HT_{2B}R+β_2-AR+Dox group to 5-HT_{2B}R+β_2-AR+Dox+MNF group and β-gal+Dox+MNF group to 5-HT_{2B}R+β_2-AR+Dox+MNF group in D (middle panel). (E) Schematic diagram. Dox and H_2O_2 produce oxidative stress leading to cardiomyocyte injury and death. MNF binds to β_2-AR and promotes heterodimerization of β_2-AR and 5-HT_{2B}R. The agonist-stimulated 5-HT_{2B}R/β_2-AR heterodimer elicits a pro-survival G_i-Akt-eNOS signaling to prevent cardiomyocyte death. Key: 5-HT_{2B}R: 5-HT_{2B} receptor, Akt: protein kinase B, β_2-AR: β_2-adrenoceptor, Dox: doxorubicin, eNOS: endothelial nitric oxide synthase, G_i: inhibitory G protein, H_2O_2: hydrogen peroxide, MNF: (*R,R'*)-4-methoxy-1-naphthylfenoterol, ROS: reactive oxygen species.

NOVELTY AND SIGNIFICANCE

What Is Known?

- Stimulation of the β_2-AR is beneficial to humans with heart failure likely by activation of the downstream G_i-PI3K-Akt cell survival pathway.
- Both the β_2-adrenoceptor (β_2-AR) and the 5-hydroxytrptamine receptor 2B (5-HT_{2B}R) are expressed in cardiomyocytes and have been suggested to form dimers with other G protein-coupled receptors (GPCRs).
- The β_2-AR couples to both G_s and G_i proteins, and protein kinase A-phosphorylation of the β_2-AR has been proposed as a key mechanism for the coupling of the β_2-AR to G_i proteins.

What New Information Does This Article Contribute?

- β_2-ARs and 5-HT_{2B}Rs can form heterodimers, and β_2-agonists enhance the heterodimer formation.
- (*R,R'*)-4-methoxy-1-naphthylfenoterol (MNF), a selective β_2-agonist, attenuates mouse myocardial injury and mortality in a 5-HT_{2B}R-dependent manner.
- The 5-HT_{2B}R/β_2-AR heterodimer rather than the individual receptors alone is sufficient to mediate the β_2-agonist-induced cardioprotective G_i signaling.

GPCR heterodimerization not only modulates receptor function but also alters G protein-coupling preference. The present study has shown, for the first time, that β_2-ARs heterodimerize with 5-HT_{2B}Rs, and that the heterodimerization is essential for the β_2-AR-stimulated cardioprotective G_i signaling. This finding provides a new information about coupling of the cardiac β_2-AR to G_i and suggest the potential importance of cardiac-specific expression variability of the 5-HT_{2B}R for heart failure prognosis. Consistent with this hypothesis, our findings show that the the β_2-AR agonist, MNF, protects the heart against doxorubicin- and oxidative stress-induced myocardial injuries. As MNF also possesses antitumor properties, it may have a role in reduction of cardiotoxicity in the setting of malignancy.

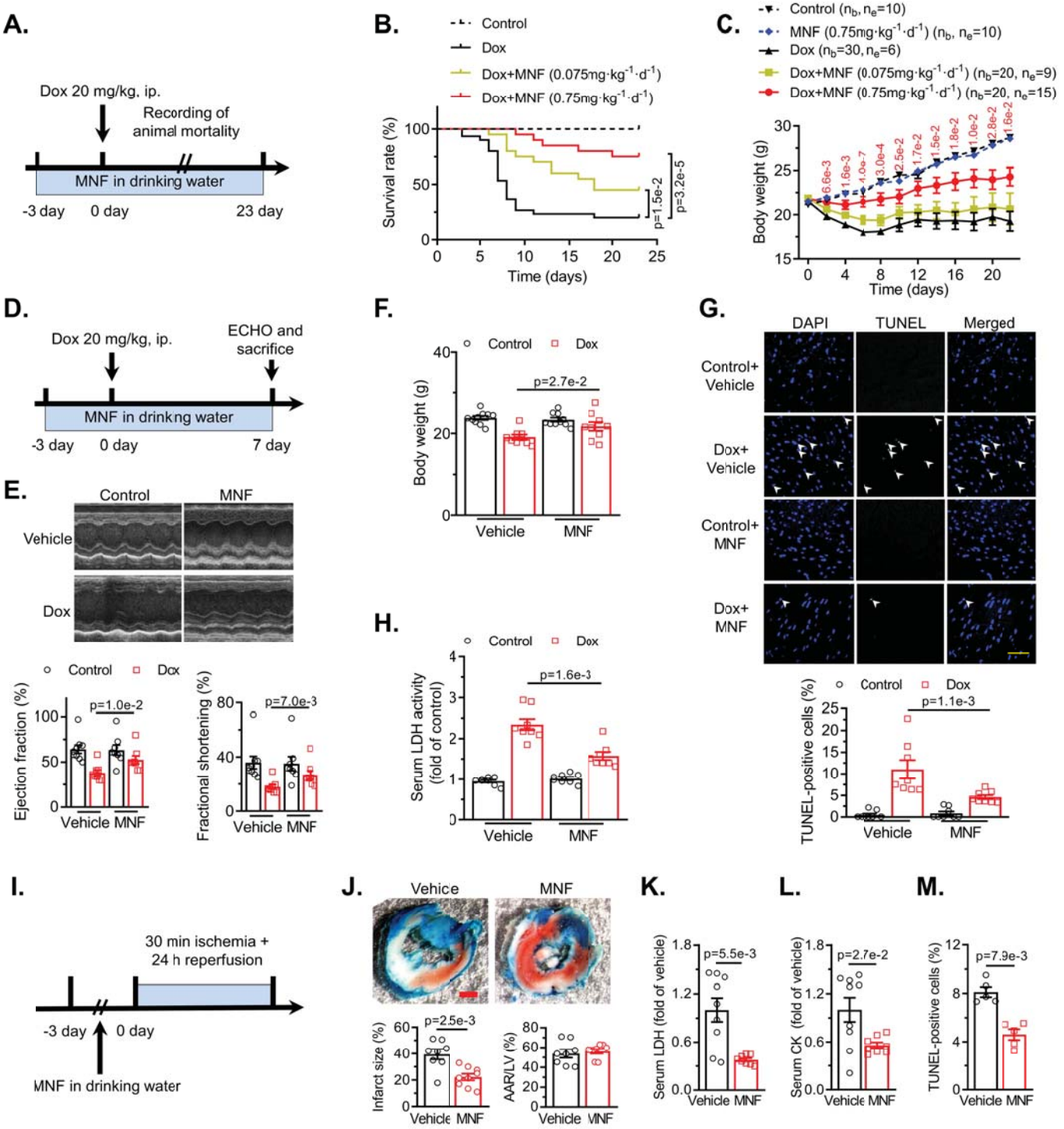

FIGURE 1