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Mitochondrial production of reactive oxygen species contributes to the β-adrenergic stimulation of mouse cardiomycytes

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Non-technical summary   When under stress, the heart beat becomes stronger, in part due to enhanced fluxes of Ca^{2+} at the level of the cardiac cell. It is known that this effect is mediated by activation of β-receptors on the cardiac cell surface. This leads to modifications of intracellular proteins that in turn increase the flux of Ca^{2+} within the cell. In this study we show that activation of β-receptors increases the production of reactive oxygen species (ROS) in the heart cell. These ROS generate enhanced Ca^{2+} fluxes and more vigorous contraction. This finding shows a new cellular signalling route for regulating the power of the heart beat and might contribute to our understanding of diseases with defective cardiac contraction, such as heart failure.

Abstract   The sympathetic adrenergic system plays a central role in stress signalling and stress is often associated with increased production of reactive oxygen species (ROS). Furthermore, the sympathetic adrenergic system is intimately involved in the regulation of cardiomyocyte Ca^{2+} handling and contractility. In this study we hypothesize that endogenously produced ROS contribute to the inotropic mechanism of β-adrenergic stimulation in mouse cardio-myocytes. Cytoplasmic Ca^{2+} transients, cell shortening and ROS production were measured in freshly isolated cardiomyocytes using confocal microscopy and fluorescent indicators. As a marker of oxidative stress, malondialdehyde (MDA) modification of proteins was detected with Western blotting. Isoproterenol (ISO), a β-adrenergic agonist, increased mitochondrial ROS production in cardiomyocytes in a concentration- and cAMP–protein kinase A-dependent but Ca^{2+}-independent manner. Hearts perfused with ISO showed a twofold increase in MDA protein adducts relative to control. ISO increased Ca^{2+} transient amplitude, contraction and L-type Ca^{2+} current densities (measured with whole-cell patch-clamp) in cardiomyocytes and these increases were diminished by application of the general antioxidant *N*-acetylcysteine (NAC) or the mitochondria-targeted antioxidant SS31. In conclusion, increased mitochondrial ROS production plays an integral role in the acute inotropic response of cardiomyocytes to β-adrenergic stimulation. On the other hand, chronically sustained adrenergic stress is associated with the development of heart failure and cardiac arrhythmias and prolonged increases in ROS may contribute to these defects.

Introduction

The sympathetic adrenergic system plays a central role in our ability to rapidly respond to various types of threats. One important target of adrenergic stimulation is the heart, where activation of β-adrenergic receptors causes increases in heart rate (chronotropy), relaxation speed (lusitropy) and contractility (inotropy). β-Adrenergic-induced increases in relaxation speed and contractility are considered to be a consequence of cAMP-dependent protein kinase A (PKA)-mediated phosphorylation of proteins involved in cardiomyocyte Ca^{2+} handling (Bers, 2002). Two important Ca^{2+} handling proteins that are targeted by this phosphorylation are phospholamban and the sarcoplasmic reticulum (SR) Ca^{2+} release channel (the ryanodine receptor, RyR). Phosphorylation of phospholamban leads to increased activity of the SR Ca^{2+}-ATPase (SERCA), which accelerates SR Ca^{2+} uptake and hence increases SR Ca^{2+} loading (MacLennan & Kranias, 2003). SR Ca^{2+} release can be enhanced by RyR phosphorylation that increases the Ca^{2+} channel open probability (Zalk *et al.* 2007). Moreover, Ca^{2+} entry into the cardiac cell is increased by PKA-mediated effects on the L-type Ca^{2+} channel and increased Ca^{2+} entry through this channel will further amplify the release of SR Ca^{2+} via the Ca^{2+}-induced Ca^{2+} release mechanism (Bassani *et al.* 1995). The $Ca_v1.2$-subunit of the L-type Ca^{2+} channel can be phosphorylated on Ser1928 and this is widely considered to be mediated by PKA (Davare & Hell, 2003). However, it is uncertain whether this phosphorylation is the direct cause of enhanced L-type Ca^{2+} current (I_{CaL}) following β-adrenergic stimulation because the response is still present in knock-in mice with targeted mutation of Ser1928 to alanine, which abolishes the PKA-induced phosphorylation (Lemke *et al.* 2008). Furthermore, a recent study identified Ser1700 as the primary regulatory site for the response to β-adrenergic stimulation (Fuller *et al.* 2010).

The faster and larger Ca^{2+} transients following β-adrenergic stimulation result in enhanced myofilament contractions. Adrenergic signalling in the heart is thus of critical importance for acute increases of cardiac output. However, chronically sustained adrenergic stress is associated with the development of heart failure and cardiac arrhythmias (Clark & Cleland, 2000; Marx *et al.* 2000; Curran *et al.* 2007).

Various pathological states have been coupled to increased cellular production of reactive oxygen species (ROS), which include: superoxide ($O_2 \cdot^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$) (Houstis *et al.* 2006; Moylan & Reid, 2007). However, ROS are not only involved in pathological processes, but also participate in normal physiological signalling (Droge, 2002; Linnane & Eastwood, 2006). For instance, Ca^{2+} handling in cardiac cells has been shown to be altered by changes in ROS, where ROS activates the RyR and inhibits SR Ca^{2+} uptake (Zima & Blatter, 2006). Moreover, the cardiac I_{CaL} has been shown to be redox sensitive (Sims & Harvey, 2004; Hool, 2008). In this context, we recently showed that the saturated fatty acid palmitate disturbs Ca^{2+} handling in healthy cardiomyocytes via a mechanism involving markedly increased ROS production (Fauconnier *et al.* 2007).

In the present study, we measured mitochondrial ROS production and cellular Ca^{2+} handling in isolated mouse cardiomyocytes. We tested the following hypotheses: (i) β-adrenergic stimulation increases mitochondrial ROS production in cardiomyocytes; (ii) this endogenous ROS production alters cellular Ca^{2+} handling such that it effectuates cardiac inotropy; (iii) the effect involves altered I_{CaL}. Our results are in accordance with the hypotheses. Thus, application of the β-adrenergic agonist isoproterenol (ISO) increased mitochondrial ROS production in a cAMP–PKA-dependent manner and the stimulatory effect of ISO on cytoplasmic Ca^{2+} transients, cell shortening and I_{CaL} was blunted by pre-exposure to antioxidants.

Methods

Ethical approval

All experiments complied with the Swedish Animal Welfare Act, the Swedish Animal Welfare ordinance, and applicable regulations and recommendations from Swedish authorities. The study was approved by the Stockholm North Ethical Committee on Animal Experiments. The experiments comply with the policies and regulations of *The Journal of Physiology* (Drummond, 2009).

Cell isolation and stimulation

We used ∼4-month-old C57BL6 mice; in total 41 were used. The mice were killed by rapid neck disarticulation and the heart was excised. Single cardiac cells were isolated from the ventricles following the protocols developed by the Alliance for Cellular Signalling (AfCS Procedure Protocol ID PP00000 125) (Sambrano *et al.* 2002). After being loaded with fluorescent indicators, cardiomyocytes were attached to the bottom of a stimulation chamber, superfused with standard Tyrode solution at room temperature (∼24°C) and stimulated at 1 Hz with 1 ms current pulses (Fauconnier *et al.* 2007). The signals of fluorescent indicators were measured with confocal microscopy using a Bio-Rad MRC 1024 unit attached to a Nikon Diaphot inverted microscope. Measurements were performed on the largest possible in-focus areas of individual cells.

Mitochondrial ROS production

Changes in mitochondrial $O_2 \cdot^-$ production were monitored using MitoSOX Red (Invitrogen/Molecular Probes) and confocal microscopy (Fauconnier *et al.* 2007). Isolated cardiomyocytes were loaded with MitoSOX Red (5 μM) for 30 min at room temperature, followed by washout. MitoSOX Red was excited with light at 488 nm while measuring the emitted light collected through a 585 nm long-pass filter. Confocal images were obtained at the start, after 10 min of 1 Hz stimulation and after 10 min at 1 Hz stimulation in the presence of the following drugs: the β-adrenergic agonist isoproterenol (ISO, 1–100 nM; Sigma); the adenylyl cyclase activator forskolin (1 μM; Sigma), or the L-type Ca^{2+} channel agonist (–)-Bay K 8644 (1 μM; Sigma). Before application of ISO (100 nM), some cells were exposed to the PKA inhibitor H89 (5 μM; Calbiochem) or the cell-permeant myristoylated PKA inhibitor 14–22 amide (PKI, 5 μM; Calbiochem) (Xiao *et al.* 2006). In some experiments with ISO exposure, a cell-permeant form of the Ca^{2+} buffer BAPTA (BAPTA AM, 50 μM; Invitrogen/Molecular Probes) was added together with the fluorescent indicator. The signal from each cell was normalized to that immediately before application of a drug.

Changes in ROS induced by application of a high concentration of H_2O_2 (1 mM) were monitored with MitoSOX Red as described above but confocal images were obtained at 2 min intervals. Cardiomyocytes were stimulated at 1 Hz for 20 min before H_2O_2 application with the last 10 min in the absence or presence of the mitochondrial respiratory complex I inhibitor rotenone (2.4 μM; Sigma).

The fluorescence of endogenous flavin adenine dinucleotide (FAD)-linked proteins can alter with changes in mitochondrial energy metabolism and redox state in cardiomyocytes (Zima *et al.* 2003). The FAD-linked autofluorescence overlaps in wavelengths with the MitoSOX Red fluorescence and changes in autofluorescence might then interfere with the MitoSOX Red measurements. We therefore performed experiments with application of 100 nM ISO (same protocol as above) using cardiomyocytes not loaded with any fluorescent dye.

Mitochondrial membrane potential

Tetramethylrhodamine, ethyl ester, (TMRE; Invitrogen/ Molecular Probes) was used to measure mitochondrial membrane potential ($\Delta\Psi_m$) (Duchen *et al.* 1998). Isolated cardiomyocytes were loaded with TMRE (10 nM) for 15 min at room temperature, followed by washout. Confocal images of TMRE fluorescence were obtained by excitation at 488 nm while measuring the emitted light at $\geq$585 nm. Images were taken every 5 min and fluorescence signals were normalized to the fluorescence measured in each cell at the start of the experiment, which was set to 100%. At the end of each experiment, cells were depolarized by exposure to the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 10 μM).

Mitochondrial Ca²⁺ concentration

Rhod-2 (Invitrogen/Molecular Probes) was used to assess changes in the mitochondrial Ca^{2+} concentration. Isolated cells were loaded with rhod-2 (10 μM) for 30 min at 4°C, followed by 60 min washout at room temperature. Confocal images were obtained with excitation at 531 nm while measuring the emitted light at $\geq$585 nm.

Western blotting

Whole hearts were excised and immediately perfused in a Langendorff set-up with Tyrode solution in the presence or absence of ISO or H_2O_2. Hearts were then snap-frozen and analysed for malondialdehyde (MDA) protein adducts using Western blot and rabbit anti-MDA (Academy Bio-Medical, no. MD20A-R1a) and donkey anti-rabbit horseradish peroxidase (HRP) secondary antibody (Bio-Rad, no. 170-6515) as described previously (Aydin *et al.* 2009).

Cytosolic Ca²⁺ and cell shortening

The cytosolic Ca^{2+} concentration was measured with the fluorescent Ca^{2+} indicator fluo-3 and confocal microscopy (Fauconnier *et al.* 2007). Confocal images were obtained by line scanning along the long axis, with focus in the middle of the cell. Stored confocal images were analysed with ImageJ (National Institutes of Health; available at http://rsb.info.nih.gov/ij). To enable comparisons between cells, the amplitude of Ca^{2+} transients was assessed as the change in the fluo-3 fluorescence signal (ΔF) divided by the fluorescence immediately before a stimulation pulse given under control conditions (F_0). The decay rate of Ca^{2+} transients was assessed by measuring the time constant of the exponential part of the decay phase, ignoring the initial phase that often diverges from a monoexponential function. In some experiments the edges of the fluorescence images were used to measure cell length in the rested and the maximally contracted state and shortening is expressed as a percentage of the resting cell length.

The relationship between the free cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and fluorescence intensity becomes steeper at high $[Ca^{2+}]_i$ as fluo-3 approaches saturation. To assess the importance of this non-linearity of the fluo-3 signal, we used a pseudo-ratio to calculate $[Ca^{2+}]_i$ (Santiago *et al.*

2010):

$$[Ca^{2+}]_i = K_D F/F_0 / (K_D/[Ca^{2+}]_{dia} - F/F_0 + 1),$$

where K_D is the dissociation constant of fluo-3, which was set to 1.1 μM, and $[Ca^{2+}]_{dia}$ is the diastolic $[Ca^{2+}]_i$, which was set to 100 nM.

In some experiments the effect of Ca^{2+} transient facilitating agents (ISO, forskolin or (−)-Bay K 8644) were tested in the presence of the general antioxidant *N*-acetylcystein (NAC; 5 mM) or the mitochondria-targeted peptide antioxidant SS31 (200 nM) (Zhao *et al.* 2004; Szeto, 2006). Cardiomyocytes were then exposed to the antioxidant for 20 min before applying one of the Ca^{2+} transient facilitating agents.

L-type Ca^{2+} current (I_{CaL}) measurements

Whole-cell patch-clamp experiments were performed on isolated cardiomyocytes at room temperature (∼24°C) using an Axopatch 200B amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA).
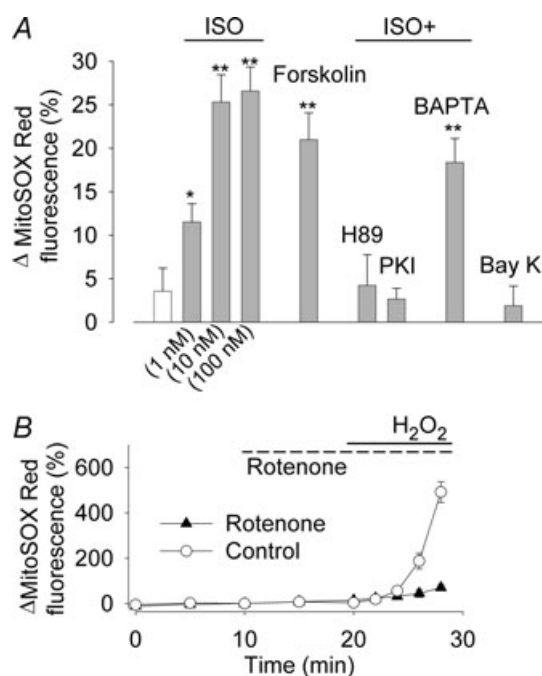

Figure 1. β-Adrenergic stimulation of mouse cardiomyocytes results in increased mitochondrial ROS production
A, mean data (±SEM) of the relative increase in mitochondrial superoxide production measured with MitoSOX Red following 10 min of 1 Hz stimulation under control conditions (white bar) or exposure to: ISO (1–100 nM as indicated); forskolin (1 μM); ISO (100 nM) in cells pre-exposed to H89 (5 μM), PKI (5 μM) or BAPTA AM (50 μM); (−)-Bay K 8644 (1 μM). Data in each group were obtained from ≥8 cells from at least 2 mice. *$P < 0.05$ and **$P < 0.001$ vs. the control group. *B*, MitoSOX Red fluorescence in cardiomyocytes exposed to H_2O_2 (1 mM) in the presence or absence (Control) of the respiratory chain inhibitor rotenone (2.4 μM) ($n \geq 13$ cells).

Currents, recorded with 2–3 MΩ patch pipettes, were normalized to cell membrane capacitance and expressed as current densities (pA pF^{-1}). To record I_{CaL}, the following pipette solution was used (mM): 120 CsCl, 6.8 MgCl$_2$, 5 Na$_2$ATP, 5 sodium creatine phosphate, 0.4 Na$_2$GTP, 11 EGTA, 4.7 CaCl$_2$ (120 nM free $[Ca^{2+}]$), and 20 Hepes; pH was adjusted with CsOH to 7.2. The bath solution contained (mM): 135 TEA-Cl, 2 MgCl$_2$, 10 glucose, 10 Hepes, 1.8 CaCl$_2$; pH adjusted to 7.4 with TEAOH. The current–voltage relationship was obtained by giving test pulses varying from −80 mV to +60 mV from a holding potential of −80 mV. Electrophysiological data acquisition and analyses were performed using pCLAMP (v. 6, Axon Instruments/Molecular Devices).

Statistics

Student's *t* test for paired or unpaired data was used when two measurements were compared. One-way ANOVA was used for multiple comparisons *vs.* a control group. One-way repeated measures ANOVA was used for repeated measurements. The Holm–Sidak *post hoc* test was used with both ANOVAs. $P < 0.05$ was considered significant. Data are expressed as means ± SEM.

Results

β-Adrenergic stimulation increases mitochondrial ROS production in cardiomyocytes

The effect of the β-adrenergic agonist ISO on mitochondrial ROS production was studied in freshly isolated cardiomyocytes using the fluorescent indicator MitoSOX Red (Fauconnier *et al.* 2007; Martins *et al.* 2008). Cardiomyocytes were paced at 1 Hz for 10 min in standard Tyrode solution and then exposed to various drugs for 10 min. FAD-linked autofluorescence may interfere with the MitoSOX Red signal and control experiments were therefore performed on cells not loaded with any dye. The fluorescence of unloaded cells amounted to ∼20% of the fluorescence in cells loaded with MitoSOX Red and the signal was not affected by exposure to 100 nM ISO for 10 min (Δfluorescence 0 ± 3%; $n = 9$ cells). Thus, FAD-linked autofluorescence would not affect the MitoSOX Red measurements. Experiments with cells not exposed to any drug showed no significant ($P > 0.05$) increase in MitoSOX Red fluorescence (Fig. 1*A*). Application of ISO caused a concentration-dependent increase in the MitoSOX Red fluorescence, with ∼10% increase at 1 nM ISO (Fig. 2*A*) and a maximum increase of ∼25% at ≥10 nM ISO. Application of forskolin, which directly stimulates cAMP formation, resulted in an increase in the MitoSOX Red fluorescence similar to the

maximum response with ISO. Moreover, ISO did not increase the MitoSOX Red fluorescence in cardiomyocytes pre-exposed to the PKA inhibitors H89 or PKI. These results indicate that the increased mitochondrial ROS production induced by β-adrenergic stimulation occurred via cAMP–PKA-dependent signalling.

Previous studies on intact and permeabilized cardiomyocytes have shown large increases in mitochondrial ROS production to be accompanied by depolarized $\Delta\Psi_m$ (Fauconnier et al. 2007; Nagasaka et al. 2007). We used TMRE to assess whether exposure to ISO induces a change in $\Delta\Psi_m$ and confocal images from one such experiment are shown in Fig. 2B. Exposure to 100 nM ISO for 10 min had no effect on the TMRE fluorescence ($112 \pm 5\%$ of the pre-exposure control; $n = 10$ cells from 2 mice, $P > 0.05$), whereas the subsequent exposure to the mitochondrial uncoupler FCCP (10μM) resulted in a marked depolarization of $\Delta\Psi_m$ with the TMRE signal decreasing to $29 \pm 10\%$ of the control ($P < 0.01$).

The ISO-induced increase in mitochondrial ROS production may be a consequence of increases in SR Ca^{2+} release and/or the amplitude of cytosolic Ca^{2+} transients. Therefore experiments were performed where the Ca^{2+} transient amplitude was increased by application of the L-type Ca^{2+} channel agonist (–)-Bay K 8644, but this did not increase the MitoSOX Red fluorescence (Fig. 1A). Experiments were also performed on cardiomyocytes pre-loaded with the Ca^{2+} buffer BAPTA. Although Ca^{2+} transients were minimal ($\Delta F/F_0 = 0.25 \pm 0.09$, $n = 5$) and contractions undetectable in these BAPTA-loaded cells, ISO still induced an increase in the MitoSOX Red fluorescence (Fig. 1A). Thus, these experiments indicate that the increase in mitochondrial ROS production induced by β-adrenergic stimulation is not a consequence of increases in SR Ca^{2+} release or amplitude of cytosolic Ca^{2+} transients.

It has been suggested that there are Ca^{2+} microdomains through which Ca^{2+} may be directly channelled between spatially associated SR Ca^{2+} release sites and mitochondria (Maack & O'Rourke, 2007). These tentative Ca^{2+} micro-domains might then allow an ISO-induced stimulation of SR Ca^{2+} release to increase the mitochondrial Ca^{2+} concentration, and hence affect mitochondrial ROS, even when the cytosolic Ca^{2+} is buffered by BAPTA. We therefore measured the mitochondrial Ca^{2+} concentration with rhod-2 before and after exposing BAPTA-loaded cardiomyocytes to ISO (100 nM) and confocal images from one such experiment are shown in Fig. 2C. The results showed no ISO-induced change in the rhod-2 fluorescence ($105 \pm 3\%$ of the pre-exposure control; $n = 11$ cells from one mouse, $P > 0.05$).

Control experiments were performed to assess the extent of ROS increase induced by β-adrenergic stimulation. Cardiomyocytes paced at 1 Hz were then exposed to 1 mM H$_2$O$_2$, which would increase the

mitochondrial [O$_2\cdot^-$] by inducing product inhibition of the superoxide dismutase in the mitochondrial matrix (SOD2) and thereby inhibit conversion of O$_2\cdot^-$ to H$_2$O$_2$ (McAdam et al. 1977; Hearn et al. 1999; Aydin et al. 2009). Application of H$_2$O$_2$ resulted in $\sim$500% increase in the MitoSOX Red fluorescence and this increase depended on mitochondrial respiration, because it was markedly smaller when the mitochondrial respiratory complex I was inhibited by rotenone (Fig. 1B). Thus, the increase in MitoSOX Red fluorescence obtained with a high concentration of H$_2$O$_2$ was markedly larger than that observed with β-adrenergic stimulation.

β-Adrenergic stimulation increases oxidation-induced protein modifications

Increased ROS production is associated with decomposition of polyunsaturated fatty acids, which leads to the formation of reactive carbonyl species that
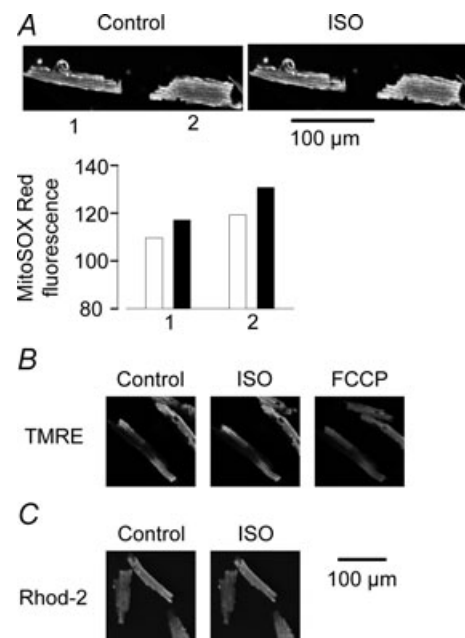

Figure 2. β-Adrenergic stimulation increases mitochondrial ROS, whereas it does not affect mitochondrial membrane potential ($\Delta\Psi_m$) or mitochondrial [Ca^{2+}]
A, representative confocal images showing two MitoSOX Red-loaded cardiomyocytes under control conditions and after exposure to 1 nM ISO. Measurements show $\sim$10% increase in fluorescence in both cells after ISO exposure (black bars). B, confocal images showing three cardiomyocytes loaded with TMRE to measure $\Delta\Psi_m$. Images were obtained under control conditions, after exposure to ISO (100 nM), and after dissipating $\Delta\Psi_m$ with FCCP (10μM). There was no noticeable effect of ISO exposure, whereas the fluorescence decreased after application of FCCP. C, confocal images of three cardiomyocytes loaded with rhod-2 to measure mitochondrial [Ca^{2+}] under control conditions and after 10 min exposure to ISO (100 nM), which had no noticeable effect on the fluorescence. Common scale bar for B and C.

can bind to protein (Aldini *et al.* 2007). To further address the question whether β-adrenergic stimulation increased ROS production, we used Western blotting and an antibody specific for protein binding of one such carbonyl species, malondialdehyde (MDA). Whole hearts were perfused in a Langendorff set-up for 30 min with Tyrode solution containing or lacking ISO (100 nM) and then frozen and analysed for MDA protein modifications. Hearts perfused with ISO showed a ~twofold increase of MDA protein adducts relative to controls (Fig. 3*A* and *B*). The relative magnitude of this change was assessed by perfusing hearts with H_2O_2 (0.2 mM) and this resulted in a ~sevenfold increase in MDA protein adducts (Fig. 3*C* and *D*), i.e. an increase much larger than that with ISO exposure. Thus, these results concur with the MitoSOX Red results presented above and imply that β-adrenergic stimulation triggers a robust, but not excessive, increase in mitochondrial ROS production.

Decreased inotropic response to β-adrenergic stimulation in cardiomyocytes in the presence of antioxidant

ROS and the redox state are known to alter SR Ca^{2+} cycling at multiple sites, including the release

and uptake machineries (Zima & Blatter, 2006). Since β-adrenergic stimulation resulted in an increased ROS production in cardiomyocytes, we investigated whether ISO-induced changes in cardiomyocyte Ca^{2+} handling are affected by the general antioxidant NAC. Ca^{2+} transient properties and contractility (cell shortening) were not affected by application of NAC on its own (Fig. 4). Application of ISO (100 nM) induced larger and faster Ca^{2+} transients and increased the contractility (cell shortening). The ISO-induced increase of the Ca^{2+} transient amplitude and cell shortening was reduced, but not abolished, in the presence of NAC, whereas the rate of Ca^{2+} decline was unaffected (Fig. 4). The actual size of the ISO-induced increase in Ca^{2+} transient amplitude, as well as the inhibitory effect of NAC on this increase, is underestimated when expressed as $\Delta F/F_0$ because fluo-3 approaches saturation (cf. Fig. 4*B* and *C*). Thus, the ISO-induced increase in Ca^{2+} transient amplitude was ~60% when expressed as $\Delta F/F_0$ and ~150% when converted to $[Ca^{2+}]_i$. When forskolin was
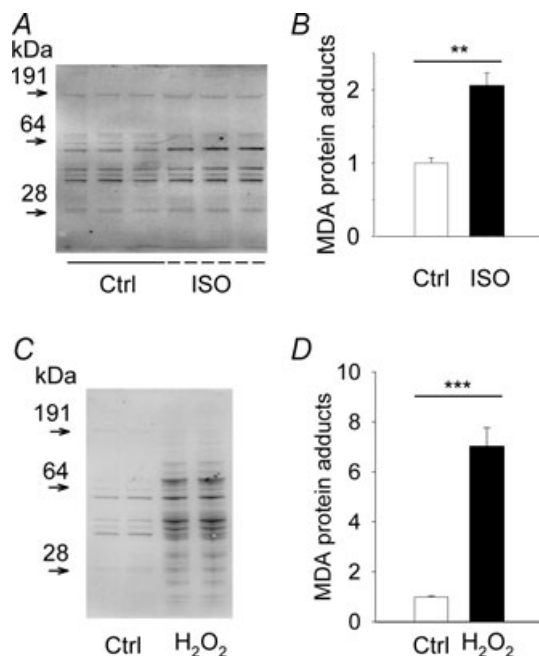

Figure 3. β-Adrenergic stimulation of mouse hearts increases MDA protein adducts
Western blots and mean (±SEM) densitometric quantifications of total MDA protein adducts in hearts perfused under control conditions (Ctrl) or in the presence of ISO (100 nM; *A* and *B*) or H_2O_2 (0.2 mM; *C* and *D*). Data normalized to the mean value in control, which was set to 1.0; $n = 3$–4 hearts in each group. **$P < 0.01$, ***$P < 0.001$.
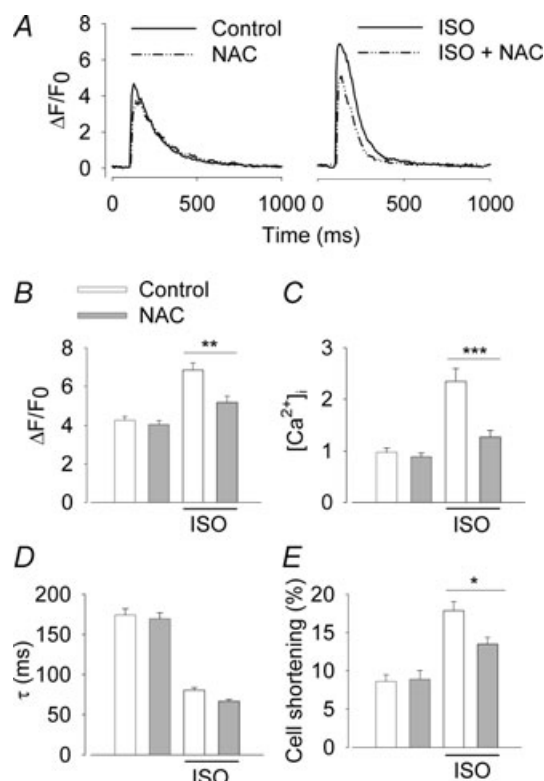

Figure 4. The β-adrenergic stimulatory effect on cardiomyocyte Ca^{2+} transient amplitude and cell shortening is blunted by the antioxidant NAC
A, representative Ca^{2+} transients from cardiomyocytes in the presence of ISO (100 nM) and/or NAC (5 mM) as indicated. Average (±SEM; $n \geq 19$ cells from at least three mice) amplitude of Ca^{2+} transients expressed as $\Delta F/F_0$ (*B*) or translated into $[Ca^{2+}]_i$ (*C*), Ca^{2+} transient decay time constant (*D*), and fractional cell shortening (*E*) without and with ISO as indicated and in the absence (white bars) or presence (grey bars) of NAC. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

used to directly activate the cAMP–PKA pathway, NAC decreased the Ca^{2+} transient amplitude (from $\Delta F/F_0$ 6.22 ± 0.23 to 5.46 ± 0.26 (representing ~30% decrease in [Ca^{2+}]$_i$; $P < 0.05$) and cell shortening (from 16.0 ± 0.6 to 10.8 ± 1.1% of the cell length; $P < 0.001$), whereas it did not affect the time constant of Ca^{2+} decline (from 101 ± 8 to 95 ± 6 ms; $P > 0.05$) (data obtained from ≥ 12 cells from at least two mice). On the other hand, the response to application of the L-type Ca^{2+} channel agonist (–)-Bay K 8644 was not significantly different ($P > 0.05$) in the absence and presence of NAC: Ca^{2+} transient amplitude ($\Delta F/F_0$) 6.13 ± 0.33 vs. 6.31 ± 0.32; cell shortening 17.7 ± 1.9 vs. 17.2 ± 1.4% of the cell length; time constant of Ca^{2+} decline 180 ± 16 vs. 162 ± 15 ms (data obtained from ≥ 10 cells from at least two mice). To sum up, the increases in Ca^{2+} transient amplitude and cell shortening induced by β-adrenergic stimulation or by direct activation of the cAMP–PKA pathway are blunted by the general antioxidant NAC.

In an additional series of experiments we tested the effect of SS31, a novel cell-permeant peptide antioxidant targeted to mitochondria (Zhao et al. 2004; Szeto, 2006), on the Ca^{2+} transient changes induced by β-adrenergic stimulation. Application of SS31 (200 nM) had no effect on Ca^{2+} transients under control conditions, whereas it decreased the Ca^{2+} transient amplitude in the presence of ISO (100 nM) by ~30% (Fig. 5). SS31 had no effect on the time constant of Ca^{2+} decline either in the absence or presence of ISO. Thus, these results with a mitochondria-targeted antioxidant are very similar to those obtained with the general antioxidant NAC.

To directly test whether increased mitochondrial ROS production affects Ca^{2+} transients, cardiomycytes were exposed to the mitochondrial complex III inhibitor anti-mycin A, which is known to increase ROS production (Chen et al. 2003). Initial experiments were performed with application of 25 μM antimycin A but this resulted in irregular contractions and cells stopped contracting in response to electrical stimulation after ~5 min exposure. We interpret this deleterious effect as a consequence of antimycin A-induced inhibition of mitochondrial respiration, because cardiomyocytes are heavily dependent on aerobic metabolism during repeated contractions. In subsequent experiments we therefore used a lower concentration of antimycin A (5 μM) and this concentration gave a 7.4 ± 1.9% increase in MitoSOX Red fluorescence ($P < 0.01$, $n = 27$ cells from two mice). The Ca^{2+} transient amplitude ($\Delta F/F_0$) increased from 4.06 ± 0.17 under control conditions to 4.63 ± 0.13 in the presence of antimycin A ($P < 0.01$; representing an increase in [Ca^{2+}]$_i$ of ~20%), whereas the time constant of Ca^{2+} decline was not affected (170 ± 8 vs. 168 ± 7 ms) (data obtained from > 30 cells from two mice).

β-Adrenergic stimulation is known to increase I_{CaL} in ventricular cardiomyocytes and this effect may not depend on PKA-induced phosphorylation of the channel (Lemke et al. 2008). Moreover, the cardiac I_{CaL} has been shown to be redox sensitive (Sims & Harvey, 2004; Hool, 2008). We therefore tested whether the NAC-induced inhibition of ISO-mediated inotropy in cardiomyocytes involves a ROS-dependent effect on the L-type Ca^{2+} channels. Whole cell patch clamp of isolated cardiomyocytes was used to measure I_{CaL}. Superfusing the cells with ISO (100 nM) caused a marked increase in I_{CaL} and a leftward shift of the I–V curve (Fig. 6A). The ISO-induced stimulation of I_{CaL} was almost fully abolished in the presence of NAC (Fig. 6B).

Discussion

The effects of β-adrenergic stimulation are generally mediated via cAMP and PKA. It was recently shown
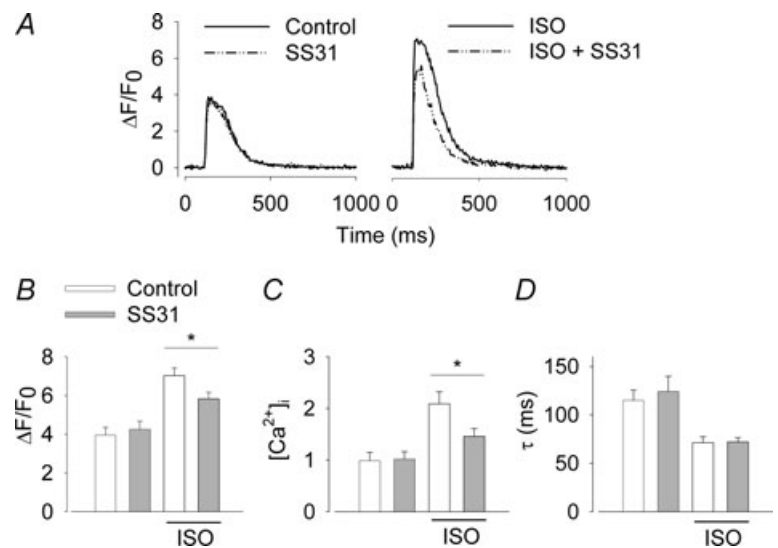

Figure 5. The β-adrenergic stimulatory effect on cardiomyocyte Ca^{2+} transient amplitude is blunted by the mitochondria-targeted antioxidant SS31
A, representative Ca^{2+} transients from cardiomyocytes in the presence of ISO (100 nM) and/or SS31 (200 nM) as indicated. Average ($\pm$SEM; $n \geq 10$ cells from at least two mice) amplitude of Ca^{2+} transients expressed as $\Delta F/F_0$ (B) or translated into [Ca^{2+}]$_i$ (C), and Ca^{2+} transient decay time constant (D) without and with ISO as indicated and in the absence (white bars) or presence (grey bars) of SS31. *$P < 0.05$.

that cAMP can be generated within the mitochondria (Acin-Perez *et al.* 2009). Moreover, PKA, as well as PKA anchoring proteins, can be localized in the mitochondria (Papa *et al.* 2002). Prolonged (24 h) β-adrenergic stimulation has been shown to induce mitochondrial membrane depolarization and apoptosis in adult rat cardiomyocytes (Remondino *et al.* 2003; Menon *et al.* 2006). This apoptosis was inhibited by SOD/catalase mimetics and by overexpression of catalase, which indicates that the apoptotic signalling induced by β-adrenergic stimulation involves increased ROS production (Remondino *et al.* 2003). A recent study on permeabilized rat cardiomyocytes shows that addition of activated PKA results in a rapid and reversible increase in mitochondrial ROS production (Nagasaka *et al.* 2007). In accordance with these results, we now show that acute β-adrenergic stimulation with ISO induces a cAMP- and PKA-dependent increase in mitochondrial ROS production in intact ventricular mouse cardiomyocytes. In addition, we show that this increase in ROS plays a crucial role in the β-adrenergic inotropic effect, because the ISO-induced increases in Ca^{2+} transient amplitude is diminished in the presence of the general antioxidant NAC as well as the mitochondria-targeted antioxidant SS31.

The increase in mitochondrial ROS production induced by β-adrenergic stimulation might be a direct effect of activation of the cAMP–PKA signalling pathway. Alternatively, it can occur as a consequence of the increased Ca^{2+} transients in the presence of β-adrenergic stimulation. To study these possibilities we used forskolin to directly activate cAMP signalling, H89 or PKI to inhibit ISO-induced PKA signalling and (−)-Bay K 8644 or BAPTA to independently increase or decrease Ca^{2+} transients, respectively. The results show that mitochondrial

ROS production was increased by application of forskolin and the ISO-induced increase was inhibited by H89 and PKI. On the other hand, buffering of Ca^{2+} transients with BAPTA had no significant impact on the ISO-induced increase in ROS production and increasing the amplitude of Ca^{2+} transients with (−)-Bay K 8644 did not increase ROS production. Moreover, the increases in Ca^{2+} transient amplitude and cell shortening induced by ISO and forskolin were diminished by NAC, whereas NAC had no effect on the (−)-Bay K 8644-induced increases. Taken together, these results demonstrate that the increased mitochondrial ROS production with β-adrenergic stimulation occurs as a direct consequence of cAMP–PKA-dependent signalling and not as an indirect effect of increased Ca^{2+} transient amplitudes. Our results also show that the increased mitochondrial ROS production induced by β-adrenergic stimulation is not dependent on mitochondrial Ca^{2+} accumulation or membrane depolarization (see Fig. 3). However, the direct mechanism(s) by which activation of the cAMP–PKA signalling pathway leads to increased mitochondrial ROS production remains to be elucidated. Furthermore, the effects of ISO on cardiomyocyte Ca^{2+} handling have recently been shown to also depend on nitric oxide (Collins & Rodrigo, 2010) and possible interactions between reactive nitrogen species and ROS requires further attention.

The ROS-mediated increase in Ca^{2+} transient amplitude could, in principle, be due to either increased Ca^{2+} entry over the plasma membrane or increased Ca^{2+} release from intracellular Ca^{2+} stores. Our data support a mechanism whereby ROS, induced by β-adrenergic stimulation, facilitate Ca^{2+} entry through L-type Ca^{2+} channels. An increase in L-type Ca^{2+} entry would not only in itself increase cytosolic Ca^{2+}, but also enhance RyR Ca^{2+}
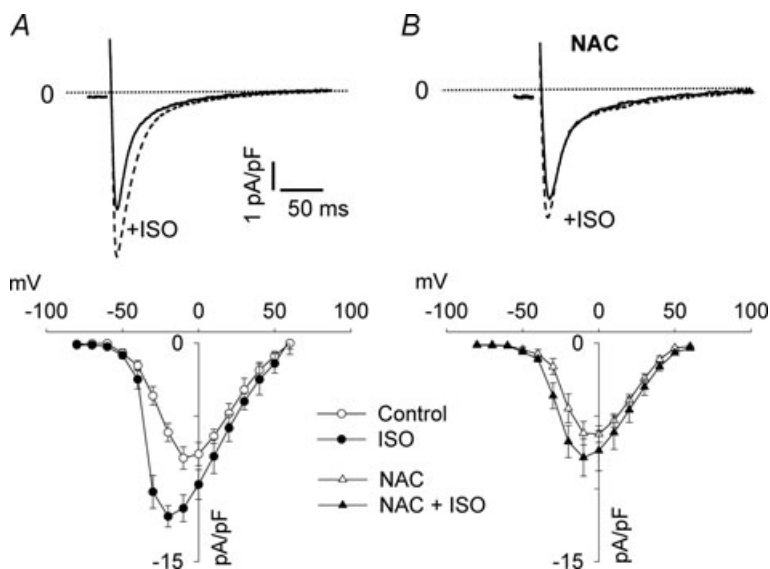

Figure 6. The β-adrenergic stimulation of cardiomyocyte L-type Ca^{2+} currents is inhibited by the antioxidant NAC
A, representative records of the L-type Ca^{2+} current (I_{CaL}) in cardiomyocytes ±100 nM ISO (upper panel, voltage step from −80 mV holding potential to −10 mV) and mean data (±SEM) of the peak current density (lower panel) (*n* = 6 cells in each group). *B*, same as *A* but in the presence of 5 mM NAC (*n* = 7 cells in each group).

release via increasing Ca^{2+}-induced Ca^{2+} release (Bassani *et al.* 1995).

Our results show faster Ca^{2+} transient decays with both ISO and forskolin and this effect, which is considered to be due to phosphorylation of phospholamban resulting in faster SR Ca^{2+} uptake (MacLennan & Kranias, 2003), was not affected by NAC or SS31. This shows that not all effects of β-adrenergic stimulation are ROS dependent. There are two subtypes of β-adrenergic receptors (β_1ARs and β_2ARs). β_1ARs are distributed across the entire surface of cardiomyocytes and activation of these is required for PKA-induced phosphorylation of phospholamban and acceleration of the decay of Ca^{2+} transients (Xiao, 2001; Nikolaev *et al.* 2010). β_2ARs, on the other hand, are specifically localized to the transverse tubules and hence more prone to affect plasma membrane proteins, such as L-type Ca^{2+} channels (Xiao, 2001; Nikolaev *et al.* 2010). Thus, localized signalling induced by activation of β_2ARs in the transverse tubules may involve increased ROS production, whereas this does not appear to be the case for the generalized signalling mediated via β_1ARs.

In the present study we exposed cardiomyocytes to antimycin A (5 μM) to directly stimulated mitochondrial ROS production and this resulted in ∼7% increase in MitoSOX Red fluorescence and ∼20% increase in [Ca^{2+}]$_i$ transient amplitude, which compares to increases of ∼25% and ∼150% after application of ISO. This indicates a dose-dependent ROS-induced stimulation of Ca^{2+} transients; unfortunately a higher concentration of antimycin A (25 μM) could not be used because of deleterious effects on cardiomyocyte function. On the other hand, we previously showed that application of palmitate resulted in a markedly increased ROS production in wild-type cardiomyocytes, which was accompanied by *decreased* Ca^{2+} transient amplitude and contractility, i.e. opposite to the present findings (Fauconnier *et al.* 2007). The opposite effect on Ca^{2+} transients can be explained by the fact that the ROS production was larger with palmitate than with ISO or antimycin A; the increase in MitoSOX Red fluorescence was ∼50% during 10 min exposure to palmitate compared to ∼25% with ISO and 7% with antimycin A. Moreover, application of palmitate caused a marked depolarization of $\Delta\Psi_m$ (Fauconnier *et al.* 2007), whereas the present results show no effect of ISO on $\Delta\Psi_m$. On the other hand, a marked depolarization of $\Delta\Psi_m$ was observed in rat cardiomyocytes after prolonged (24 h) exposure to ISO (Menon *et al.* 2006). Thus, these findings represent examples of the dual effects of ROS where an acute limited increase in ROS acts as a beneficial integral part in normal physiological signalling, whereas a large or prolonged increases can have adverse effects and contributes to pathological changes (Andrade *et al.* 2001; Goldstein *et al.* 2005; Rojas *et al.* 2006; Zima & Blatter, 2006).

Conclusions

Our results show that increased mitochondrial ROS production plays an integral role in the acute β-adrenergic-induced inotropic response of cardiomyocytes. This stimulatory effect is mediated via cAMP–PKA-dependent and Ca^{2+}-independent signalling. On the other hand, prolonged increases in ROS can have deleterious effects and may contribute to the development of heart failure and cardiac arrhythmias associated with chronically sustained adrenergic stress.

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Author contributions

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