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PRECLINICAL RESEARCH

Carvedilol Prevents Redox Inactivation of Cardiomyocyte B_1-Adrenergic Receptors

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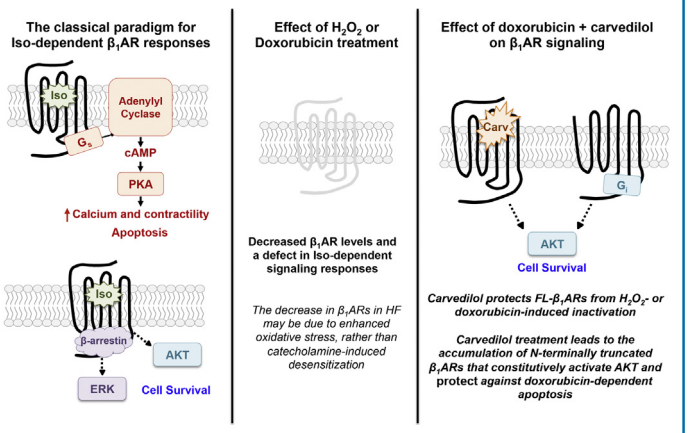
VISUAL ABSTRACT



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HIGHLIGHTS

- **Oxidative stress induced by acute H_2O_2 or chronic doxorubicin treatment leads to a decrease in β_1AR expression and isoproterenol responsiveness in cardiomyocytes.**
- **The redox-dependent disruption of the β_1AR signaling pathway, which could explain the defect in catecholamine responsiveness that characteristically develops in heart failure, is prevented by the novel protein kinase C inhibitor GFX109203X or carvedilol.**
- **Carvedilol treatment leads to the accumulation of a truncated β_1AR species whose signaling properties can be distinguished from full-length β_1ARs; truncated β_1ARs constitutively activate protein kinase B and protect against doxorubicin-induced apoptosis.**
- **These results identify a novel β_1AR-dependent mechanism that contributes to carvedilol-induced cardioprotection.**

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ABBREVIATIONS AND ACRONYMS

βAR = β-adrenergic receptor

AKT = protein kinase B

cAMP = cyclic adenosine monophosphate

CREB = cyclic adenosine monophosphate binding response element protein

ERK = extracellular regulated kinase

FL = full-length

GFX = GF109203X

GRK = G protein-coupled receptor kinase

HF = heart failure

PKA = protein kinase A

PKC = protein kinase C

PTX = pertussis toxin

ROS = reactive oxygen species

SUMMARY

The mechanism that leads to a decrease in β_1-adrenergic receptor (β_1AR) expression in the failing heart remains uncertain. This study shows that cardiomyocyte β_1AR expression and isoproterenol responsiveness decrease in response to oxidative stress. Studies of mechanisms show that the redox-dependent decrease in β_1AR expression is uniquely prevented by carvedilol and not other βAR ligands. Carvedilol also promotes the accumulation of N-terminally truncated β_1ARs that confer protection against doxorubicin-induced apoptosis in association with activation of protein kinase B. The redox-induced molecular controls for cardiomyocyte β_1ARs and pharmacologic properties of carvedilol identified in this study have important clinical and therapeutic implications. (J Am Coll Cardiol Basic Trans Science 2018;3:521–32)

Catecholamines enhance the mechanical performance of the heart by activating cardiac β-adrenergic receptors (βARs). Although cardiomyocytes co-express β_1AR and β_2ARs, the β_1AR is the predominant subtype and principal driver of catecholamine-driven sympathetic responses in the healthy heart. β_1ARs couple to a G_s-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway that phosphorylates substrates that enhance excitation/contraction coupling and provide hemodynamic support in the setting of an acute stress. Although β_1ARs also couple to cardioprotective G_s-independent mechanisms such as extracellular regulated kinase (ERK) (1), chronic/persistent β_1AR activation leads to a spectrum of changes (including cardiomyocyte hypertrophy/apoptosis, interstitial fibrosis, and contractile dysfunction) that contribute to the pathogenesis of heart failure (HF) (2). βAR inhibitors that prevent maladaptive cAMP-driven βAR responses have become mainstays of HF therapy.

Although βAR activation provides hemodynamic support and compensates for the contractile dysfunction that develops in HF, chronic HF leads to a loss of cardiac reserve due to desensitization and/or down-regulation of βARs (2). Decreased β_1AR density is a hallmark of HF, but the mechanism underlying this adaptive mechanism remains uncertain because the classic paradigms for βAR regulation are based largely on studies of the β_2AR subtype. Current models hold that agonists stabilize βARs in an active conformation that is phosphorylated by G protein-coupled receptor kinases (GRKs). GRK-phosphorylated βARs then recruit β-arrestin, which functions both to initiate desensitization and facilitate clathrin-mediated βAR internalization (3). However, the general assumption that this mechanism applies equally to β_1AR and β_2AR subtypes is at odds with evidence that these receptor subtypes are regulated differently in the setting of HF; HF leads to a relatively selective down-regulation of β_1ARs that is not accompanied by a commensurate loss of β_2ARs (4).

The prevailing assumption that decreased β_1AR expression in the failing heart is attributable to chronic catecholamine-induced, GRK/β-arrestin-dependent receptor desensitization is also at odds with cell-based studies showing that β_1ARs are relatively resistant to agonist-induced, GRK-dependent phosphorylation; they engage β-arrestin only weakly; and they show little-to-no agonist-induced internalization (5,6). These differences should not be surprising because β_1AR and β_2ARs share only 54% overall homology at the amino acid level, with sequence conservation confined largely to transmembrane/ligand-binding regions; β_1AR and β_2AR intracellular loops and C-termini (regions that serve as substrates for GRK phosphorylation and/or docking sites for β-arrestin) are divergent (7). This study identifies an alternative redox-activated mechanism that selectively decreases cardiomyocyte β_1ARs.

In the course of these studies, we also identified a heretofore unrecognized property of carvedilol, a βAR inhibitor that reportedly offers survival advantages over other βAR inhibitors in the treatment of HF (8). Earlier studies have argued that this finding might be attributable to carvedilol's ancillary properties as an antioxidant (9) or its unique pharmacologic profile; carvedilol acts as an inverse agonist for the βAR- G_s-cAMP pathway (i.e., it prevents catecholaminergic-induced cardiotoxicity) and a biased agonist for β-arrestin-mediated signaling to ERK and potentially other cardioprotective pathways (10,11). Our studies show that carvedilol prevents β_1AR redox inactivation and that it also triggers a novel β_1AR-dependent cardioprotective mechanism. We recently found that β_1ARs are detected as both full-length (FL) and

N-terminally truncated species that differ in their signal bias to effector pathways (12). This study shows that carvedilol increases the abundance of N-terminally truncated β_1ARs which constitutively activate protein kinase B (AKT) and confer protection against doxorubicin-induced apoptosis.

METHODS

MATERIALS. A full description of the antibodies and chemical reagents can be found in the Supplemental Methods.

CARDIOMYOCYTE CULTURE AND ADENOVIRAL INFECTIONS. Cardiomyocytes were isolated from the ventricles of 2-day-old Wistar rats by a trypsin dispersion technique using a differential attachment procedure to enrich for cardiomyocytes followed by irradiation as described previously (12). Methods to infect cardiomyocytes with adenoviruses that drive expression of FL or N-terminally truncated forms of human β_1AR (Ad-FL-β_1AR and Ad-Δ2-52-β_1AR) were published previously.

IMMUNOBLOTTING. Immunoblotting was performed on cell extracts according to methods described previously (12) or manufacturer's instructions as detailed in the Supplemental Methods. All results were replicated in at least 3 experiments on separate culture preparations.

MEASUREMENTS OF βAR DENSITY AND cAMP ACCUMULATION. βAR density and cAMP accumulation were measured according to standard methods as described previously (12).

STATISTICAL ANALYSIS. Results are shown as mean ± SEM and were analyzed by using Student's *t*-test or analysis of variance for multiple comparisons; p values < 0.05 were considered statistically significant.

RESULTS

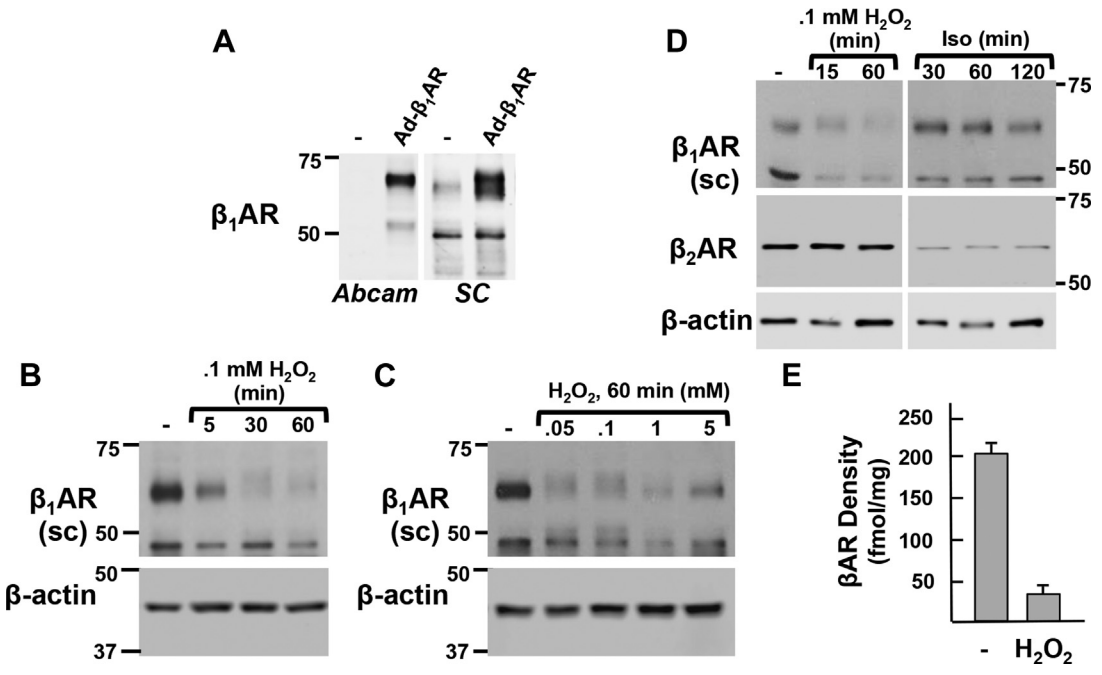
H_2O_2 DECREASES β_1AR EXPRESSION IN CARDIOMYOCYTES. Reactive oxygen species (ROS) contribute to the pathogenesis of HF and other cardiomyopathic syndromes. Although oxidative stress leads to changes in signaling by many components of the G_s/adenylyl cyclase/PKA pathway, the notion that the β_1AR itself is a ROS-sensitive element in this signaling pathway (i.e., that a ROS-dependent event localized to the β_1AR itself can contribute to evolution of HF) has never been considered. Our initial studies used immunoblotting to track H_2O_2-dependent effects on β_1AR immunoreactivity.

Because the specificity of various commercially available anti-G protein-coupled receptor antibodies has been questioned (13,14), preliminary studies were designed to rigorously characterize the anti-β_1AR antibodies from Abcam and Santa Cruz (SC) that were used for the analysis. **Figure 1A** (left) shows that the Abcam anti-β_1AR antibody detects human β_1ARs heterologously overexpressed in rat cardiomyocytes as a major ~69-kDa band and a minor ~55-kDa species. We recently identified a similar immunoreactive profile for this transgene in 2 different model cell lines and showed that the ~69-kDa species corresponds to FL fully glycosylated β_1ARs; the ~55-kDa species corresponds to N-terminally truncated glycosylation-defective β_1ARs (12). We also showed that N-terminal truncation does not alter β_1AR-binding affinity for the antagonist ligand iodocyanopindolol or lead to changes in basal cAMP/PKA or ERK; instead, the N-terminally truncated β_1AR is stabilized in a conformation that results in enhanced agonist-dependent activation of cAMP/PKA and reduced agonist-dependent activation of ERK.

The Abcam anti-β_1AR antibody does not detect endogenous β_1ARs in cardiomyocyte cultures (**Figure 1A**, left). However, consistent with previous studies showing that the SC anti-β_1AR antibody recognizes endogenous mouse β_1ARs in lysates from wild-type, but not β_1AR knockout, hearts (15,16), **Figure 1A** (right) shows that the SC anti-β_1AR antibody detects a band corresponding to the FL-β_1AR in uninfected rat cardiomyocyte cultures and that the abundance of this band increases in the context of β_1AR overexpression. The SC anti-β_1AR antibody also detects a band with considerably faster electrophoretic mobility. Although this species might represent endogenous N-terminally truncated β_1ARs, it could not unambiguously be identified as a bona fide β_1AR species because it does not increase with β_1AR overexpression. Because this smaller band could represent nonspecific immunoreactivity, it is not considered further in the analysis.

Initial studies used the SC anti-β_1AR antibody to track H_2O_2-dependent regulation of the native β_1AR in cardiomyocyte cultures. **Figure 1B** shows that treatment with 0.1 mM H_2O_2 leads to a time-dependent decrease in β_1AR immunoreactivity. The decrease in overall β_1AR immunoreactivity is detected in cultures treated with a range of H_2O_2 concentrations (0.05 to 5 mM) (**Figure 1C**). Experiments using low H_2O_2 concentrations (0.05 to 0.1 mM) also captured a decrease in FL-β_1AR electrophoretic mobility (which could suggest an H_2O_2-dependent increase in β_1AR phosphorylation), as well as the accumulation of small amounts of a ~50-kDa species that is presumed to represent a β_1AR cleavage product. The H_2O_2-dependent decrease in β_1AR immunoreactivity is specific and is

FIGURE 1 H_2O_2 Decreases β_1AR Expression in Cardiomyocytes



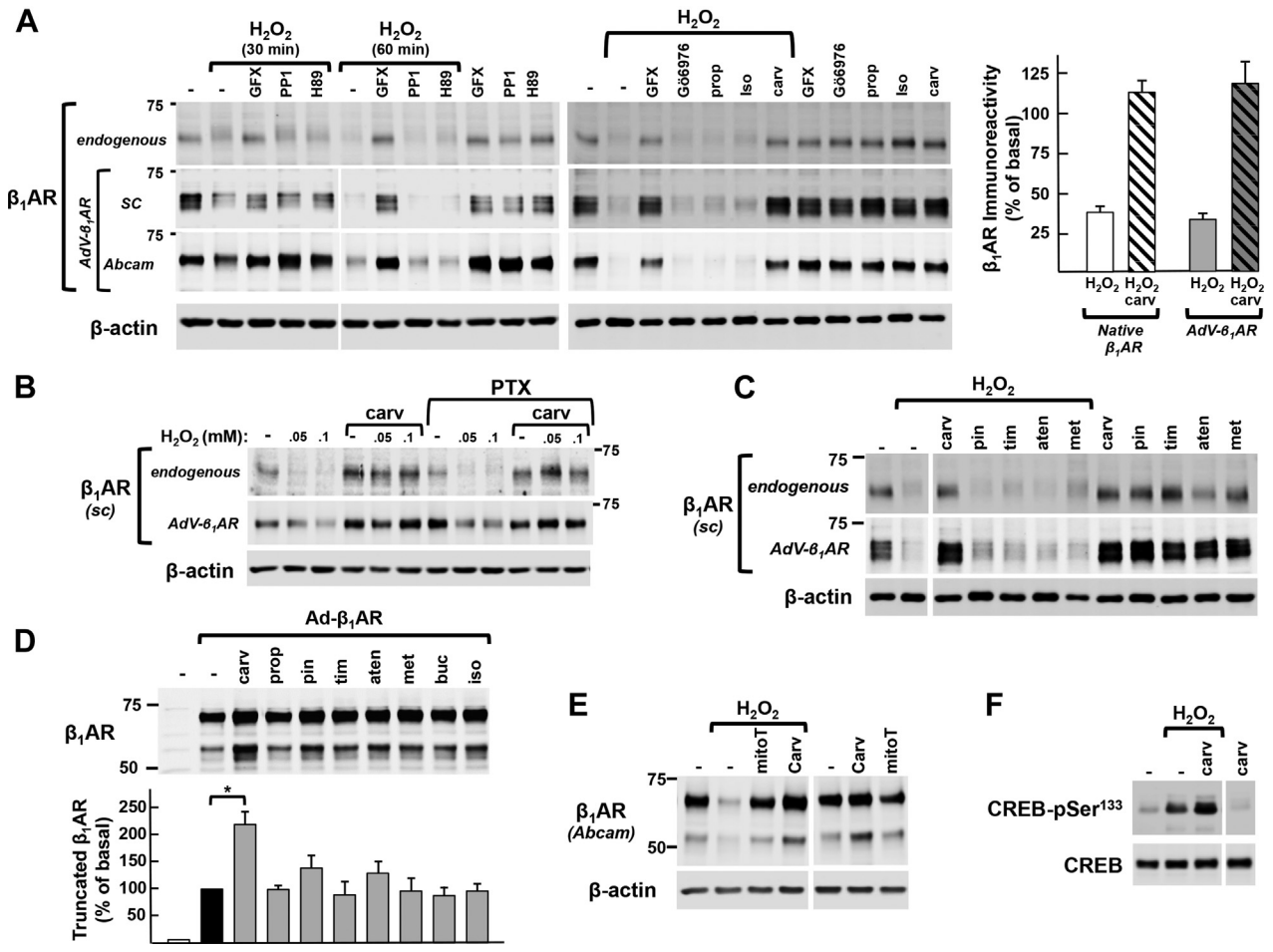
(A) Abcam and Santa Cruz (SC) anti-β_1-adrenergic receptor (β_1AR) antibodies were used to detect native or heterologously overexpressed β_1ARs in cardiomyocytes. **(B to D)** Lysates from cardiomyocyte cultures treated with the specified H_2O_2 concentrations or 1 μM isoproterenol (for the indicated time intervals) were probed with the SC anti-β_1AR antibody to track endogenous β_1AR expression (with β-actin immunoreactivity serving as a protein loading control). Results in **D** are from a single set of gels exposed for a uniform duration, with white space inserted where data from 2 regions of the same gel were merged for presentation purposes. All results in **A to D** were replicated in 3 to 5 separate experiments. **(E)** βAR density (as defined by saturation binding experiments with ^{125}I-iodocyanopindolol) in β_1AR overexpressing cardiomyocytes treated with vehicle or 0.1 mM H_2O_2 for 30 min (n = 3).

not accompanied by a change in cardiomyocyte β_2ARs (Figure 1D). Importantly, the response to H_2O_2 contrasts markedly with the response to chronic isoproterenol stimulation (30 to 120 min), which leads to a profound down-regulation of β_2ARs and no change in the abundance of the β_1AR subtype.

Because β_1AR immunoreactivity could in theory be disrupted by an H_2O_2-dependent post-translational modification at the β_1AR C-tail (which harbors the recognition epitopes for both anti-β_1AR antibodies), we also performed radioligand binding experiments with ^{125}I-iodocyanopindolol, a high-affinity βAR antagonist that does not discriminate between β_1ARs and β_2ARs. Figure 1E shows that this alternative method also identifies an H_2O_2-dependent decrease in βAR density.

THE H_2O_2-DEPENDENT DECREASE IN β_1AR IMMUNOREACTIVITY IS PREVENTED BY GF109203X AND CARVEDILOL. Cardiomyocytes were challenged with H_2O_2 in the presence of compounds that inhibit various signaling enzymes implicated as downstream components of the β_1AR signaling response as an initial strategy to identify mechanism. Studies were performed in parallel on uninfected cardiomyocytes (to track H_2O_2-dependent effects on the native rat β_1AR) as well as cardiomyocytes that heterologously overexpress human β_1ARs. This approach allowed us to screen for possible differences in H_2O_2-dependent regulation of Gly^{49} (rodent) versus Ser^{49} (human) β_1AR variants. Immunoblotting studies on overexpressed human β_1ARs performed in parallel with Abcam and SC anti-β_1AR antibodies also allowed us to further validate the specificity of these reagents. Figure 2A shows that H_2O_2 treatment leads to similar changes in native and heterologously overexpressed β_1AR immunoreactivity. In each case, H_2O_2 treatment led to an initial decrease in β_1AR electrophoretic mobility (best detected at the 30-min time point) followed by a decrease in β_1AR immunoreactivity (detected at 60 min). Figure 2A shows that the H_2O_2-dependent mobility shift (at 30 min) and the

FIGURE 2 The H_2O_2-dependent Decrease in β_1AR Immunoreactivity Is Prevented by GFX or Carvedilol



(A and C) Cardiomyocytes were pretreated for 1 h with 10 μM GF109203X (GFX), 10 μM PP1, 10 μM H89, 10 μM Gö6976, 0.1 μM propranolol (prop), 1 μM isoproterenol (Iso), 1 μM carvedilol (carv), 10 μM pindolol (pin), 10 μM timolol (tim), 10 μM atenolol (aten), or 10 μM metoprolol (met) as indicated and then challenged with vehicle or 0.1 mM H_2O_2 for 60 min (unless indicated otherwise). **(B)** Treatment with vehicle or the indicated concentrations H_2O_2 (in the absence or presence of 1 μM carvedilol) followed a 24-h pre-incubation with 100 ng/ml pertussis toxin (PTX). Experiments in **A to C** were performed in parallel on cardiomyocyte cultures that did or did not overexpress the β_1AR transgene to compare stimulus-induced changes in native rat β_1ARs (tracked with SC anti-β_1AR antibody) and heterologously overexpressed human β_1ARs (tracked with SC and/or Abcam anti-β_1AR antibodies as indicated). Because β-actin immunoreactivity was not altered by β_1AR overexpression, a single β-actin blot from uninfected cultures is depicted in the figures as a protein loading control. H_2O_2 and carvedilol-dependent changes in endogenous or heterologously overexpressed β_1AR immunoreactivity are quantified in **A**, **right** ($n = 6$). **(D)** β_1AR-overexpressing cardiomyocytes were treated for 24 h with a panel of β_1AR ligands (at concentrations stipulated in **A**). Effects on β_1AR transgene abundance are depicted on **top**, with results for 3 separate experiments on different culture preparations quantified on the **bottom** (*$p < 0.05$ by analysis of variance followed by a Tukey post hoc analysis). For quantification of immunoreactivity (which is expressed as arbitrary units), levels of the truncated β_1AR species in ligand-treated cultures **(gray bars)** were normalized to the level of the truncated β_1AR species in the corresponding vehicle-treated culture **(black bar)**, which was set to 100%. **(E)** β_1AR-overexpressing cardiomyocytes were pretreated for 24 h with vehicle, 1 μM carvedilol, or 5 μM mitoTempo (mitoT, Sigma-Aldrich, St. Louis, Missouri) and then challenged with 100 μM H_2O_2 as indicated. Lysates were probed for β_1AR immunoreactivity with β-actin immunoreactivity included as a loading control. The experiment is representative of data obtained in 4 separate experiments on different culture preparations. **(F)** Lysates from cardiomyocytes treated for 1 h with vehicle or 100 μM H_2O_2 (following a 1-h pretreatment with vehicle or 1 μM carvedilol as indicated) were probed for cyclic adenosine monophosphate binding response element-protein (CREB) phosphorylation and CREB protein expression. All immunoblotting data represent results obtained in 3 to 5 separate experiments. Abbreviations as in Figure 1.

decrease in β_1AR immunoreactivity (at 60 min) are both completely abrogated by GF109203X (GFX; a general protein kinase C [PKC] inhibitor) but not by Gö6976 (which selectively blocks calcium-sensitive PKC isoforms or protein kinase D), the PKA inhibitor H-89, or the Src kinase inhibitor PP1.

Although there is precedent for a switch in the β_1AR's G protein-coupling specificity from G_s to G_i

under certain stimulatory conditions (17), the observation that the H_2O_2-dependent decrease in native or heterologously overexpressed β_1AR immunoreactivity is preserved in pertussis toxin (PTX)-pretreated cardiomyocytes (**Figure 2B**) indicates that H_2O_2-dependent regulation of β_1ARs is not through a G_i-dependent mechanism. Rather, these results indicate that H_2O_2 regulates endogenous rodent β_1ARs and heterologously overexpressed human β_1ARs in a similar manner and that H_2O_2-dependent regulation of β_1ARs is via a mechanism that requires a novel PKC isoform activity.

H_2O_2 treatments were also performed in the presence of various adrenergic receptor ligands to determine whether redox sensitivity is influenced by the activation state or conformation of the β_1AR. **Figures 2A and 2B** show that H_2O_2-dependent decreases in native and heterologously overexpressed β_1ARs are completely abrogated by carvedilol but not by isoproterenol or propranolol. The protective effect of carvedilol is also preserved in PTX-treated cardiomyocytes, and it is specific; various other βAR antagonists (pindolol, timolol, atenolol, and metoprolol) do not share this action (**Figure 2C**). Studies performed on cardiomyocytes that heterologously overexpressed human β_1ARs (where both FL and N-terminally truncated β_1ARs species could unambiguously be tracked with the Abcam antibody) also exposed an additional effect of carvedilol to increase basal levels of the more rapidly migrating N-terminally truncated β_1AR species (**Figure 2D**). This action is also unique to carvedilol; the abundance of the N-terminally truncated β_1AR species is not influenced by other βAR ligands.

These unique actions of carvedilol to protect β_1ARs from H_2O_2-dependent inactivation and increase expression of N-terminally truncated β_1ARs are intriguing, given reports that carvedilol might offer survival advantages over other βAR blockers in patients with HF (8). Although some have argued that carvedilol might exert distinct clinical actions by virtue of its unique pharmacologic profile (carvedilol acts as an inverse agonist for the G_s-PKA pathway but a biased agonist for non-G protein/β-arrestin-dependent signaling [11]), carvedilol also possesses antioxidant properties (9). Therefore, it was important to consider whether carvedilol protects β_1ARs from H_2O_2-dependent inactivation by limiting oxidative stress, in essence mimicking the actions of the mitochondrial-targeted antioxidant mitoTEMPO (Sigma-Aldrich, St. Louis, Missouri, which prevents the H_2O_2-depedent decrease in β_1AR (**Figure 2E**). The observations that the effect of carvedilol to increase the abundance of the more rapidly migrating N-terminally-truncated β_1AR species is not mimicked by mitoT (**Figure 2E**) and that carvedilol treatment does not block the H_2O_2 -dependent increase cAMP binding response element protein (CREB) phosphorylation at Ser^{133} (a signaling response that results from the activation of several H_2O_2 -sensitive signaling kinases that cooperate to phosphorylate CREB (18) (**Figure 2F**) argue that the β_1AR-regulatory actions of carvedilol cannot simply be ascribed to its antioxidant properties.

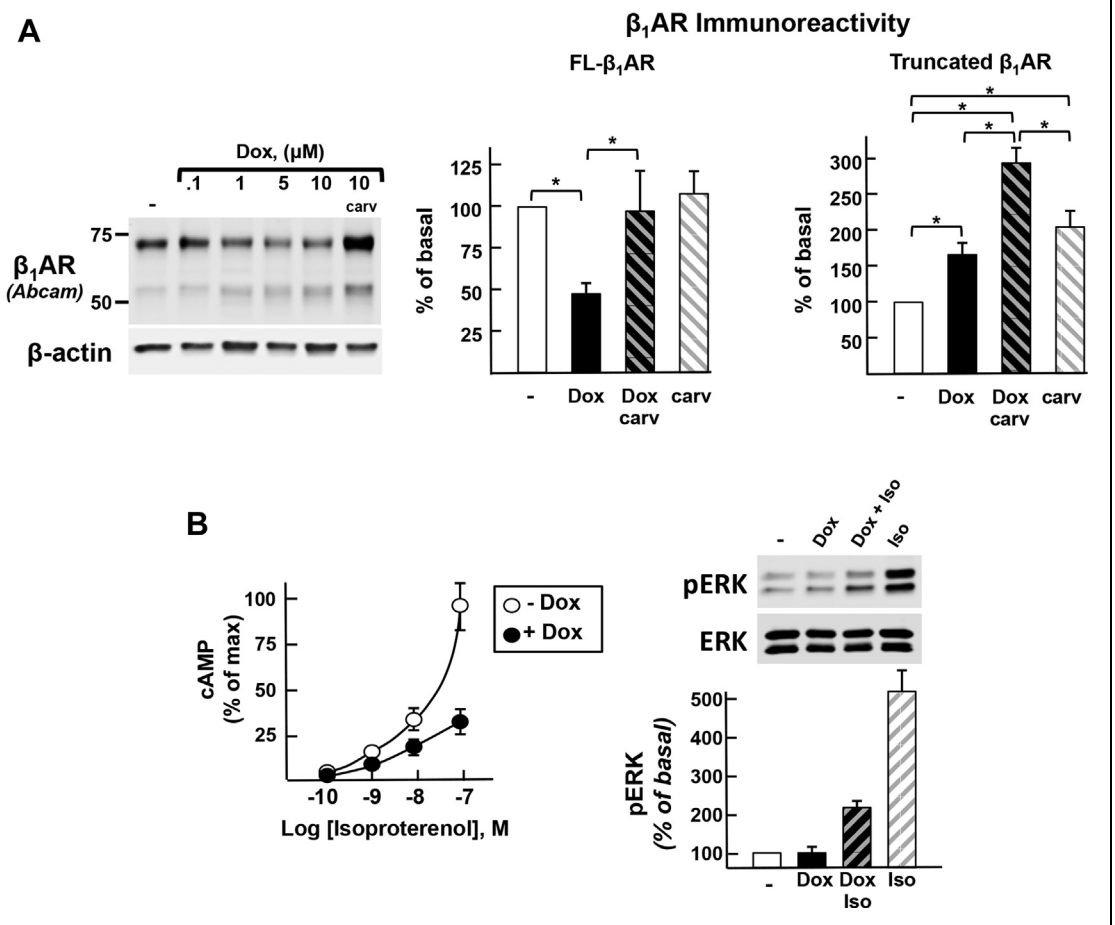
DOXORUBICIN DECREASES β_1AR LEVELS IN CARDIOMYOCYTES; CARVEDILOL PREVENTS THE DOXORUBICIN-INDUCED DECREASE IN β_1ARs. Doxorubicin is a chemotherapeutic agent that is highly effective in the treatment of various hematologic and solid tissue malignancies. Although the anticancer effects of doxorubicin derive primarily from its actions to intercalate into nucleic acid side chains and disrupt deoxyribonucleic acid/ribonucleic acid synthesis and repair, doxorubicin treatment also leads to the generation of ROS species that contribute to doxorubicin-induced cardiotoxicity (19). We therefore examined whether doxorubicin treatment influences β_1ARs.

Figure 3A shows that doxorubicin treatment leads to a dose-dependent decrease in FL-β_1AR immunoreactivity and that this action is associated with the predicted defect in βAR-signaling responses; isoproterenol-dependent increases in cAMP accumulation and ERK phosphorylation are blunted in doxorubicin-treated cardiomyocytes (**Figure 3B**). Of note, doxorubicin specifically regulates the β_1AR subtype; doxorubicin treatment does not lead to a change in β_2AR immunoreactivity (**Figure 4A**).

The doxorubicin-dependent decrease in β_1AR immunoreactivity is prevented by carvedilol (**Figure 3A**), much like the response to an acute challenge with H_2O_2. However, chronic 24-h doxorubicin treatment also leads to the accumulation of the smaller ~55-kDa β_1AR species. This band, which increases in cardiomyocytes treated with carvedilol alone, becomes prominent in cardiomyocytes treated with doxorubicin in the presence of carvedilol.

N-TERMINALLY TRUNCATED β_1ARs THAT ACCUMULATE IN CARVEDILOL-TREATED CARDIOMYOCYTES CONSTITUTIVELY ACTIVATE AKT AND CONFER PROTECTION AGAINST DOXORUBICIN-INDUCED APOPTOSIS. Carvedilol has been characterized as an antagonist for the classic G_s-cAMP pathway and a biased agonist for the GRK/β-arrestin pathway that activates ERK and potentially other cardioprotective pathways (11,20); thus, the carvedilol-rescued β_1AR

FIGURE 3 Doxorubicin Treatment Leads to a Decrease in β_1AR Expression and Action in Cardiomyocytes

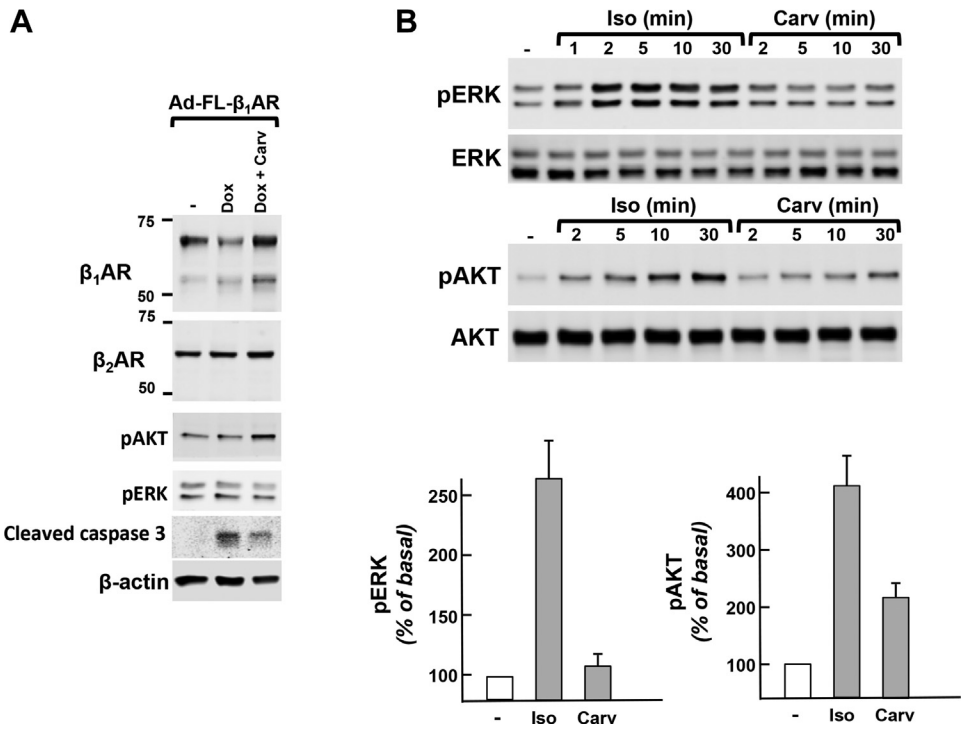


(A) β_1AR-overexpressing cardiomyocyte cultures were treated for 24 h with doxorubicin (Dox) in the absence or presence of 1 μM carvedilol as indicated, and lysates were probed for β_1AR expression, with β-actin immunoreactivity used as a loading control. A representative experiment is depicted on the **left**, with effects of doxorubicin (10 μM) and/or carvedilol on the abundance of the larger full-length (FL) and smaller truncated β_1AR species quantified on the **right** ($n = 7$; *$p < 0.05$ by analysis of variance). **(B)** Cardiomyocytes were pretreated for 24 h with vehicle or 10 μM doxorubicin and then challenged for 5 min with vehicle or isoproterenol (1 μM, unless indicated otherwise). Doxorubicin treatment resulted in a significant decrease in isoproterenol-dependent cyclic adenosine monophosphate (cAMP) accumulation and extracellular regulated kinase (ERK) phosphorylation ($p < 0.05$; $n = 4$). Abbreviations as in Figure 1.

could in theory confer protection against doxorubicin-induced apoptosis. In fact, Figure 4 shows that doxorubicin treatment leads to the accumulation of a caspase-3 cleavage product and that the level of this apoptosis marker is reduced when doxorubicin treatment is in the presence of carvedilol. Of note, this antiapoptotic action of carvedilol is associated with a modest increase in the phosphorylation of AKT but no detectable increase in ERK phosphorylation.

The failure to identify a carvedilol-dependent increase in ERK phosphorylation was somewhat surprising, given previous evidence that carvedilol acts as a biased agonist for the β-arrestin-ERK pathway (11). However, previous conclusions were based almost exclusively on experiments performed in model cell lines that heterologously overexpress epidermal growth factor receptors; evidence that carvedilol activates ERK in cardiomyocytes is conspicuously absent. In fact, Figure 4B shows that isoproterenol induces a rapid increase in ERK phosphorylation (at 2 to 5 min), whereas carvedilol-activated β_1ARs do not increase ERK phosphorylation under these conditions in neonatal rat cardiomyocyte cultures. Rather, carvedilol (much like isoproterenol) activates AKT; this response is detected after more prolonged agonist stimulations (at 30 min).

FIGURE 4 Carvedilol Activates AKT, Prevents the Doxorubicin-dependent Loss of β_1AR Expression, and Protects Against Doxorubicin-Induced Apoptosis

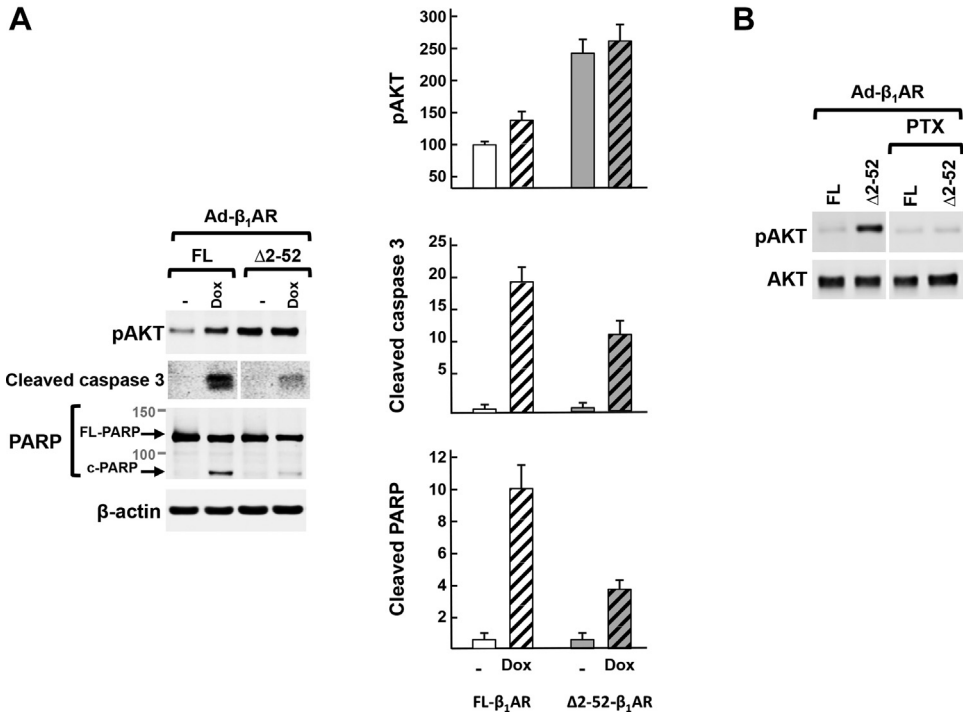


(A) Lysates from β_1AR-overexpressing cardiomyocytes treated for 24 h with vehicle, 10 μM doxorubicin alone, or 10 μM doxorubicin plus 1 μM carvedilol were probed for β_1AR and β_2AR expression, protein kinase B (AKT) and ERK phosphorylation, and caspase-3 cleavage (with β-actin immunoreactivity used as a loading control). Results are representative of data obtained in 4 separate experiments on separate culture preparations. **(B)** Lysates from cardiomyocytes treated with 0.1 μM isoproterenol or 1 μM carvedilol for the indicated intervals were probed for ERK and AKT phosphorylation, with immunoreactivity for the cognate protein serving as a loading control. A representative experiment is depicted on **top,** with maximal agonist-dependent phosphorylation responses for ERK (at 5 min, n = 5) or AKT (at 30 min, n = 3) quantified at the **bottom**, with the basal value in each individual experiment set to 100% as a reference **(open bar)** and agonist-dependent increases in immunoreactivity expressed relative to basal **(shaded bars)**. Abbreviations as in Figures 1 and 3.

These results raise the intriguing hypothesis that carvedilol rescues cardiomyocytes from doxorubicin-induced apoptosis by activating a cardioprotective AKT phosphorylation pathway. Two mechanisms are possible. In theory, carvedilol might induce cardioprotection by preventing redox inactivation of FL-β_1ARs because the carvedilol-rescued FL-β_1ARs would be stabilized in a conformation that activates AKT. Alternatively, the actions of carvedilol to increase expression of the truncated ~55-kDa β_1AR species might be cardioprotective, if the N-terminally truncated β_1AR species displays signaling bias to AKT or other antiapoptotic pathways. The N-terminally truncated Δ2-52-β_1AR species (designed to mimic the β_1AR cleavage product that accumulates in carvedilol-treated cardiomyocytes) was heterologously overexpressed in cardiomyocytes to resolve these alternative mechanisms. We previously showed that Δ2-52-β_1AR overexpression does not result in changes in basal cAMP levels or ERK phosphorylation in cardiomyocytes (12). However, **Figure 5A** shows that Δ2-52-β_1AR overexpression leads to the constitutive activation of AKT and reduced doxorubicin-dependent apoptosis (tracked as caspase-3 and poly [ADP-ribose] polymerase cleavage).

The G protein-independent/β-arrestin-dependent pathway that links G protein-coupled receptors to ERK activations can also lead to the activation of AKT; however, a β-arrestin-dependent Δ2-52-β_1AR-AKT activation pathway seemed unlikely given previous evidence that Δ2-52-β_1ARs display reduced agonist-dependent activation of ERK (12). Rather, **Figure 5B** shows that the increase in basal AKT phosphorylation in Δ2-52-β_1AR overexpressing cardiomyocytes is completely abrogated by PTX, implicating a PTX-sensitive G_i protein in this pathway. Collectively,

FIGURE 5 The N-terminally Truncated β_1AR That Accumulates in Carvedilol-Treated Cardiomyocytes Constitutively Activates AKT and Confers Protection Against Doxorubicin-Induced Apoptosis



(A) Cardiomyocytes that heterologously overexpress similar levels of FL or N-terminally truncated β_1ARs (Δ2-52) (as validated according to both immunoblot analysis and radioligand binding with ^{125}I-iodocyanopindolol, based on protocols detailed elsewhere [12]) were treated for 24 h with vehicle or doxorubicin. Lysates were probed for β_1AR expression, AKT phosphorylation, and caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage, with β-actin immunoreactivity as a loading control. A representative experiment is depicted on the **left**; results for pAKT (n = 9), caspase-3 cleavage (n = 4), or PARP cleavage (n = 6) are quantified on the **right** (with all results normalized to immunoreactivity in corresponding vehicle-treated FL-β_1AR expressing cardiomyocytes). **(B)** Effects of PTX (100 ng/ml) on AKT phosphorylation in FL-β_1AR or Δ2-52-β_1AR overexpressing cardiomyocytes. The data are representative of results obtained in 3 separate culture preparations. Abbreviations as in Figures 1 to 4.

these results identify a novel role for N-terminally truncated β_1ARs that accumulate in carvedilol-treated cardiomyocytes as activators of a G_i-AKT pathway and mediators of cardioprotection.

DISCUSSION

The factors that regulate βAR responsiveness, which provide hemodynamic support in response to stress but also contribute to the pathogenesis of HF, have been the focus of extensive investigation. The published data historically has focused on homologous desensitization mechanisms involving GRKs and β-arrestins that prevent βAR coupling to G proteins, promote βAR internalization, and terminate signaling via the cAMP pathway. However, cell-based studies showing that β_1ARs are relatively refractory to this form of desensitization raise questions as to the mechanism underlying the defect in β_1AR responsiveness that characteristically develops in HF (5,6). This dilemma is not necessarily resolved by studies that identify alternative mechanisms to influence cardiac catecholamine responsiveness because these mechanisms alter signaling by the β_2AR subtype (21–26). Similarly, although there is evidence that doxorubicin treatment leads to a selective decrease in cardiac β_1AR expression without an associated decrease in β_2ARs (27,28), these previous experiments do not specifically address the underlying mechanism (and the role of oxidative stress) because the changes in β_1AR expression occur in the context of a doxorubicin-induced contractile defect that alone would be predicted to impair βAR responsiveness. Studies reported herein provide novel evidence that oxidative stress, a stimulus that contributes to the

pathogenesis of HF, acts as a direct regulator of β_1AR expression and catecholamine responsiveness in cardiomyocytes.

In an attempt to identify mechanisms, we found that the H_2O_2-dependent decrease in β_1AR expression is abrogated by GFX and carvedilol. The mechanism underlying the protection afforded by GFX remains uncertain. Although the β_1AR third intracellular loop contains a consensus phosphorylation motif for basophilic kinases (and could in theory serve as a substrate for PKC), phosphorylation at this site has previously been attributed to PKA (and not PKC). However, the β_1AR extreme C-terminus conforms to a PDZ motif that interacts with synapse-associated protein SAP97 (29), a scaffolding protein that serves as a platform to anchor higher order macromolecular complexes involving β_1ARs and signaling partners such as PKC (30,31). Studies to determine whether this interaction provides a mechanism for PKC regulation of β_1ARs and to determine the identity of the novel PKC isoform that prevents the H_2O_2-dependent decrease in β_1AR levels are ongoing.

The mechanism underlying carvedilol's ability to protect β_1ARs from H_2O_2-dependent inactivation also is not directly addressed by our studies but may be more explainable. Carvedilol contains a bulky aromatic amine substitution that is not present in other adrenergic ligands. Structural studies indicate that this bulky side group makes unique contacts with an extended β_1AR ligand-binding pocket that includes the redox-sensitive cysteines in extracellular loop 2 (32), the presumptive redox-sensitive molecular determinants on the β_1AR extracellular surface. It is tempting to speculate that carvedilol prevents H_2O_2- or doxorubicin-dependent decreases in β_1ARs by directly shielding these cysteines from redox inactivation, producing a conformational rearrangement of the extracellular surface so as to bury the redox-sensitive disulfide bonds within the receptor structure, or stabilizing the structure of the reduced receptor.

Previous published data showed that lipophilic ligands (e.g., alprenolol, carvedilol) act as pharmacologic chaperones to increase levels of immature, smaller 47- to 55-kDa forms of the β_1AR that lack core glycans (and presumably represent N-terminally truncated β_1ARs), which otherwise are retained in the endoplasmic reticulum and targeted for degradation (33); these could also explain carvedilol's actions to increase levels of the N-terminally truncated β_1AR species. Our results suggest that levels of the N-terminally truncated β_1AR (the minor β_1AR species in most cardiomyocyte preparations) are limited by endoplasmic reticulum quality control systems that recognize truncated β_1ARs as improperly or incompletely folded proteins; these findings also suggest that carvedilol stabilizes N-terminally truncated β_1ARs in a conformation that facilitates their exit from the endoplasmic reticulum and trafficking to their site of action.

Carvedilol abrogates H_2O_2- and doxorubicin-induced decreases in FL-β_1AR expression and enhances expression of a cardioprotective N-terminally truncated β_1AR species; these findings suggest 2 possible mechanisms that could contribute to carvedilol's antiapoptotic/cardioprotective actions in animal models of ischemia/reperfusion injury and acute myocardial infarction (34) as well as to its actions to protect against doxorubicin-induced cardiotoxicity in the clinic (35–38). First, the carvedilol-rescued FL-β_1AR would be stabilized in a conformation that activates cardioprotective signaling pathways but not cAMP. Although previous studies in model cell lines showed that carvedilol-activated β_1ARs stimulate ERK, our studies identified AKT as a downstream effector of carvedilol-activated β_1ARs in cardiomyocytes. These findings resonate with the recent observation that carvedilol activates a β_1AR/β-arrestin–dependent pathway that stimulates the processing of certain micro-ribonucleic acids that activate AKT in cardiomyocytes (39). Second, N-terminally truncated β_1ARs that constitutively activate AKT accumulate in carvedilol-treated cardiomyocytes and would protect against doxorubicin-induced apoptosis. Of note, carvedilol is reported to protect bone marrow stem cells against H_2O_2-induced cell death, attenuate 6-hydroxydopamine–induced cell death in PC12 cells, and prevent doxorubicin-induced cardiomyopathy, in each case in association with the activation of AKT (40–42). These results suggest that a β_1AR-AKT pathway plays a more general role in mediating carvedilol's cytoprotective actions in different cell types.

The observation that the actions of carvedilol to prevent redox inactivation of FL-β_1ARs and enhance expression of N-terminally truncated β_1ARs (i.e., promote the accumulation of 2 β_1AR species that activate a cardioprotective AKT pathway) are not shared by various other β-blockers also deserves emphasis. Although the notion that β-blockers provide clinical benefit for patients with HF is not disputed, uncertainties as to whether β-blockers exert a class effect (i.e., can be used interchangeably in the treatment of patients with HF), or whether carvedilol offers superior clinical efficacy, has never been fully resolved. The incremental survival advantage afforded by carvedilol over metoprolol in COMET

(Carvedilol Or Metoprolol European Trial) (one of the few published large-scale, head-to-head randomized comparisons of carvedilol vs. another β-blocker) has variably been attributed to carvedilol's unique pharmacologic actions or should be dismissed as a feature of the study design and a possible difference in the efficacy of β_1-blockade (8,43). Our results identify a unique β_1AR-dependent cardioprotective action of carvedilol that may be pertinent to this controversy because it is predicted to offer a meaningful survival advantage.

Finally, the observation that N-terminally truncated β_1ARs constitutively activate AKT through a PTX-sensitive G_i-dependent mechanism was surprising. Although there are isolated reports that describe βAR signaling via PTX-sensitive G_i proteins, β_1AR-dependent responses traditionally have been attributed to G_s or G protein-independent, β-arrrestin-dependent pathways. However, there is recent evidence that carvedilol stabilizes β_1ARs in a conformation that initiates $G\alpha_i$-dependent β-arrestin biased signaling responses (44) and that β-arrestin recruitment may be dispensable for βAR activation of ERK in certain model cell types (45,46). Collectively, these results serve to challenge prevailing dogma regarding the molecular transducers that link βARs to cardioprotective ERK or AKT pathways. The role of GRKs or β-arrestins in H_2O_2- or doxorubicin-mediated decreases in β_1AR signaling or the signaling responses evoked by N-terminally truncated β_1ARs that attenuate doxorubicin-induced apoptosis are the focus of ongoing studies.

CONCLUSIONS

These studies identify a novel redox-induced mechanism that controls cardiac β_1AR responsiveness. The studies also identify a novel β_1AR regulatory action for carvedilol, providing a framework to use carvedilol as a prototype for the design of next-generation β_1AR-selective compounds with unique β_1AR regulatory cardioprotective properties.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: A decrease in β_1AR expression and defective catecholamine-dependent responses are hallmarks of HF, but the precise mechanisms that drive HF-induced changes in β_1AR responsiveness remain uncertain because β_1ARs are relatively refractory to agonist-dependent desensitization in cell-based studies. Similarly, βAR inhibitors have become mainstays in the therapy of HF; however, the factors that contribute to their seemingly counterintuitive cardioprotective actions (whether they act by inhibiting maladaptive responses induced by sustained β_1AR activation or conversely by resensitizing cardiac βARs and restoring catecholamine responses) remain uncertain. This study expands current β_1AR signaling paradigms to show that oxidative stress disrupts β_1AR-dependent signaling responses in cardiomyocytes. We also show that certain βAR inhibitors protect β_1ARs from redox inactivation and promote the accumulation of an N-terminally truncated form of the β_1AR that displays a unique cardioprotective action.

TRANSLATIONAL OUTLOOK: Current guidelines recommend βAR inhibitors as first-line HF therapy, but controversies as to whether carvedilol (a nonspecific α_1, β_1, and β_2AR blocker with unique pharmacologic properties) offers survival advantage over other currently available βAR blockers have never been fully resolved. This study identifies novel cardioprotective actions for carvedilol that are not shared by other βAR blockers, showing that carvedilol prevents redox inactivation of the β_1AR, and it promotes the accumulation of N-terminally truncated β_1ARs that constitutively activate AKT and prevent doxorubicin-induced apoptosis. These unique β_1AR-regulatory cardioprotective properties are predicted to offer meaningful survival advantages.

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APPENDIX For an expanded Methods section, please see the online version of this paper.