Transient Exposure to Hydrogen Peroxide Causes an Increase in Mitochondria-Derived Superoxide As a Result of Sustained Alteration in L-Type Ca\textsuperscript{2+} Channel Function in the Absence of Apoptosis in Ventricular Myocytes

Helena M. Viola, Peter G. Arthur, Livia C. Hool

Abstract—We sought to understand the effect of a transient exposure of cardiac myocytes to H\textsubscript{2}O\textsubscript{2} at a concentration that did not induce apoptosis. Myocytes were exposed to 30 \textmu\text{mol}/L H\textsubscript{2}O\textsubscript{2} for 5 minutes followed by 10 U/mL catalase for 5 minutes to degrade the H\textsubscript{2}O\textsubscript{2}. Cellular superoxide was measured using dihydroethidium. Transient exposure to H\textsubscript{2}O\textsubscript{2} caused a 66.4\% increase in dihydroethidium signal compared with controls exposed to only catalase, without activation of caspase 3 or evidence of necrosis. The increase in dihydroethidium signal was attenuated by the mitochondrial inhibitors myxothiazol or carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone and when calcium uptake by the mitochondria was inhibited with Ru360. We investigated the L-type Ca\textsuperscript{2+} channel (I\textsubscript{Ca-L}) as a source of calcium influx. Nisoldipine, an inhibitor of I\textsubscript{Ca-L}, attenuated the increase in superoxide. Basal channel activity increased from 5.4 to 8.9 pA/pF. Diastolic calcium was significantly increased in quiescent and contracting myocytes after H\textsubscript{2}O\textsubscript{2}. The response of I\textsubscript{Ca-L} to \beta-adrenergic receptor stimulation was used as a functional reporter because decreasing intracellular H\textsubscript{2}O\textsubscript{2} alters the sensitivity of I\textsubscript{Ca-L} to isoproterenol. H\textsubscript{2}O\textsubscript{2} increased the \textit{K}_0.5 required for activation of I\textsubscript{Ca-L} by isoproterenol from 5.8 to 27.8 nmol/L. This effect and the increase in basal current density persisted for several hours after H\textsubscript{2}O\textsubscript{2}. We propose that extracellular H\textsubscript{2}O\textsubscript{2} is associated with an increase in superoxide from the mitochondria caused by an increase in Ca\textsuperscript{2+} influx from I\textsubscript{Ca-L}. The effect persists because a positive feedback exists among increased basal channel activity, elevated intracellular calcium, and superoxide production by the mitochondria. (Circ Res. 2007;100:1036-1044.)

Key Words: hydrogen peroxide \textbullet \ mitochondria \textbullet \ L-type Ca\textsuperscript{2+} channel

Reactive oxygen species (ROS) can act as signaling molecules able to stimulate and modulate a variety of biochemical and genetic systems, including the regulation of signal transduction pathways, gene expression, proliferation, and cell death by apoptosis.\textsuperscript{1} The regulation of signaling pathways by hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and superoxide has been linked to the development of various cardiovascular diseases including ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy, and congestive heart failure.\textsuperscript{2-4}

Mitochondria play an integral role in cellular metabolism and oxidative phosphorylation but are also a source of superoxide and an important determinant of the fate of a cell. Increased ROS production by mitochondria has been reported after exposing mitochondria to ROS in cardiac myocytes.\textsuperscript{5,6} The synchronized release of ROS by the mitochondria has been shown to induce oscillations in action-potential duration and life-threatening postischemic arrhythmias.\textsuperscript{5,7,8} A persistent increase in intracellular ROS is associated with pathological remodeling and myocardial dysfunction.\textsuperscript{2,4,9}

It has been suggested that increases in mitochondria-derived ROS are attributable to a direct effect of ROS on mitochondrial function.\textsuperscript{5,6} Another possible explanation for increased production of ROS by the mitochondrion is enhanced Ca\textsuperscript{2+} uptake attributable to altered L-type Ca\textsuperscript{2+} channel (I\textsubscript{Ca-L}) function. It is reasonable to postulate an involvement of the channel in oxidative stress because the \alpha\textsubscript{1C} subunit of the channel protein contains a number of cysteines that could be modified under oxidizing conditions. In support of this, channel function can be acutely modified by thiol-oxidizing compounds including H\textsubscript{2}O\textsubscript{2}.\textsuperscript{10-13} Acute exposure to H\textsubscript{2}O\textsubscript{2} or thiol-oxidizing agents has been shown to increase macroscopic basal I\textsubscript{Ca-L}.\textsuperscript{10,11,13,14} In addition, adrenergic regulation of the channel is modified in response to alterations in
the ROS production of the cell. A decrease in cellular production of superoxide or H$_2$O$_2$ has been shown to increase the sensitivity of the channel to β-adrenergic receptor stimulation.\textsuperscript{10,11,15} Therefore, there is good evidence that the activity of $I_{\text{Ca,L}}$ is responsive to alterations in the redox state of the cell.

In this study, we sought to understand the effects of H$_2$O$_2$ on myocyte function at a concentration insufficient to cause apoptosis or necrosis. We found that transient exposure to H$_2$O$_2$ induces an increase in mitochondria-derived superoxide in ventricular myocytes. The increase in cellular superoxide is reversible and is associated with increased intracellular Ca$^{2+}$ influx of Ca$^{2+}$ into the mitochondria as a result of an increase in basal $I_{\text{Ca,L}}$ density. The increase in basal $I_{\text{Ca,L}}$ persisted for several hours after exposure to H$_2$O$_2$. We propose a model to explain the data and the persistent response. We suggest that this may be a possible mechanism for pathophysiological remodeling associated with transient oxidative stress that involves elevated intracellular Ca$^{2+}$ and ROS.

**Materials and Methods**

**Detection of Superoxide**
Guinea pig ventricular myocytes and rat neonatal ventricular myocytes were isolated using a collagenase digestion method, as previously described.\textsuperscript{10,16} Generation of superoxide was assessed in 24-hour guinea pig myocytes or 36-hour contracting neonatal myocytes using the fluorescent indicator dihydroethidium (DHE) (5 μmol/L, 515- to 560-nm excitation filter, 590 long pass emission; Molecular Probes), as previously described.\textsuperscript{17} Application of the superoxide scavengers N-tert-butyl-a-phenyl-nitrone and superoxide dismutase significantly decreased DHE signal, confirming specificity of the indicator for superoxide (n=4).\textsuperscript{17} Comparisons using inhibitors were made with cells exposed to 30 μmol/L H$_2$O$_2$ for 5 minutes followed by 10 U/mL catalase for 5 minutes and were performed on the same day. For further details regarding cell isolation and detection of superoxide, see the online data supplement, available at http://circres.ahajournals.org.

**Quantitation of Apoptosis/Caspase 3 Activity Assay**
Caspase 3 activity was measured using Ac-DEVD-AMC (Promega) as a substrate.\textsuperscript{18,19} For further details, see the online data supplement.

**Determination of Intracellular Ca$^{2+}$**
Intracellular Ca$^{2+}$ was monitored using the fluorescent indicator Fura-2 acetoxymethyl ester (1 μmol/L, Molecular Probes). Fluorescent ratios at 340/380 nm excitation, 510 nm emission were measured over 50 ms at 1-minute intervals on a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. Metamorph 6.3 was used to quantify the signal by manually tracing myocytes. An equivalent region not containing cells was used for background subtraction. The fluorescent ratios recorded over 3 minutes before and immediately after addition of H$_2$O$_2$ and catalase were averaged. We performed calibrations to determine intracellular Ca$^{2+}$ concentrations in guinea pig ventricular myocytes as previously described (see the online data supplement).

**Data Acquisition for Patch-Clip Studies**
The whole-cell configuration of the patch-clip technique was used to record L-type Ca$^{2+}$ currents up to 9 hours after isolation of myocytes as described previously.\textsuperscript{10} For further details, see the online data supplement.

![Image](https://example.com/image)

**Results**

**Transient Exposure to H$_2$O$_2$ Is Associated With an Increase in DHE Signal**
We examined the effect of a transient exposure to H$_2$O$_2$ on DHE signal in adult ventricular myocytes. We chose DHE...
because it is a good indicator of changes in intracellular superoxide and does not interact with H$_2$O$_2$. However, intracellular superoxide is rapidly dismutated to H$_2$O$_2$, and we have shown that increases in cellular superoxide parallel increases in cellular H$_2$O$_2$. We began the experiments by titrating increasing concentrations of H$_2$O$_2$ until we detected an increase in cellular superoxide. We found that 20 to 30 $\mu$mol/L H$_2$O$_2$ was the minimum concentration required to reproducibly induce an increase in cellular superoxide. Myocytes were exposed to 30 $\mu$mol/L H$_2$O$_2$ for 5 minutes followed by 10 U/mL catalase for 5 minutes to degrade the peroxide. The concentration of catalase was sufficient to completely degrade extracellular H$_2$O$_2$ (n=5; see the online data supplement). In 45 cells, 30 $\mu$mol/L H$_2$O$_2$ caused a 66.4±8.6% increase in DHE signal (Figure 1A and 1B). The increase in superoxide did not cause necrosis in any of the 45 cells tested (assessed with propidium iodide uptake) or apoptosis determined by caspase 3 assay (Figure 1C).

Source of Increased Production of Superoxide Is the Mitochondria
We examined different sources for the increase in superoxide. In vascular smooth muscle cells, NAD(P)H oxidase is a prominent source of superoxide. Cells were exposed to 50 $\mu$mol/L gp91ds-tat peptide, a concentration of the peptide that is sufficient to inhibit angiotensin II–induced superoxide production in aortic rings by preventing association of gp47phox with gp91phox in NAD(P)H oxidase. The cells were then exposed to 30 $\mu$mol/L H$_2$O$_2$ followed by catalase and from another guinea pig ventricular myocyte before and after exposure to 50 $\mu$mol/L gp91ds-tat peptide followed by H$_2$O$_2$, as indicated. Means±SE of the ratio of fluorescence for guinea pig ventricular myocytes exposed to H$_2$O$_2$ or gp91ds-tat peptide, as indicated, are shown at right. B, DHE recorded from a guinea pig ventricular myocyte before and after exposure to 30 $\mu$mol/L H$_2$O$_2$ followed by catalase and from another guinea pig ventricular myocyte before and after exposure to 50 $\mu$mol/L allopurinol followed by H$_2$O$_2$, as indicated. Means±SE of the ratio of fluorescence for guinea pig ventricular myocytes exposed to H$_2$O$_2$ or allopurinol, as indicated, are shown at right. C, DHE recorded from a guinea pig ventricular myocyte before and after exposure to 30 $\mu$mol/L H$_2$O$_2$ followed by catalase and from another guinea pig ventricular myocyte before and after exposure to 100 $\mu$mol/L L-NAME followed by H$_2$O$_2$, as indicated. Means±SE of the ratio of fluorescence for guinea pig ventricular myocytes exposed to H$_2$O$_2$ or L-NAME, as indicated, are shown at right.
We examined whether xanthine oxidase was a possible source of superoxide by exposing the cells to 50 \(\mu\)mol/L allopurinol before \(H_2O_2\). In 7 cells, 30 \(\mu\)mol/L \(H_2O_2\) caused a 49.6 \(\pm\) 5.5% increase in DHE signal that was not significantly different from the increase in DHE signal recorded in the absence of allopurinol (66.4 \(\pm\) 8.6%, \(n=45\); Figure 2B). Similarly when cells were exposed to the nitric oxide synthase inhibitor \(N^G\)-nitro-L-arginine methyl ester (L-NAME) (100 \(\mu\)mol/L), there was no change in the DHE signal compared with cells exposed to \(H_2O_2\) in the absence of L-NAME (74.4 \(\pm\) 22.6%, \(n=7\) versus 66.4 \(\pm\) 8.6%, \(n=45\); \(P=NS\); Figure 2C).

We examined whether mitochondria were the source of superoxide by partially reducing mitochondrial membrane potential with 2 nmol/L carbonyl cyanide \(p\)-(trifluoromethoxy) phenyl-hydrazone (FCCP), an uncoupler of oxidative phosphorylation. In 10 cells, FCCP significantly attenuated the increase in DHE signal (\(P<0.05\); Figure 3A). In addition, exposure of myocytes to 30 \(\mu\)mol/L \(H_2O_2\) for 5 minutes followed by 10 U/mL catalase for 5 minutes caused a 11.6 \(\pm\) 5.6% increase in mitochondrial membrane potential assessed with the fluorescent indicator JC-1 (\(n=13\); Figure 3B; see also the online data supplement). We performed additional experiments in which we partially inhibited the electron transport chain with 7 nmol/L myxothiazol. In 14 cells, myxothiazol significantly decreased DHE signal (\(P<0.05\); Figure 3C). Consistent with previously published data, FCCP and myxothiazol decreased DHE signal in the absence of \(H_2O_2\) (Figure 3A and 3C, inset, right). These data strongly suggest that the source of increase in superoxide following exposure to \(H_2O_2\) is the mitochondria.

**Increase in Cellular Superoxide Requires an Increase in Uptake of Ca\(^{2+}\) by the Mitochondria**

We investigated whether an uptake of Ca\(^{2+}\) into the mitochondria is a requirement for the increase in superoxide. Ru360 (2 \(\mu\)mol/L), an inhibitor of the mitochondrial Ca\(^{2+}\) uniporter, applied before or after 30 \(\mu\)mol/L \(H_2O_2\) significantly attenuated the increase in DHE signal (\(P<0.05\); Figure 4A). To investigate the source of calcium, we exposed cells to the \(I_{Ca-L}\) inhibitor nisoldipine (2 \(\mu\)mol/L) before or after 30 \(\mu\)mol/L \(H_2O_2\). Nisoldipine significantly attenuated the increase in DHE signal (\(P<0.05\); Figure 4B), suggesting that calcium influx through \(I_{Ca-L}\) is a requirement for the increase in superoxide.
in cellular superoxide. Oxidative stress can cause Ca\(^{2+}\) release from ryanodine receptors.\(^{23}\) However preventing ryanodine receptor activation with 20 \(\mu\)mol/L dantrolene did not change the DHE signal \((n=6; \*P=NS versus exposure to \(H_2O_2\) in the absence of dantrolene; Figure 4C).

**Transient Exposure to \(H_2O_2\) Is Associated With Sustained Alteration in L-Type Ca\(^{2+}\) Channel Function**

Alters in cellular production of \(H_2O_2\) influence \(I_{Ca-L}\). Exposing cardiac myocytes to hypoxia is associated with a decrease in cellular production of superoxide and \(H_2O_2\) by the mitochondria that results in a decrease in basal channel activity, while increasing the sensitivity of the channel to \(\beta\)-adrenergic receptor stimulation.\(^{10,11,15,17}\) In addition, perfusing myocytes intracellularly with catalase (that specifically converts \(H_2O_2\) to \(H_2O\) and \(O_2\)) mimics the increase in sensitivity of the channel to \(\beta\)-adrenergic receptor stimulation during hypoxia.\(^{15}\) We therefore used the response of the channel to \(\beta\)-adrenergic receptor stimulation as a functional reporter of a persistent oxidative stress. We exposed the cells to 30 \(\mu\)mol/L \(H_2O_2\) for 5 minutes followed by 10 U/mL catalase for 5 minutes to degrade extracellular \(H_2O_2\) and recorded the sensitivity of the channel to increasing concentrations of the \(\beta\)-adrenergic receptor agonist isoproterenol (Iso). In the absence of \(H_2O_2\), 0.003 \(\mu\)mol/L and 0.01 \(\mu\)mol/L Iso elicited currents that were 28.8±7.1 and 74.1±8.6% of the current elicited by 1 \(\mu\)mol/L Iso, a maximally stimulating concentration of the \(\beta\)-adrenergic receptor agonist within the same cell \((n=5);\) Figure 5A). However, after exposure to \(H_2O_2\), 0.003 \(\mu\)mol/L and 0.01 \(\mu\)mol/L Iso elicited currents that were only 0.67±0.4 and 22.4±9.3% of the current recorded in response to 1 \(\mu\)mol/L Iso \((n=9);\) Figure 5B). The \(K_{0.5}\) for activation of the channel by Iso was significantly increased from 5.8±0.3 to 27.8±0.1 nmol/L \((P<0.05);\) Figure 6A). There was no difference in the response of the channel to a maximally stimulating concentration of Iso (1 \(\mu\)mol/L) before or after exposure to \(H_2O_2\) (Figure 6B). However, the activity of \(I_{Ca-L}\) under non-\(\beta\)-adrenergic-stimulated conditions (basal channel activity) was significantly increased from 5.4±0.5 \((n=7)\) to 8.9±0.7 pA/pF \((n=25)\) at +10 mV following exposure to \(H_2O_2\) \((P<0.05)\). These data were
recorded, on average, 4 hours after exposure to H$_2$O$_2$. The decrease in sensitivity of the channel to Iso and the increase in basal current activity persisted for at least 9 hours after exposure to H$_2$O$_2$ (and degradation of extracellular H$_2$O$_2$ with 10 U/mL catalase). These data suggest that a transient exposure to H$_2$O$_2$ is associated with a persistent alteration in $I_{\text{Ca-L}}$. It would appear that existing channels are persistently activated with increased current after exposure to H$_2$O$_2$.

Persistently Altered $I_{\text{Ca-L}}$ Is Mediated by Superoxide Produced by the Mitochondria

The persistently altered $I_{\text{Ca-L}}$ could have been attributable to irreversible oxidation of the channel protein following a transient exposure to H$_2$O$_2$. Alternatively, it may have been maintained in an oxidized state as a result of increased extracellular H$_2$O$_2$ with 10 U/mL catalase). These data suggest that a transient exposure to H$_2$O$_2$ is associated with a persistent alteration in $I_{\text{Ca-L}}$. It would appear that existing channels are persistently activated with increased current after exposure to H$_2$O$_2$.

Iso and the increase in basal current activity (see the online data supplement). These data suggest that after a transient exposure to H$_2$O$_2$, channel function is altered as a result of elevated cellular H$_2$O$_2$.

We confirmed that the mitochondria were the source of production of superoxide. Following exposure to 30 μmol/L H$_2$O$_2$, when cells were perfused intracellularly with FCCP or myxothiazol, the decrease in sensitivity of the channel to Iso and increase in basal current were significantly attenuated (see the online data supplement). In addition, the source of superoxide did not involve NAD(P)H oxidase, xanthine oxidase, or nitric oxide (see the online data supplement). These results confirm that channel function is persistently altered after a transient exposure to H$_2$O$_2$ but can be reversed when mitochondrial production of superoxide is inhibited.
Transient Exposure to H₂O₂ Is Associated With Elevated Intracellular Ca²⁺

We measured intracellular Ca²⁺ before and after exposure of guinea pig ventricular myocytes to 30 μmol/L H₂O₂ using the fluorescent indicator Fura-2. Figure 7A illustrates the persistent increase in intracellular Ca²⁺ after exposure to H₂O₂ and catalase in a guinea pig myocyte. Exposure of cells to H₂O₂ caused a significant increase in 340/380 fluorescence that was attenuated by 2 μmol/L nisoldipine before exposure to H₂O₂ but not with prior exposure to 2 μmol/L Ru360 (P<0.05; Figure 7A, inset at right), indicating the source of calcium was I₉Ca-L.

Activation of I₉Ca-L Is Required for an Increase in Mitochondria-Derived Superoxide

We examined whether activation of I₉Ca-L was sufficient to increase intracellular superoxide. Application of 2 μmol/L Bay K, an L-type Ca²⁺ channel agonist, caused a 79.2±14.4% increase in DHE signal (n=7). This was comparable to the increase in DHE recorded after exposure to H₂O₂ (Figure 1A and 1B). The increase in DHE could be attenuated by application of 2 μmol/L Ru360 or 2 μmol/L nisoldipine before exposure to Bay K (Figure 7B). These data indicate that activation of I₉Ca-L is sufficient for an increase in mitochondrial uptake of calcium and increased superoxide production by the mitochondria.

Transient Exposure to H₂O₂ Is Associated With an Increase in DHE Signal and Elevated Intracellular Ca²⁺ in Active Cycling Myocytes

We examined the effect of a transient exposure to H₂O₂ on DHE signal in spontaneously contracting neonatal rat ventricular myocytes. Exposure to 30 μmol/L H₂O₂ for 5 minutes followed by 10 U/mL catalase for 5 minutes caused a 8.1-fold increase in DHE signal that could be attenuated by exposure of cells to 2 μmol/L Ru360 before H₂O₂ (Figure 8A). In addition, exposure of spontaneously contracting myocytes to 30 μmol/L H₂O₂ for 5 minutes resulted in a small increase in diastolic 340/380 fluorescence (10.5±5.1% increase in 4 of 19 cells), but after 10 minutes, the diastolic 340/380 fluorescence was increased 71.1±9.5% (Figure 8B), without causing apoptosis or necrosis, as determined by caspase 3 assay and lactate dehydrogenase release 24 hours later (see Figure III in the online data supplement). Application of 2 μmol/L nisoldipine...
dipine before 30 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \) prevented the increase in diastolic 340/380 fluorescence (Figure 8B). Consistent with the results recorded in quiescent guinea pig ventricular myocytes, a transient exposure to \( \text{H}_2\text{O}_2 \) is associated with a significant increase in cellular superoxide and diastolic \( \text{Ca}^{2+} \) in active calcium cycling myocytes.

**Discussion**

In this study, we examined the effects of a brief exposure of cardiac myocytes to \( \text{H}_2\text{O}_2 \) at a concentration that did not cause apoptosis or necrosis. We found that 5 minutes of exposure of cardiac myocytes to 30 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \) was sufficient to cause persistent alterations in cellular superoxide production, \( I_{\text{Ca,L}} \), and cellular \( \text{Ca}^{2+} \). The increase in cellular superoxide was dependent on activation of \( I_{\text{Ca,L}} \), and it was reversible. One current view regarding the increased production of ROS by mitochondria following oxidative stress is that the mitochondria are the target of ROS. Our results differ from these studies in that we specifically applied an oxidative stress externally that is likely to mimic a burst of ROS associated with ischemia/reperfusion in vivo. We attenuated the oxidative stress with catalase and examined the effect on cellular function. At low concentrations (30 \( \mu \text{mol/L} \) of \( \text{H}_2\text{O}_2 \), an increase in the activity of \( I_{\text{Ca,L}} \) was required for the increase in mitochondrial ROS production. Because Bay K alone increased cellular superoxide (Figure 7B), it would appear that the increase in superoxide occurs solely because of an increase in basal \( I_{\text{Ca,L}} \) activity and that low concentrations of \( \text{H}_2\text{O}_2 \) do not have to directly affect mitochondria. Persistently elevated intracellular \( \text{Ca}^{2+} \) and ROS are associated with induction of calmodulin and NFAT pathways that lead to pathological states such as cardiac hypertrophy and failure. Chronic in vitro exposure to low concentrations of \( \text{H}_2\text{O}_2 \) (10 to 30 \( \mu \text{mol/L} \)) have been shown to induce protein synthesis in adult cardiac myocytes, without affecting survival.

Our results may represent the mechanisms that contribute to the development of cardiac pathology.

The L-type \( \text{Ca}^{2+} \) channel is responsive to alterations in cellular redox state. When intracellular \( \text{H}_2\text{O}_2 \) is decreased with exposure of myocytes to hypoxia or intracellular application of catalase, the sensitivity of the channel to \( \beta \)-adrenergic receptor stimulation is increased.

This response is mimicked when cells are exposed to the thiol-specific reducing agent dithiothreitol, suggesting that direct redox modification of the channel or redox modification of a signaling intermediate such as protein kinase A is responsible for the change in channel sensitivity.
for the response. We found that the $K_{ass}$ for activation of the channel by Iso significantly increased after exposure to $H_2O_2$, consistent with a response of the channel to an oxidized cellular environment (Figures 5B and 6A). The response persisted for many hours after the insult as a result of a persistent increase in production of superoxide by the mitochondria because the mitochondria inhibitors myxothiazol and FCCP attenuated the decrease in sensitivity of the channel to Iso. If direct oxidation of the channel is necessary for the increase in basal current density (as acute exposure to hydrogen peroxide or thiol-oxidizing agents would suggest), then the persistent increase in cellular superoxide appears to be necessary to maintain the channel in an oxidized state. In support of this, we found that basal current density was increased many hours after the transient exposure to $H_2O_2$ (Figure 6B), and this was attenuated by intracellular catalase, FCCP, and myxothiazol.

Consistent with persistent L-type $Ca^{2+}$ channel activation, intracellular $Ca^{2+}$ was increased in the myocytes (Figures 7A and 8B). Our data indicate that $Ca^{2+}$ influx through the L-type $Ca^{2+}$ channel is required for the increase in superoxide by the mitochondria because nisoldipine attenuated the increase in DHE signal (Figure 4B) and the increase in intracellular $Ca^{2+}$ (Figures 7A and 8B). In addition Bay K alone was sufficient to increase $Ca^{2+}$ uptake into the mitochondria and increase superoxide (Figure 7B). Dantrolene, an inhibitor of ryanodine release of $Ca^{2+}$ from sarcoplasmic reticulum stores, did not alter the increase in DHE signal after exposure to $H_2O_2$ (Figure 4C). We propose therefore that the increase in intracellular $Ca^{2+}$ and superoxide persists because a positive feedback exists between increased basal channel activity and superoxide production by the mitochondria (Figure 8C). The model does not preclude a direct effect of $H_2O_2$ on the mitochondria. Our data show that increased L-type $Ca^{2+}$ channel activity is required for the response at low $H_2O_2$ concentrations. The L-type $Ca^{2+}$ channel appears to be an important regulator of cellular $Ca^{2+}$ and cellular superoxide production under conditions of oxidative stress that do not involve apoptosis or necrosis and have the potential to mediate cardiac pathology.

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Disclosures
None.

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A transient exposure to hydrogen peroxide causes an increase in mitochondrial-derived superoxide as a result of sustained alteration in L-type Ca\(^{2+}\) channel function in the absence of apoptosis in ventricular myocytes

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Expanded Materials and Methods

Guinea pig ventricular myocyte isolation procedure

For all studies involving quiescent myocytes adult Tricolor guinea pigs (cavia porcellis) weighing between 200 and 250g were used. A total number of 133 guinea pigs were anesthetized with intraperitoneal injection of pentobarbitone sodium (240 mg/kg) prior to excision of the heart as approved by The Animal Ethics Committee of The University of Western Australia in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NH&MRC, 7th Edition, 2004). Guinea pig ventricular myocytes were isolated and cultured as described previously \(^1\)\(^2\). For experiments involving detection of superoxide or determination of intracellular calcium freshly isolated guinea pig myocytes were allowed to become adherent in culture overnight and were used the next day (24 hour old myocytes).

Isolation of ventricular myocytes from rat neonates

Neonatal ventricular myocytes were isolated as described previously \(^3\). Hearts were excised from a total of 48 neonatal rats as approved by The Animal
Detection of superoxide

Just prior to experimentation the culture dish media was changed to a HEPES-buffered solution containing (in mmol/L) KCl 5.33, MgSO₄ 0.41, NaCl 139, Na₂HPO₄ 5.63, glucose 5, HEPES 20, glutamine 2, Ca(NO₃)₂ 2.5 and 1ml/100ml penicillin/streptomycin (pH adjusted to 7.4 with NaOH). Generation of superoxide was assessed using the fluorescent indicator dihydroethidium (DHE, 5 µmol/L, 515-560 nm ex filter, 590 long pass em; Molecular Probes). Fluorescent signal was measured on a MTI-DAGE video camera attached to an inverted Nikon TE300 microscope. Metamorph 5.2 was used to quantify the signal by manually tracing myocytes. An equivalent region not containing cells was used for background and was subtracted. The ratio of fluorescence was reported as the slope of the signal measured at 30-50 minutes over the slope of the signal measured at 0-20 minutes.

Quantitation of Apoptosis / Caspase 3 activity assay

Caspase 3 activity was measured using Ac-DEVD-AMC (Promega) as a substrate. Adherent myocytes were treated in the presence or absence of 30 µmol/L H₂O₂ (5 min) then 10 U/ml catalase (5 min) or 2 µmol/L doxorubicin
for 24 hr (as positive control) \(^6\) and allowed to recover at 37°C in 5\%O\(_2\)/95\%CO\(_2\) for 24 hours. Culture media was removed and contents scraped on ice in 400\(\mu\)l 25\% ICE buffer (50 mmol/L Tris, 0.5mmol/L EDTA, 20\% glycerol, pH 7). Cell lysates were sonicated on ice and centrifuged. Supernatant (50 \(\mu\)l) was assayed in 50 \(\mu\)l of assay reagent (3 ml 100\% ICE buffer, 1.8 mg DTT and 3 \(\mu\)l of 20 mmol/L Ac-DEVD-AMC) with and without caspase-3 inhibitor VAD-fmk (20 \(\mu\)mol/L) \(^6\) to confirm apoptosis. The rate of increase in caspase 3 activity was measured over 90 minutes using a fluorescent plate reader (Fluostar, ex 355, em 460nm) and expressed as a ratio of total protein. Each \(n\) represents one dish of cells.

**Lactate Dehydrogenase Activity (LDH) assay in contracting neonatal ventricular myocytes**

Contracting adherent neonatal myocytes (0.8-1 million cells per dish) were treated in the presence or absence of 30 \(\mu\)mol/L H\(_2\)O\(_2\) (5 min) then 10 U/ml catalase (5min), or 200 \(\mu\)mol H\(_2\)O\(_2\) (5 min) then 10 U/ml catalase (5 min) (positive control) \(^7\) and allowed to recover at 37°C in 5\%O\(_2\)/95\%CO\(_2\) for 24 hours. Cells were then lysed in 750 \(\mu\)l Lysis Buffer (1\% Triton-X in HBS) before being incubated at 37°C for 30 minutes, followed by centrifugation at 10,000 RPM for 5 min. The supernatant was then removed and stored at 4°C. Assay reagent (50 mmol/L imidazole pH=7, 375 \(\mu\)mol/L NADH, 1 mmol/L pyruvate, and 0.05\% BSA) (50 \(\mu\)l) was added to samples of culture media and cell lysates (150 \(\mu\)l) and the rate of decrease of absorbance was measured at 340 nm in a Spectra Max 340 over 15 min at 25°C. Release of LDH into the
culture media was expressed as a percentage of total LDH activity. Each \( n \) represents one dish of cells.

**Calibrations to determine intracellular \( \text{Ca}^{2+} \) concentration**

Resting \( \text{Ca}^{2+} \) fluorescence was recorded in 5 guinea pig ventricular myocytes loaded with Fura-2 in (2.5 mmol/L \( \text{Ca}^{2+} \)-containing) HBS. Media was then replaced with \( \text{Ca}^{2+} \)-free HBS containing ionomycin (10 \( \mu \text{mol/L} \)) and EGTA (3 mmol/L), and intracellular \( \text{Ca}^{2+} \) fell to \( R_{\text{min}} \) within 30 minutes. Rotenone (4 \( \mu \text{mol/L} \)), FCCP (2 \( \mu \text{mol/L} \)) and 5 mmol/L \( \text{Ca}^{2+} \) were then added to produce \( R_{\text{max}} \). HBS containing ionomycin and MnCl\(_2\) (1 mmol/L) was added to quench the excitation spectra. Intracellular \( \text{Ca}^{2+} \) was determined according to the equation \(^{10}\):

\[
[\text{Ca}] = K_d \cdot b \cdot (R - R_{\text{min}}) / (R_{\text{max}} - R)
\]

Where \( R_{\text{min}} = 0.111 \pm 0.002 \), \( R_{\text{max}} = 1.898 \pm 0.234 \), \( b (=5.5216) \) is the fluorescent intensity during illumination at 380 nm with 0 mmol/L \( \text{Ca}^{2+} \) and 5 mmol/L \( \text{Ca}^{2+} \), and dissociation constant \( (K_d) = 371 \) nmol/L as determined previously \(^8\). In the 5 myocytes intracellular resting calcium was determined as 84.6 ± 9.6 nmol/L.

**Fluorescent detection of mitochondrial membrane potential**

Mitochondrial membrane potential was recorded using the fluorescent indicator 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (Molecular Probes). JC-1 (200 nmol/L) was loaded into cells by pre-incubating for 3 hour at 37°C. Imaging was carried out using inverted microscopy (Nikon TE-300 Inverted Microscope and MTI-DAGE camera). Individual cardiomyocytes were traced manually and fluorescent signal
(excitation 480 nm, emission at 520 nm and 590 nm) quantified using Metamorph 5.2. Mitochondrial membrane potential was expressed as a ratio of fluorescent emission at 590 nm/520 nm. To establish that the JC-1 signal was indicative of mitochondrial membrane potential, 20 µmol/L oligomycin and 4 µmol/L FCCP were added at the end of each experiment to collapse mitochondrial membrane potential.

Measuring catalase activity

Cells containing 30 µmol/L H$_2$O$_2$ in the presence or absence of 10 U/ml catalase were incubated at 37°C for 5 minutes after addition of catalase. After inhibiting catalase with 15 mmol/L sodium azide (NaN$_3$), 10 µl of sample was incubated with assay reagent (200 µl) containing 2 U/ml horseradish peroxidase and 5 µmol/L N-Acetyl-3,7-Dihydroxyphenoxazine in 200 mmol/L sodium phosphate (pH=7.4). Following addition of 150 mmol/L NaN$_3$ and measurement on a fluorescent plate reader (Fluostar, ex 544, em 590), H$_2$O$_2$ concentration was calculated from a standard H$_2$O$_2$ curve. 10 U/ml catalase was sufficient to degrade extracellular H$_2$O$_2$ 98.4±0.5% (n=5).

Data acquisition for patch-clamp studies

Microelectrodes with tip diameters of 3-5 µm and resistances of 0.5 - 1.5 MΩ contained (in mmol/L): CsCl 115, HEPES 10, EGTA 10, tetraethylammonium chloride 20, MgATP 5, Tris-GTP 0.1, phosphocreatine 10, CaCl$_2$ 1 (pH adjusted to 7.05 at 37°C with CsOH). All experiments were performed at 37°C. Currents were measured in extracellular modified Tyrode’s solution that contained (in mmol/L): NaCl 140, CsCl 5.4, CaCl$_2$ 2.5,
MgCl₂ 0.5, HEPES 5.5 and glucose 11 (pH adjusted to 7.4 with NaOH).

Macroscopic currents were recorded using an Axopatch 200B voltage-clamp amplifier (Axon Instruments) and an IBM compatible computer with a Digidata 1200 interface and pClamp software (Axon Instruments). A Ag/AgCl electrode was used to ground the bath. Once the whole-cell configuration was achieved, the holding potential was set at –80 mV. Na⁺ channels and T-type Ca²⁺ channels were inactivated by applying a 50 ms prepulse to –30 mV immediately before each test pulse. The time course of changes in Ca²⁺ conductance was monitored by applying a 75 ms test pulse to +10 mV once every 10 seconds.

To minimize the possibility of oxidative degradation, solutions containing isoproterenol were prepared fresh and changed every 60-90 min. Experiments were performed on freshly isolated guinea pig ventricular myocytes up to 9 hours after isolation. We found no difference in the density of I_{Ca-L} or the sensitivity of I_{Ca-L} to Iso between freshly isolated myocytes and 24 hr old cultured myocytes in 5 cells tested.

**Statistical analysis**

Results are reported as means ± SE. Statistical comparisons of responses between unpaired data were made using the student’s t-test or between groups of cells using one-way ANOVA and the Tukey’s posthoc test (GraphPad Prism version 3.02).
Results

Additional supporting data

Acute exposure of guinea pig ventricular myocytes to H2O2 increases basal $I_{\text{Ca-L}}$

The effect of acute exposure of $H_2O_2$ on basal channel activity was examined. Consistent with previous studies showing an activation of basal current $^{11,12}$, exposure of guinea pig ventricular myocytes to 30 µmol/L $H_2O_2$ caused a 30.1 ± 4.5% increase in basal current that could be reversed upon washout of $H_2O_2$ (n=7, Online Data Supplement Figure 1A). The increase in peak basal current was not associated with a shift in the current-voltage relationship for activation of the channel (inset at right Online Data Supplement Figure 1A). Basal current density was increased from 5.4 ± 0.6 to 8.1 ± 0.7 pA/pF (Online Data Supplement Figure 1B).

The persistently altered $I_{\text{Ca-L}}$ is mediated by superoxide produced by the mitochondria

We perfused cells intracellularly with 2000U/ml catalase (that converts $H_2O_2$ to $H_2O$ and $O_2$) and measured the response of the channel to Iso. After exposure to $H_2O_2$, 0.003 and 0.01 µmol/L Iso elicited currents that were 18.8±3.6 and 41.6±3.5% of the current recorded in response to 1 µmol/L Iso in the same cell (n=6; Online Data Supplement Figure 2A). These currents were significantly greater than the currents elicited by the same concentrations of Iso without catalase in the pipette after exposure to 30 µmol/L $H_2O_2$ ($P<0.05$, Figures 5B and 6A). In addition, basal current activity was significantly decreased from 8.9±0.7 pA/pF in 25 cells exposed to $H_2O_2$ in the absence of...
intracellular catalase to 5.1±0.5 pA/pF in cells exposed to H₂O₂ and then perfused intracellularly with catalase (n=14, P<0.01). These data suggest that after a transient exposure to H₂O₂ channel function is altered as a result of elevated cellular H₂O₂.

We confirmed that the mitochondria were the source of production of superoxide. Following exposure to 30 µmol/L H₂O₂, cells were perfused intracellularly with 7 nmol/L myxothiazol and the response of the channel to Iso was recorded. In 7 cells, 0.003 and 0.01 µmol/L Iso elicited currents that were 53.9±14.9 and 84.8±10.9% of the current recorded in response to 1 µmol/L Iso in the same cell (Online Data Supplement Figure 2B). These were significantly greater than currents elicited by the same concentrations of Iso after exposure to H₂O₂ in the absence of myxothiazol (Figures 5B and 6A). In addition basal current activity was significantly decreased in the 7 cells perfused with myxothiazol (4.3±0.8 versus 8.9±0.7 pA/pF in cells not exposed to myxothiazol after H₂O₂, n=25, P<0.01). We perfused cells intracellularly with 5 µmol/L FCCP and recorded the response of I_{Ca-L} to Iso after the cells had been exposed to 30 µmol/L H₂O₂ for 5 min followed by 10 U/ml catalase for 5 min. In 6 cells, 0.003 and 0.01 µmol/L Iso elicited currents that were 17.3±5.5 and 59.5±12.4% of the current recorded in response to 1 µmol/L Iso in the same cell. These were significantly greater than the currents elicited by the same concentrations of Iso without FCCP in the pipette after exposure to 30 µmol/L H₂O₂ (P<0.05, Figures 5B and 6A). In addition, basal current activity was significantly decreased from 8.9±0.7 pA/pF in 25 cells exposed to H₂O₂ in the absence of intracellular FCCP to 4.3±0.4 pA/pF in cells exposed to H₂O₂ and then perfused intracellularly with FCCP (n=6, P<0.01). These
data confirm that $I_{Ca-L}$ is persistently altered as a result of persistent production of superoxide by the mitochondria.

We also exposed cells to the NAD(P)H-oxidase peptide inhibitor gp91ds-tat (50 µmol/L) before exposure to 30 µmol/L H$_2$O$_2$ for 5 min followed by 10 U/ml catalase for 5 min and then recorded the response of $I_{Ca-L}$ to Iso. In 5 cells, 0.003 and 0.01 µmol/L Iso elicited currents that were $4.4\pm2.1$ and $21.1\pm10.9\%$ of the current recorded in response to 1 µmol/L Iso in the same cell. These were similar to currents elicited by the same concentrations of Iso in the absence of the peptide after exposure to 30 µmol/L H$_2$O$_2$ ($P=NS$, Figures 5B and 6A). In addition, basal current density was unchanged (8.9±0.7 pA/pF in the absence of the peptide after H$_2$O$_2$, n=25 vs 7.3±0.4 pA/pF in the presence of the peptide after H$_2$O$_2$, n=5; $P=NS$). Similarly when cells were exposed to the NAD(P)H-oxidase inhibitor apocynin (300 µmol/L) before exposure to 30 µmol/L H$_2$O$_2$ for 5 min followed by 10 U/ml catalase for 5 min there was no difference in the response of $I_{Ca-L}$ to Iso. In 5 cells, 0.01 and 0.1 µmol/L Iso elicited currents that were $20.4\pm4.8$ and $82.5\pm2.9\%$ of the current recorded in response to 1 µmol/L Iso in the same cell. These were similar to currents elicited by the same concentrations of Iso in the absence of apocynin after exposure to 30 µmol/L H$_2$O$_2$ ($P=NS$, Figure 6A). In addition, basal current density was unchanged (8.9±0.7 pA/pF in the absence of apocynin after H$_2$O$_2$, n=25 vs 7.1±1.3 pA/pF in the presence of apocynin after H$_2$O$_2$, n=5; $P=NS$). Consistent with the DHE results (Figure 2A) these data suggest that the source of superoxide is not NAD(P)H-oxidase.

To rule out an involvement of xanthine oxidase, we exposed cells to 50 µmol/L allopurinol before exposure to 30 µmol/L H$_2$O$_2$ for 5 min followed by 10
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U/ml catalase for 5 min and then recorded the response of I\textsubscript{Ca-L} to Iso. In 5 cells, 0.01 and 0.1 µmol/L Iso elicited currents that were 21.8±6.1 and 72.5±13.2% of the current recorded in response to 1 µmol/L Iso in the same cell. These were similar to currents elicited by the same concentrations of Iso in the absence of allopurinol after exposure to 30 µmol/L H\textsubscript{2}O\textsubscript{2} (P=NS, Figure 6A). In addition, basal current density was unchanged (8.9±0.7 pA/pF in the absence of allopurinol after H\textsubscript{2}O\textsubscript{2}, n=25 vs 7.8±1.1 pA/pF in the presence of allopurinol after H\textsubscript{2}O\textsubscript{2}, n=5; P=NS). Consistent with the DHE results (Figure 2B) these data suggest that the source of superoxide is not xanthine oxidase.

Finally cells were exposed to 100 µmol/L L-NAME before exposure to 30 µmol/L H\textsubscript{2}O\textsubscript{2} for 5 min followed by 10 U/ml catalase for 5 min and the response of I\textsubscript{Ca-L} to Iso was recorded. In 5 cells, 0.01 and 0.1 µmol/L Iso elicited currents that were 18.1±5.2 and 51.3±13.8% of the current recorded in response to 1 µmol/L Iso in the same cell. These were similar to currents elicited by the same concentrations of Iso in the absence of L-NAME after exposure to 30 µmol/L H\textsubscript{2}O\textsubscript{2} (P=NS, Figure 6A). In addition, basal current density was unchanged (8.9±0.7 pA/pF in the absence of L-NAME after H\textsubscript{2}O\textsubscript{2}, n=25 vs 8.9±1.6 pA/pF in the presence of L-NAME after H\textsubscript{2}O\textsubscript{2}, n=5; P=NS).

Consistent with the DHE results (Figure 2C) these data suggest that the source of superoxide is not nitric oxide.

**Transient exposure of contracting neonatal rat ventricular myocytes to H\textsubscript{2}O\textsubscript{2} does not induce apoptosis or necrosis**

Adherent contracting myocytes were treated in the presence or absence of 30 µmol/L H\textsubscript{2}O\textsubscript{2} (5 min) then 10 U/ml catalase (5 min) or 2 µmol/L doxorubicin
for 24 hr (as positive control)\textsuperscript{6}. Caspase 3 activity was assessed as described in Methods section. Exposure of contracting myocytes to 30 µmol/L H\textsubscript{2}O\textsubscript{2} for 5 min did not increase caspase 3 activity whereas exposure to doxorubicin caused a 5 fold increase in activity (Online Data Supplement Figure 3A). Lactate dehydrogenase (LDH) release was assessed in the contracting myocytes. Exposure of contracting myocytes to 30 µmol/L H\textsubscript{2}O\textsubscript{2} for 5 min then 10 U/ml catalase for 5 min did not increase LDH activity whereas treatment of cells with 200 µmol/L H\textsubscript{2}O\textsubscript{2} significantly increased LDH release (Online Data Supplement Figure 3B). These data strongly suggest that transient exposure of contracting neonatal rat myocytes to H\textsubscript{2}O does not induce cell necrosis or apoptosis.
References


5. Arthur PG, Lim SC, Meloni BP, Munns SE, Chan A, Knuckey NW. The protective effect of hypoxic preconditioning on cortical neuronal cultures is associated with increases in the activity of several antioxidant enzymes. Brain Res. 2004;1017:146-54.


**Figure Legends**

**Online Fig 1.** Acute exposure of guinea pig ventricular myocytes to H$_2$O$_2$ results in an increase in basal I$_{Ca-L}$ that is reversed upon washout of H$_2$O$_2$. 

**A,** Time course of changes in membrane current recorded in a guinea pig ventricular myocyte during exposure to 30 µmol/L H$_2$O$_2$ including membrane currents recorded at the time points indicated (inset left). Current-voltage (I-V) relationship measured in the guinea pig ventricular myocyte during voltage steps from –60 mV to + 80 mV in the absence and presence of 30 µmol/L H$_2$O$_2$ is shown inset at right. 

**B,** Mean ± SE basal current density before (Pre H$_2$O$_2$) and during (H$_2$O$_2$) exposure to 30 µmol/L H$_2$O$_2$.

**Online Fig 2.** A, Perfusing guinea pig ventricular myocytes intracellularly with catalase attenuates the decrease in sensitivity of I$_{Ca-L}$ to Iso. Time course of changes in membrane current recorded in a guinea pig ventricular myocyte during exposure to 0.003, 0.01 and 1 µmol/L Iso after 5 min exposure to 30 µmol/L H$_2$O$_2$ then 5 min exposure to 10 U/ml catalase while being perfused intracellularly with 2000 U/ml catalase including membrane currents recorded at the time points indicated (inset left). Current-voltage (I-V) relationship measured in the cell during voltage steps from –60 mV to + 80 mV is shown (inset right). 

**B,** Perfusing guinea pig ventricular myocytes intracellularly with myxothiazol attenuates the decrease in sensitivity of I$_{Ca-L}$ to Iso. Time course of changes in membrane current recorded in a cell during exposure to 0.003, 0.01 and 1 µmol/L Iso after 5 min exposure to 30 µmol/L H$_2$O$_2$ then 5 min exposure to 10 U/ml catalase while being perfused intracellularly with 25
nmol/L myxothiazol including membrane currents recorded at the time points indicated (inset left). Current-voltage (I-V) relationship measured in the cell during voltage steps from –60 mV to + 80 mV is shown (inset right).

Online Fig 3. Transient exposure of contracting neonatal rat ventricular myocytes to 30 µmol/L H₂O₂ does not induce necrosis or apoptosis. A, Caspase 3 activity performed in contracting neonatal rat ventricular myocytes after exposure to 0 µmol/L H₂O₂ then catalase (No H₂O₂), after exposure to 30 µmol/L H₂O₂ then catalase (Post H₂O₂) and after exposure to 2 µmol/L doxorubicin as a positive control as described in Methods. *P<0.05 compared with cells exposed to 0 µmol/L H₂O₂ then catalase (No H₂O₂). B, Lactate Dehydrogenase release in contracting neonatal rat ventricular myocytes after exposure to 0 µmol/L H₂O₂ then catalase (No H₂O₂), after exposure to 30 µmol/L H₂O₂ then catalase (Post 30 µmol/L H₂O₂), and after exposure to 200 µmol/L H₂O₂ then catalase (Post 200 µmol/L H₂O₂) as a positive control as described in Methods. *P<0.05 compared with cells exposed to 0 µmol/L H₂O₂ then catalase (No H₂O₂).
**A**

- Graph showing current (pA) plotted against time (s).
  - Two data points labeled 'a' and 'b'.

- Additional graph showing voltage (mV) plotted against current (pA).
  - Markers indicating 'Con' and '30 µmol/L H₂O₂'.

- Peak inward current at +10 mV (pA) shown.
  - Data at 30 µmol/L H₂O₂.

- Time (min) indicated on x-axis, 0 to 5.

**B**

- Bar chart showing current density (pA/pF) comparison.
  - Comparison between 'Pre H₂O₂' and 'H₂O₂'.
  - Significance level marked as 'P<0.005'.

- Sample size indicated as 'n = 7'.
A

after hydrogen peroxide exposure

Peak inward current at +10 mV (pA)

0.00 0.05 0.10

Time (s)

-2000 -1000 -1500 -2000

0 1.0

Iso (µmol/L)

0.003 0.01 1.0

B

after hydrogen peroxide exposure

Peak inward current at +10 mV (pA)

0.00 0.05 0.10

Time (s)

-1000 -500 -1000

-1000 -500 -1000

mV

0.003 1.0

Iso (µmol/L)

Online Data Supplement Figure 2
A  Caspase 3 assay

% of No H₂O₂

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B  LDH assay

% LDH release

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Online Data Supplement Figure 3