

Oxidation of RyR2 Has a Biphasic Effect on the Threshold for Store Overload-Induced Calcium Release

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ABSTRACT At the single-channel level, oxidation of the cardiac ryanodine receptor (RyR2) is known to activate and inhibit the channel depending on the level of oxidation. However, the mechanisms through which these changes alter the activity of RyR2 in a cellular setting are poorly understood. In this study, we determined the effect of oxidation on a common form of RyR2 regulation; store overload-induced Ca²⁺ release (SOICR). We found that oxidation resulted in concentration and time-dependent changes in the activation threshold for SOICR. Low concentrations of the oxidant H₂O₂ resulted in a decrease in the threshold for SOICR, which led to an increase in SOICR events. However, higher concentrations of H₂O₂, or prolonged exposure, reversed these changes and led to an increase in the threshold for SOICR. This increase in the threshold for SOICR in most cells was to such an extent that it led to the complete inhibition of SOICR. Acute exposure to high concentrations of H₂O₂ led to an initial decrease and then increase in the threshold for SOICR. In the majority of cells the increased threshold could not be reversed by the application of the reducing agent dithiothreitol. Therefore, our data suggest that low levels of RyR2 oxidation increase the channel activity by decreasing the threshold for SOICR, whereas high levels of RyR2 oxidation irreversibly increase the threshold for SOICR leading to an inhibition of RyR2. Combined, this indicates that oxidation regulates RyR2 by the same mechanism as phosphorylation, methylxanthines, and mutations, via changes in the threshold for SOICR.

INTRODUCTION

During excitation-contraction coupling, Ca²⁺ is released via the cardiac ryanodine receptor (RyR2) from the sarcoplasmic reticulum (SR) and provides the major source of Ca²⁺ for contraction. However, uncontrolled Ca²⁺ release through RyR2 is pathogenic. A number of mechanisms by which RyR2 function becomes compromised have been identified, including mutations within RyR2, mutations in proteins associated with RyR2, phosphorylation of RyR2, and oxidation of RyR2 (1). A unifying theory by which these modifications lead to disease is via an increase in store overload-induced Ca²⁺ release (SOICR), otherwise known as Ca²⁺ leak or spontaneous Ca²⁺ release (2–6). We and others have shown that SOICR occurs due to an increase in the luminal Ca²⁺ sensitivity of RyR2 and concomitant decrease in the SR Ca²⁺ concentration required to trigger RyR2 opening (2–4).

RyR2 contains ~21 free cysteine residues per monomer or ~84 thiols per channel (7), deemed to be susceptible to reac-

tive oxygen species (ROS) mediated oxidation. It has been shown that RyR2 is oxidized in a number of forms of cardiac disease and leads to enhanced Ca²⁺ leak (8–11). Oxidation-induced activation of RyR2 has been observed at the single-channel level (7,12) and in cardiac myocytes, where the application of H₂O₂ leads to a rapid increase in Ca²⁺ sparks (13). RyR2 oxidation can occur on both sides of the channel with either resulting in an increase in channel open probability. Others have also shown that the presence of a reducing agent (dithiothreitol, DTT) can rapidly reverse the oxidation-induced increase in RyR2 open probability (12,14).

However, oxidation of RyR2 appears to have a more complex effect on RyR2 function than simple activation of the channel. In addition to the rapid increase in RyR2 activity following its oxidation, some studies suggest that prolonged exposure to thiol oxidizing compounds can elicit a biphasic effect on the channel, reflected by the suppression of RyR2 activity following the initial activation (13,15,16). These data suggest that there are multiple groups of thiol-containing residues that can become modified, with those most susceptible to modification leading to an increase in RyR2

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channel activity and those that require prolonged exposure leading to a loss of RyR2 activity (12,17). Although the effect of acute oxidation of RyR2 appears to be reversed by reducing agents (12,14), the reversibility of chronic oxidation and other forms of prolonged thiol modification of RyR2 (and RyR1) is less clear (11,12,18,19).

We have recently shown that short-term exposure of cells expressing RyR2 to the oxidizing agent 2,2'-dithiodipyridine increases the activity of RyR2 by reducing the release threshold for SOICR (20). This suggests that the cellular mechanism by which oxidation leads to Ca^{2+} leak is a sensitization to luminal store Ca^{2+} . However, whether the biphasic single-channel response of RyR2, to prolonged oxidation, results in similar biphasic changes in the release threshold for SOICR in intact cells remains unknown.

In this study, we determined the effect of prolonged oxidation of RyR2 by H_2O_2 on the release threshold for SOICR. We found that consistent with our previous data, initial oxidation increases Ca^{2+} leak by decreasing the release threshold for SOICR. After this initial decrease in the release threshold, we found that prolonged oxidation led to an increase in the release threshold for SOICR. The increase in the release threshold for SOICR was to such an extent that SOICR ceased. These data suggest that prolonged oxidation of RyR2 leads to a loss of RyR2 activity due to an increase in the release threshold for SOICR. We also assessed whether the change in SOICR could be restored to normal levels after the removal of the oxidative challenge or by the application of a reducing agent. We found that even short-term oxidation of RyR2 by high levels of H_2O_2 led to an initial decrease and then irreversible increase in the release threshold for SOICR in most cells. Our data reveal that the oxidation-mediated increase and subsequent decrease in RyR2 activity in intact cells is due to corresponding changes in the release threshold for SOICR.

MATERIALS AND METHODS

Materials

All experimental materials were purchased from Sigma (Sigma Aldrich, St. Louis, MO) unless otherwise stated.

Methods

Generation of tetracycline-inducible human embryonic kidney cell lines: HEK293

Stable inducible HEK293 cells expressing RyR2 were generated as previously described (2).

Cytosolic Ca^{2+} measurements

Measurements of cytosolic Ca^{2+} were conducted in stable, inducible HEK293 cells expressing RyR2 as previously described (21). RyR2 expression was induced ~18 h before measurements commenced via the addition of tetracycline (0.1 $\mu\text{g}/\text{ml}$). Cytosolic Ca^{2+} transients were measured after loading the cells with the acetoxymethyl ester (AM) form of the Ca^{2+} indicators, Fluo-4 (Thermo Fisher Scientific, North Shore City, New Zea-

land) dissolved in anhydrous dimethyl sulfoxide and 12% (w/vol) pluronic F127 (Thermo Fisher Scientific). Loading of Fluo-4 was carried out by incubating HEK293 cells in 0 Ca^{2+} Krebs-Ringer Hepes (KRH) buffer containing 125 mM NaCl, 5 mM KCl, 25 mM HEPES, 6 mM glucose, and 1.2 mM MgCl_2 , containing bovine saline albumin (1 mg/ml) adjusted to pH 7.4 with NaOH, and Fluo-4 AM dye (2 μM) for 8 min at room temperature. Cells were then continuously superfused with KRH solution containing various concentrations of CaCl_2 (1 to 2 mM), H_2O_2 (0.01–1 mM), and DTT (10 mM) at room temperature. At the end of the experiments, 20 mM caffeine was applied to deplete the intracellular Ca^{2+} store. Fluo-4 AM dye was excited at 470 nm (40 nm bandwidth) every 2 s with an exposure time of 100 ms using a CoolLED system (Coherent Scientific Pty., Hilton, Australia). Fluorescence of Fluo-4 was detected through a longpass dichroic mirror (495 nm) and a longpass emission filter (>515 nm) by a CoolSNAP HQ2 charge-coupled device camera (Photometrics, Tucson, AZ). The cytosolic Ca^{2+} fluorescence is represented by F/F_0 , where F is the Ca^{2+} fluorescence intensity at any time and F_0 is the average fluorescence intensity recorded in 0 Ca^{2+} KRH solution.

Luminal Ca^{2+} measurements

Stable, inducible HEK293 cells expressing RyR2 as described previously were used with the addition of transfection with D1ER cDNA. D1ER transfection took place 24 h before RyR2 induction. The cells were perfused continuously at room temperature with KRH containing various concentrations of Ca^{2+} (1 and 2 mM), H_2O_2 (0.01 to 1 mM), tetracaine (2 mM to block RyR2), and caffeine (20 mM to deplete the SR store). Fluorescence images of HEK293 cells were acquired every 2 s with an exposure time of 100 ms and excitation at 436 nm (20 nm bandwidth). The emissions of yellow fluorescent protein and cyan fluorescent protein were captured with the addition of a dual dichroic beamsplitter at 535 nm (40 nm bandwidth) and 480 nm (30 nm bandwidth), respectively. Two images of 170×256 pixels each were collected with a simultaneous dual-channel imaging system. The amount of fluorescence resonance energy transfer (FRET) was determined from the ratio of the emissions at 535 and 480 nm.

Statistical analysis

Results are presented as mean \pm SE. For statistical analysis one-way or two-way analysis of variance was applied as appropriate. Differences were considered statistically significant if $p < 0.05$. All data analysis, curve fitting, and plotting were performed using GraphPad Prism 6 (GraphPad, La Jolla, CA).

RESULTS

Oxidation has a biphasic effect on the frequency of SOICR

To assess the functional effect of RyR2 oxidation on SOICR, changes in SOICR frequency were observed in the presence of H_2O_2 (1 mM). H_2O_2 induced a biphasic effect on the activity of RyR2 (Fig. 1). Initially, within 10 min, a significant increase in the frequency of SOICR was observed, followed by a slight reduction in the frequency, and ultimately abrupt inhibition of SOICR after a prolonged time in H_2O_2 (Fig. 1 B). Within the first 5 min of H_2O_2 exposure the frequency of SOICR increased to $125 \pm 2.7\%$, $p < 0.0001$ of control. By 10 min the frequency of SOICR reached $154 \pm 3.2\%$ of pre- H_2O_2 levels ($p < 0.0001$) (Fig. 1 C). However, further exposure to H_2O_2 led to a suppression of SOICR, which is clearly seen in Fig. 1 B by the abrupt loss of SOICR events. In the majority

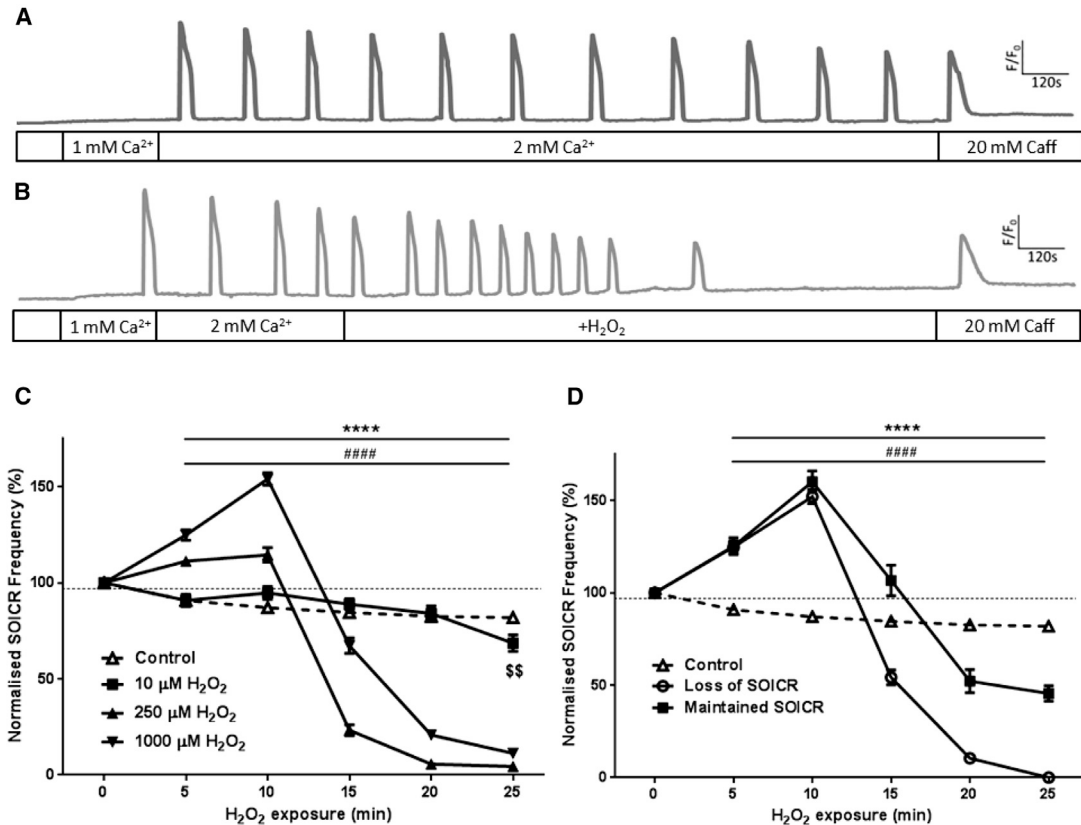


FIGURE 1 H_2O_2 increases and then decreases the occurrence of SOICR. HEK293 cells stably expressing RyR2 were loaded with Fluo-4 in KRH buffer. Cells were superfused with KRH containing 0, 1, or 2 mM Ca^{2+} , with or without various concentrations of H_2O_2 . At the end of each experiment cells were perfused with 20 mM caffeine. Representative traces from cells treated without (A) or with 1 mM H_2O_2 (B). (C) The frequency of SOICR events, per 5 min bin, normalized to the frequency of events before the application of H_2O_2 . (D) Separate frequency analysis of cells where SOICR was maintained or inhibited in cells exposed to 1 mM H_2O_2 . Control $n = 158$, 10 μM $n = 71$, 250 μM $n = 93$, 1000 μM $n = 162$. Data shown are mean \pm SE. \$\$ 10 μM $p < 0.01$; **** 250 μM , $p < 0.0001$; ##### 1000 μM , $p < 0.0001$; all compared to control (no H_2O_2).

of cells there was only a small decrease in the frequency of SOICR (from 10 min onward) before it ceased (Fig. 1 B). The apparent gradual decline in SOICR frequency between the 15 to 25 min depicted in Fig. 1 C results from the complete loss of SOICR occurring at varying time points in different cells exposed to H_2O_2 . SOICR ceased in $85.8\% \pm 6.89\%$ (mean of inhibited cells per assay) of cells exposed to 1 mM H_2O_2 by 25 min. The mean time at which SOICR events ceased in these cells was 14.41 ± 0.74 min following initial H_2O_2 exposure. Because SOICR was not inhibited in all cells exposed to 1 mM H_2O_2 , we performed separate frequency analysis on each of the two cell populations. As shown in Fig. 1 D both populations of cells showed the same initial increase in the frequency of SOICR in response to H_2O_2 , suggesting that the initial increase in SOICR is independent of whether it is inhibited. The increase in frequency peaked ($160 \pm 5.8\%$ and $152 \pm 3.9\%$ in cells where SOICR was maintained or lost, respectively) at 10 min post- H_2O_2 application. The reduction in the amplitude of SOICR depicted in Fig. 1 B is likely due to the loss and bleaching of the Fluo-4 dye rather than changes in the magnitude of Ca^{2+} release because the same trend can

be seen in the nontreated cell (Fig. 1 A). However, it is also possible that prolonged treatment with H_2O_2 leads to the gradual depletion of the Ca^{2+} store and subsequently Ca^{2+} release. Of importance, loss of SOICR in cells exposed to H_2O_2 was not due to the complete depletion of the Ca^{2+} store as the application of 20 mM caffeine was able to elicit a release of store Ca^{2+} to a level comparable to the untreated cells (Fig. 1, A and B).

Oxidation initially decreases the release threshold for SOICR in a concentration-dependent manner

To investigate the mechanism by which oxidation of RyR2 alters the frequency for SOICR, we monitored luminal Ca^{2+} directly using the lumenally expressed Ca^{2+} indicator D1ER. We have previously used this technique to monitor how point mutations within RyR2 and proarrhythmic drugs such as caffeine alter the thresholds for SOICR (3,4,22). Those experimental data have demonstrated that the occurrence and magnitude of Ca^{2+} release was determined by both the release threshold for SOICR (the intracellular

Ca^{2+} store level, expressed as a percentage of the total store, at which SOICR events occur (F_{SOICR}), governed by the sensitivity of RyR2 to SR luminal Ca^{2+} (3)), and the termination threshold (the intracellular Ca^{2+} store level, expressed as a percentage of the total store, at which SOICR events terminate (F_{Termini}), controlled by the inactivation of RyR2 due to partial depletion of SR Ca^{2+} (22,23) or luminal Ca^{2+} -dependent deactivation of RyR2 (24)). To establish a concentration-dependent effect of H_2O_2 on the release and termination threshold for SOICR, cells were exposed to a range of H_2O_2 concentrations (10 μM –1 mM) for 15 min.

Control cells (in the absence of H_2O_2) exhibited a negligible change in the release or termination thresholds throughout the duration of an experiment (data not shown). Lower concentrations of H_2O_2 (10, 25, and 50 μM) also did not significantly affect the release or termination thresholds for SOICR (Fig. 2 A). The percentage change in threshold is characterized as the difference between the baseline release threshold (before H_2O_2 application for each condition, F_{SOICR}) and the maximum change in release threshold following H_2O_2 addition (F_{SOICR}^*). As the concentration of H_2O_2 surpassed 100 μM , both the release (F_{SOICR} vs. F_{SOICR}^*) and termination (F_{Termini} vs. F_{Termini}^*) thresholds for SOICR displayed significant decreases (100 μM H_2O_2 ; release threshold: $-5.5 \pm 1.25\%$, termination threshold: $-5.4 \pm 1.28\%$; both $p < 0.01$ vs. control) (Fig. 2, D and E). Because the thresholds for release and termination fell to the same extent under each condition, there was no change in the amount of Ca^{2+} released per SOICR event (Fig. 2 F). The largest decrease in the SOICR thresholds was observed when the cells were exposed to 1 mM H_2O_2 (release threshold $-15.0 \pm 1.14\%$, termination threshold: $-16.5 \pm 1.05\%$; both $p < 0.0001$ vs. control) (Fig. 2, C, D, and E). Of importance, the maximum store content (Max) and store size (Max-Min) were unaltered by H_2O_2 suggesting that the ability of SERCA to completely fill the store was not changed by H_2O_2 (Fig. 2 G). The lack of change in the magnitude of release and maximum store size confirms that the apparent reduction in magnitude of release when using Fluo-4 (Fig. 1, A and B), is due to photobleaching or loss of the dye rather than changes in the magnitude of Ca^{2+} release. During cytosolic Ca^{2+} imaging, an increased SOICR frequency was observed by 5 min, with the maximum increase occurring at ~ 10 min (Fig. 1). These time-dependent changes in SOICR frequency coincide with the time at which H_2O_2 led to the maximum decrease in the release threshold for SOICR during luminal Ca^{2+} imaging. On average the release threshold was maximally decreased at 6.19 ± 0.218 min following 1 mM H_2O_2 addition. Interestingly, exposure to 1 mM H_2O_2 induced an additional change in the release threshold for SOICR from 10 min onward (Fig. 2 C), where both the release and termination thresholds began to increase. This effect, associated with prolonged exposure to high concentrations of H_2O_2 , was further investigated below. The secondary change in SOICR seen during luminal Ca^{2+} imaging coincides with loss of SOICR seen during cytosolic Ca^{2+}

imaging (Fig. 1). This suggests that the loss of SOICR mediated by H_2O_2 is also linked with modification of the release threshold for SOICR.

Prolonged RyR2 oxidation increases the release threshold for SOICR

Cytosolic Ca^{2+} imaging showed that prolonged oxidation (15 min onward) was associated with the complete loss of SOICR activity in most cells. Given that we observed an increase in the release threshold for release during the luminal Ca^{2+} imaging, we hypothesized that loss of SOICR was due to an increase in the release threshold of SOICR. To investigate this, luminal Ca^{2+} imaging was performed over an extended time frame (25 min). Following the initial decrease in the release threshold (F_{SOICR}^*) for SOICR, high concentrations (500 μM – 1 mM) of H_2O_2 caused both the release and termination thresholds to rise (see Fig. 3 B). This was followed by the complete loss of SOICR (F_{Inhib}) in $55.5 \pm 3.44\%$ of cells by 25 min (in 1 mM H_2O_2). In the absence of H_2O_2 there was no change in the thresholds for SOICR throughout the assay (Fig. 3 A). This suggests that prolonged oxidation can further modify the thresholds for SOICR and eventually lead to the complete loss of SOICR, which is consistent with the Fluo-4 data described previously (Fig. 1). Notably, when SOICR activity was lost, the level of luminal Ca^{2+} remains high, confirming that the loss of SOICR is not due to the depletion of luminal Ca^{2+} or inability of SERCA to fill the Ca^{2+} store. Given that SOICR is dependent on the activity of RyR2, store size, and filling, this shows that the loss of SOICR is attributable to the inactivity of RyR2. In the cells treated with high levels of H_2O_2 , the presence of H_2O_2 increased the release threshold (F_{Inhib}) to the same extent as 2 mM tetracaine (maximum store level for cells treated with 2 mM tetracaine = 0.49 ± 0.02 versus maximum store level in cells with prolonged 1 mM H_2O_2 treatment and no tetracaine = 0.45 ± 0.03 , $p = 0.3$), suggesting that prolonged RyR2 oxidation inhibits SOICR due to an increase in the release threshold for SOICR, a mechanism by which tetracaine itself inhibits RyR2 function and is also suggested to explain the loss of function of certain RyR2 mutations (2,3,25).

Brief exposure to H_2O_2 can result in an irreversible loss of SOICR

Because previous studies have found changes in RyR2 activity during oxidative stress to be partially reversible (11), we investigated if changes in SOICR mediated by short-term oxidation of RyR2 by high levels of H_2O_2 could be reversed when the oxidative challenge was removed. Cells were exposed to 1 mM H_2O_2 for 4.5 min as this previously resulted in a $\sim 25\%$ increase in the frequency of SOICR (Fig. 1). As expected all cells exhibited a significant increase

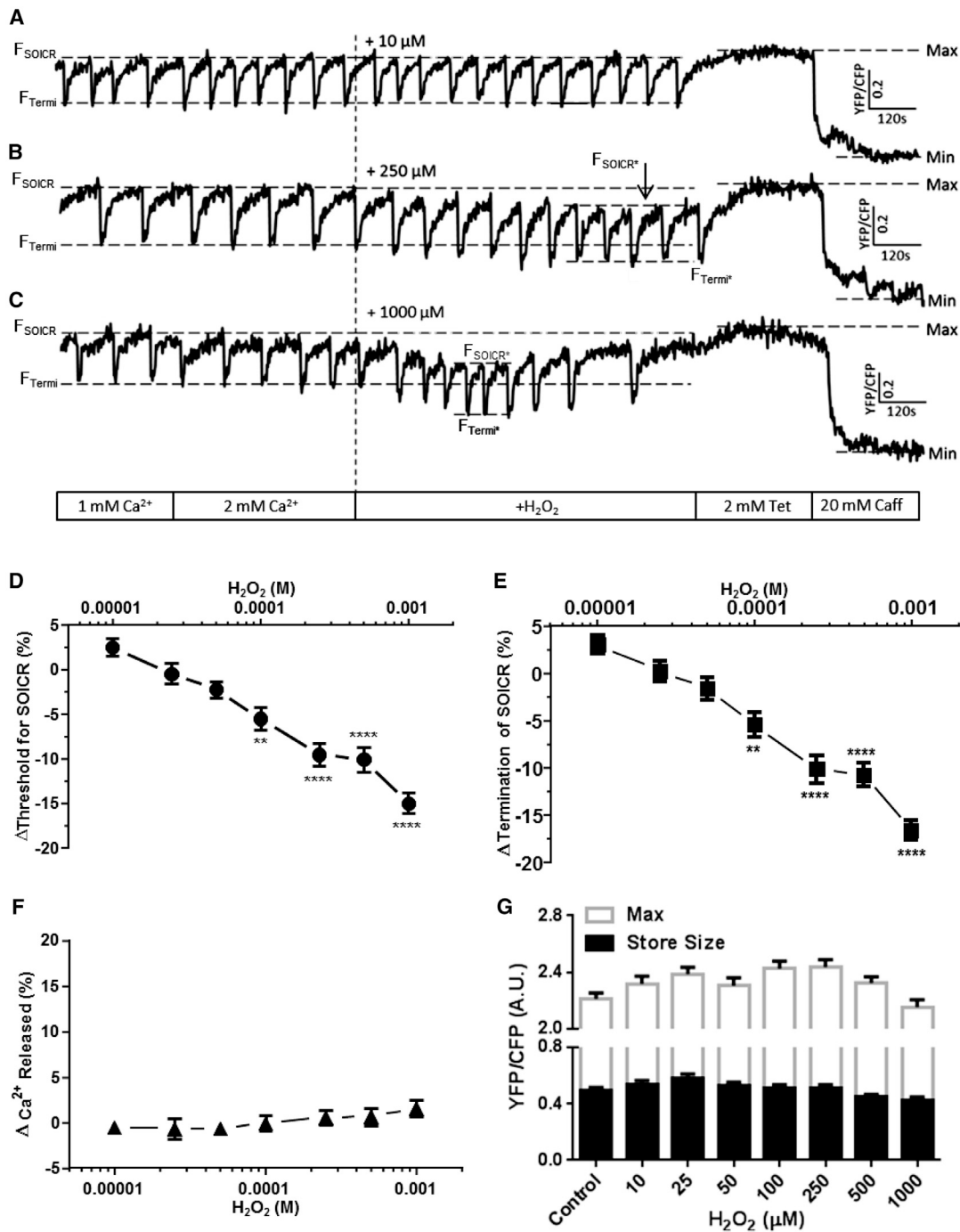


FIGURE 2 H₂O₂ initially decreases the thresholds for SOICR. HEK293 cells stably expressing RyR2 were transfected with the luminally expressed FRET-based Ca²⁺ indicator; DIER. The cells were superfused with KRH containing 1 or 2 mM Ca²⁺ and varying concentrations of H₂O₂ (10–1000 μM). At the end of each experiment cells were perfused with 2 mM tetracaine and 20 mM caffeine to determine the maximum and minimum store capacities, respectively. (A–C) Representative traces from cells exposed to 10, 250, and 1000 μM H₂O₂; dashed lines represent the release (before: F_{SOICR} and after: F_{SOICR}* H₂O₂) and termination (before: F_{Termin} and after: F_{Termin}* H₂O₂) SOICR thresholds or maximum (Max) and minimum (Min) store levels. (D–G) H₂O₂-mediated changes in the SOICR threshold for release, SOICR termination threshold, magnitude of Ca²⁺ release, and max store level and store size, respectively. Control *n* = 39, 10 μM *n* = 24, 25 μM *n* = 28, 50 μM *n* = 32, 100 μM *n* = 36, 250 μM *n* = 40, 500 μM *n* = 34, and 1000 μM *n* = 33. Data shown are mean ± SE. ** *p* < 0.01; **** *p* < 0.0001; compared to control (no H₂O₂).

in SOICR frequency during the brief exposure to 1 mM H₂O₂ (Fig. 4). Following the increase in frequency, SOICR continued in 42.6 ± 8.38% of cells, whereas SOICR was

completely inhibited in the remaining 57.4 ± 8.38% of cells (Fig. 4 C; Table 1). Again, given the distinctly different phenotypes we performed separate frequency analysis on each of

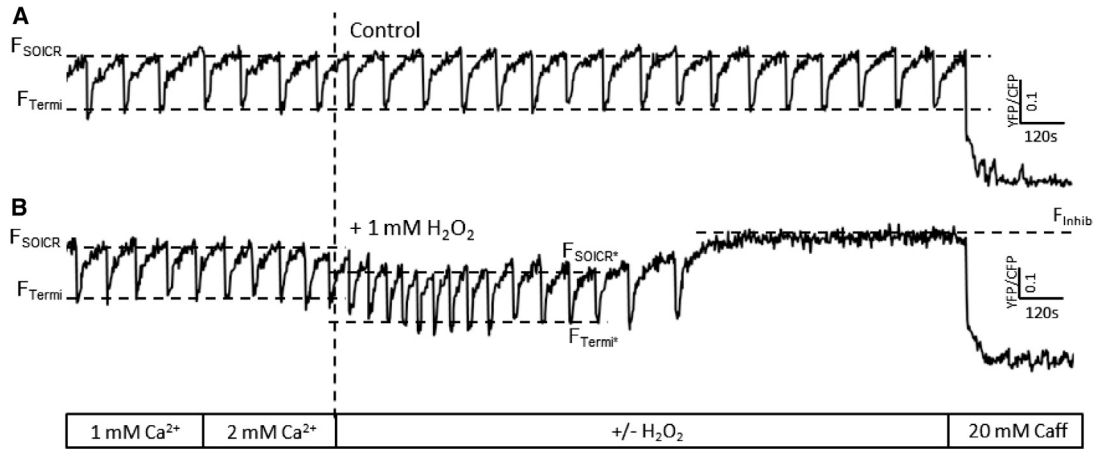


FIGURE 3 Continued exposure to high concentrations of H_2O_2 increases the thresholds for SOICR. HEK293 cells stably expressing RyR2 were transfected with the luminally expressed FRET-based Ca^{2+} indicator, D1ER. The cells were superfused with KRH containing 1 or 2 mM Ca^{2+} with or without H_2O_2 (1 mM). At the end of each experiment cells were perfused with 20 mM caffeine to illustrate functional RyR2 channels. Representative traces of cells exposed to (A) 0 or (B) 1 mM H_2O_2 ; dashed lines represent the release (before: F_{SOICR} and after: $F_{\text{SOICR}}^* \text{H}_2\text{O}_2$) and termination (before: F_{Termi} and after: $F_{\text{Termi}}^* \text{H}_2\text{O}_2$) SOICR thresholds. F_{Inhib} (B) represents the store level after SOICR was inhibited by H_2O_2 .

the two cell populations. As can be seen in Fig. 4 both populations of cells showed a similar initial increase in the frequency of SOICR in response to H_2O_2 . The increase in frequency peaked ($143 \pm 3.4\%$ and $141 \pm 3.2\%$ in cells where

SOICR continued or lost SOICR, respectively) ~5 min following the removal of H_2O_2 . This is very similar to the peak increase in frequency observed in cells continually exposed to H_2O_2 ($154 \pm 3.2\%$, Fig. 1). In those cells, which

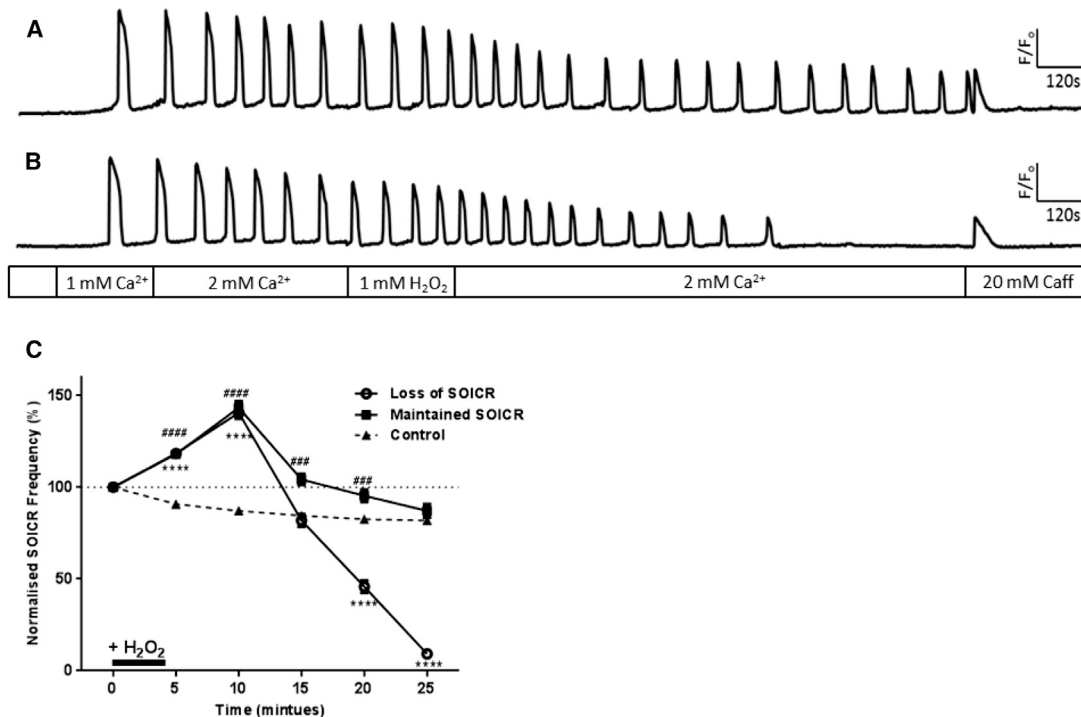


FIGURE 4 Short exposure to high $[\text{H}_2\text{O}_2]$ leads to the inhibition of SOICR. HEK293 cells stably expressing RyR2 were loaded with Fluo-4 in KRH buffer. The cells were then transiently superfused with KRH containing 0, 1, or 2 mM Ca^{2+} and 1 mM H_2O_2 (4.5 min) followed by KRH alone (21.5 min). At the end of each experiment cells were perfused with 20 mM caffeine. Representative traces from cells where SOICR (A) was maintained or (B) was inhibited. (C) The frequency of SOICR events, per 5 min bin, normalized to the frequency of events before the application of H_2O_2 . Cells treated with H_2O_2 are displayed as those where SOICR events were maintained or where SOICR ceased by the end of the experiment. Control $n = 158$, Loss of SOICR $n = 148$, Maintained SOICR $n = 106$. Data shown are mean \pm SE. **** Loss of SOICR, $p < 0.0001$; ### and ##### Maintained SOICR, $p < 0.001$ and < 0.0001 , respectively, compared to control (no H_2O_2).

TABLE 1 Summary of H₂O₂-Mediated Inhibition of SOICR

	Persistent H ₂ O ₂	Removal of H ₂ O ₂	Posttreatment with DTT	Pretreatment with DTT
Time to loss of SOICR (minutes)	14.41 ± 0.74	17.23 ± 0.64 ^a	18.15 ± 0.85 ^b	12.32 ± 0.55, not significant
Loss of SOICR (% cells)	85.8 ± 6.89 <i>n</i> = 5	57.4 ± 8.38, not significant, <i>n</i> = 5	72.52 ± 10.04, not significant, <i>n</i> = 5	73.9 ± 11.7, not significant, <i>n</i> = 8

Values are mean ± SE. Time and percentage of cells experiencing complete inhibition of SOICR. Time is given from the application of H₂O₂. Means and statistical analysis were calculated from the number of assays performed (*n* = 5 or 8) Total cells per condition were: 234, 148, 188, and 203 for persistent removal of H₂O₂, posttreatment with DTT, and pretreatment with DTT, respectively.

^a*p* < 0.05

^b*p* < 0.01 versus persistent H₂O₂

continued to experience SOICR, the frequency of SOICR returned to that of nontreated cells by 25 min post-H₂O₂ treatment. These data suggest that in roughly half of the cells, oxidation of RyR2 returned to control levels once H₂O₂ was removed, whereas in the other half the modification of RyR2 persisted.

Oxidation-induced loss of SOICR cannot be reversed or prevented by reducing agents

SOICR was inhibited in roughly half of the cells treated with H₂O₂ for 4.5 min, suggesting that endogenous cellular reducing agents were unable to reverse RyR2 oxidation. Thus, we next investigated whether the application of an exogenous reducing agent could prevent the loss of SOICR in these cells. Accordingly, cells were either treated with the reducing agent DTT immediately after H₂O₂ or were pretreated with DTT before H₂O₂.

As shown in Table 1, treatment of the cells with 10 mM DTT before or after exposure to H₂O₂ was not able to prevent the loss of SOICR with 72.52 ± 10.04% or 73.9 ± 11.7% of cells treated with DTT (post or pre, respectively) experiencing the complete loss of SOICR by 25 min. Although lower, the percentage of cells experiencing loss of SOICR was not significantly different in either condition to those cells persistently exposed to H₂O₂ (85.8 ± 6.89%, *p* > 0.05). Again, given the two distinctly different phenotypes (cells where SOICR ceased or continued) we analyzed each group separately (Figs. 5 and 6). In cells where SOICR ceased, treatment with DTT following H₂O₂ had no effect on the mean time to inhibition compared to cells where H₂O₂ was removed but no reducing agent was applied (18.15 ± 0.85 vs. 17.23 ± 0.64 min for cells treated with and without DTT, respectively, *p* > 0.05) (Table 1). However, compared to cells where H₂O₂ was present throughout the assay both removal of H₂O₂ and application of DTT delayed the mean time to inhibition of SOICR (Table 1). Interestingly, pretreatment of cells with DTT before the application of H₂O₂ did not protect against the loss of SOICR as the mean time to SOICR inhibition in the continued presence of H₂O₂ following DTT treatment was not different to cells exposed to H₂O₂ without DTT pretreatment (14.41 ± 0.74 vs. 12.32 ± 0.55 min, respectively) (Table 1). Because DTT had no effect on SOICR,

we confirmed that the concentration of DTT used (10 mM) was sufficient to reduce other intracellular proteins. This was achieved by coexpressing the oxidation sensitive fluorescent protein roGFP2-Orp1 (26). Fig. S1 in the Supporting Material shows that 10 mM DTT rapidly reduces roGFP2-Orp1, indicating that the lack of effect on SOICR is not due to insufficient DTT (reducing capacity) entering the cell.

DISCUSSION

Acute oxidation of RyR2 has recently been shown to increase Ca²⁺ leak due to a reduction in the release threshold for SOICR (20). However, how chronic oxidation of RyR2 impacts SOICR is unknown. In this study we assessed the effect of acute and prolonged exposure to H₂O₂ on cells expressing RyR2. Our data demonstrate for, to the best of knowledge, the first time that prolonged oxidation of RyR2 leads to an increase in the release threshold for SOICR, which prevents further SOICR events. Interestingly, our data also show that even brief exposure to high concentrations of H₂O₂ can lead to loss of SOICR. This oxidation-mediated inhibition of SOICR was irreversible in most cells even when treated with DTT. Our data suggest that a high level of RyR2 oxidation leads to an irreversible increase in the release threshold for SOICR and underlies the inhibition of Ca²⁺ leak seen during extreme oxidative stress.

A major finding of this study is that the previously observed biphasic effect of oxidation of RyR2 (13,15) in myocytes is due to a decrease and subsequent increase in the release threshold for SOICR. These data suggest that oxidation of RyR2 can modify RyR2 activity by a similar mechanism as mutations within RyR2, which depending on their location are known to increase or decrease the release threshold for SOICR (3,25). Several molecular models have been proposed to explain the initial increase and then decrease in channel function due to oxidation. Salama and colleagues (17) have proposed that there are three closely related thiol groups. Disulphide formation between two groups leads to an increase in channel activity, whereas further oxidation leads to a switch in disulphide formation to a different pair of the three free thiols and closes the channel. Dulhunty and colleagues (16) suggest that RyR2 contains thiol groups within the channel pore that activate the channel (SHa) and a second group of thiols

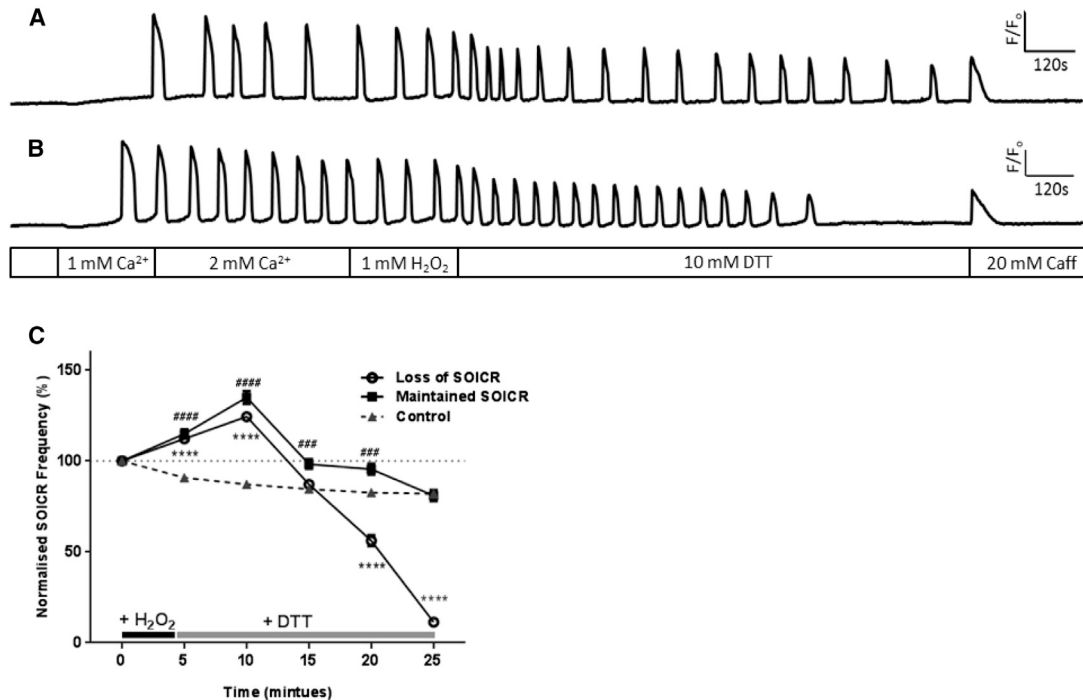


FIGURE 5 Short exposure to high $[H_2O_2]$ leads to irreversible inhibition of SOICR. HEK293 cells stably expressing RyR2 were loaded with Fluo-4 in KRH buffer. The cells were then transiently (4.5 min) superfused with KRH containing 0, 1, or 2 mM Ca^{2+} and 1 mM H_2O_2 followed by 10 mM DTT (21.5 min). At the end of each experiment cells were perfused with 20 mM caffeine. Representative traces from cells where SOICR (A) was maintained or (B) was inhibited. (C) The frequency of SOICR events, per 5 min bin, normalized to the frequency of events before the application of H_2O_2 . Cells treated with H_2O_2 and DTT are displayed as those where SOICR events were maintained or where SOICR ceased by the end of the experiment (Loss of SOICR). Control $n = 158$, Loss of SOICR $n = 188$, Maintained SOICR $n = 75$. Data shown are mean \pm SE. **** Loss of SOICR, $p < 0.0001$; ### and #### Maintained SOICR, $p < 0.001$ and < 0.0001 , respectively, compared to control (no H_2O_2).

within the transmembrane domain that inhibit the channel (SHi). Zhang et al. offer an alternative model consisting of four states. Rapid oxidation of fast but low-affinity thiols leads to an increase in activity; oxidation of slow but high-affinity thiols has no impact on channel function but primes the channel for further oxidation (27). Although different mechanisms have been proposed, these molecular models all support that continued oxidation of RyR2 can lead to closure of the channel, which is also demonstrated by this study. However, here we propose that prolonged oxidation appears to increase the release threshold for SOICR leading to an eventual inhibition of SOICR, rather than an immediate cessation of SOICR. Our data suggest that extensive oxidation of RyR2 does not close the channel, rather it increases the release threshold for SOICR to such an extent that store Ca^{2+} is no longer able to drive SOICR events. This mechanism of RyR2 channel inhibition is consistent with that of tetracaine, which also prevents SOICR by increasing the release threshold for release due to a reduction in RyR2 open probability (2,28).

The locations of the different thiol groups responsible for the changes in RyR2 activity remain elusive. The lack of crystal structure for RyR2 makes locating these sites difficult. By mutating cysteine 3602 to alanine, we were recently able to determine that this residue is not important in the

effects of oxidizing agents on RyR2 (20). However, given the predicted number of free thiols within RyR2 (at least 21 (7)) this approach would require a concerted effort to individually characterize each potential site.

A second finding of this study is that the increase in the release threshold for SOICR following prolonged exposure to H_2O_2 is irreversible. In the majority of cells the increase in the release threshold and corresponding inhibition of SOICR could not be reversed even in the presence of high concentrations of a reducing agent (DTT). The inability of DTT to reverse the effect of oxidation suggests that the structural changes mediated by oxidation are to such an extent they cannot be reversed, this may be due to the inaccessible nature of these sites (11,16). Interestingly, the inaccessibility of these sites suggests they could be buried within the protein. Methionine residues are commonly buried within a proteins structure due to the hydrophobic nature of their thiol-ether side chain; however, they are still readily oxidized by H_2O_2 in a pattern similar to that seen in vivo (29,30). Therefore, although only cysteines have been implicated in RyR2 oxidation, the role of methionines should not be overlooked. Although direct regulation of RyR2 by methionine oxidation has not been reported, calmodulin (CaM) a protein that associates and regulates RyR2 is known to undergo methionine oxidation (31). The

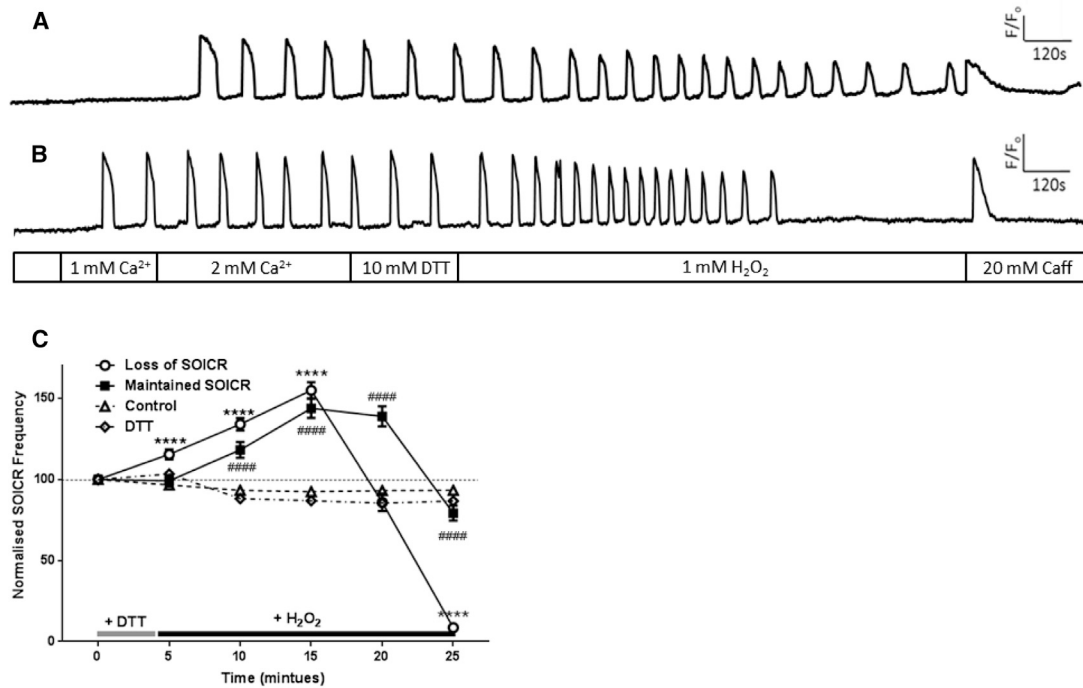


FIGURE 6 Pretreatment with DTT does not prevent H₂O₂-mediated inhibition of SOICR. HEK293 cells stably expressing RyR2 were loaded with Fluo-4 in KRH buffer. The cells were then transiently superfused with KRH containing 0, 1, or 2 mM Ca²⁺ and 10 mM DTT (4.5 min) followed by 1 mM H₂O₂ (21.5 min). Some cells were superfused throughout the assay with 10 mM DTT (DTT). At the end of each experiment cells were perfused with 20 mM caffeine. Representative traces from cells where SOICR (A) was maintained or (B) was inhibited. (C) The frequency of SOICR events, per 5 min bin, normalized to the frequency of events before the application of DTT. Cells treated with both DTT and H₂O₂ are displayed as those where SOICR events were maintained or where SOICR ceased by the end of the experiment (Loss of SOICR). Control $n = 234$, Loss of SOICR $n = 203$, Maintained $n = 82$, Persistent DTT $n = 203$. Data shown are mean \pm SE. **** Loss of SOICR, $p < 0.0001$; ### and ##### Maintained SOICR, $p < 0.001$ and < 0.0001 , respectively, compared to control (no H₂O₂).

oxidation of CaM reduces its affinity for RyR2 leading to dissociation, destabilization of RyR2, and a subsequent increase in RyR2 activity (32). HEK293 endogenously express CaM (33), which would likely be dissociated by the high concentrations of H₂O₂ in our assays. However, the association of CaM with RyR2 appears to have no effect on the release threshold for SOICR (34), suggesting it is not responsible for any of the changes reported here. Additionally, HEK293 cells do not express other RyR2 interacting proteins known to alter RyR2 function (e.g., FKBP12.6 and calsequestrin); therefore, combined with the close agreement with single-channel studies of RyR2 thiol modification, we can be confident our data unveil the direct effect of RyR2 oxidation in an intact cell model.

In this study the increase in the threshold for SOICR was primarily observed at suprphysiological concentrations of H₂O₂, similar to those used by others studying RyR1 single channel function who also observed a biphasic response (18). However, the presence of endogenous reducing agents, such as glutathione, in our intact cell model will reduce the concentration of H₂O₂ to which RyR2 is exposed. Therefore, the changes in the threshold for SOICR are likely to be occurring at lower Ca concentrations of H₂O₂ within the cell. The presence of endogenous reducing agents, particularly with respect to the reversibility of the effect of oxida-

tion, highlights the importance of extending previous single channel data on RyR2 thiol modifications (by doxorubicin (19,35)) and RyR1 oxidation (by H₂O₂ (18)) to an intact cell model, and suggests that thiol modification induced irreversible inhibition of RyRs observed at the single-channel level is not an artifact of working with purified RyR channels. Notwithstanding, the irreversible nature of oxidation-induced SOICR inhibition in situ suggests it may be a pathological form of regulation, because RyR2 activity could only be recovered through the replacement of the oxidized channels. Under normal levels of oxidative stress it is more likely to result in an increase in activity due to the decrease in release threshold. This is consistent with the greater levels of cytoplasmic Ca²⁺ seen in heart failure models where ROS production is increased. The increase in the release threshold for SOICR is only likely to occur where high level short term or prolonged exposure to oxidizing agents occurs. This effect has been reported to be one mechanism of anthracycline cardiotoxicity (35).

Another finding of our study is that even short-term exposure to high levels of oxidative stress can irreversibly inhibit SOICR. Interestingly, the timescale of the inhibition of SOICR due to the acute exposure to H₂O₂ does not appear to be much greater than that seen in cells chronically exposed to H₂O₂, occurring after the removal of H₂O₂.

Why this delay between exposure to H₂O₂ and SOICR inhibition occurs is unclear because the removal of H₂O₂ should minimize continued RyR2 oxidation. However, given the large oxidative insult applied the delayed response could be attributable to ROS-induced ROS release (RIRR) (36). RIRR is known to occur following the application of exogenous oxidizing agents to cells (37). This RIRR response to acute oxidative stress has major implications because it suggests that the duration of the oxidative injury to the heart may not need to be sustained for long periods of time for irreversible modification of RyR2 to occur.

The basal oxidized state of RyR2 is controversial. Hanna et al. have shown that to mimic in situ channel activity single-channel recordings must be performed in mildly oxidizing conditions (38). This observation suggests that the dyad contains a greater level of ROS than the bulk cytosol, which is perhaps unsurprising given the localized production of superoxide by NADPH oxidase 2 (39) and the severely restricted diffusion of the dyad (40). Our data show that the threshold for SOICR can be both increased and decreased by ROS and indicate that in vivo changes in the localized ROS content will be able to tune the activity of RyR2 by causing small increases or decreases in the threshold for SOICR. This mechanism is akin to the proposed mode of RyR2 regulation by phosphorylation. Consistent with a lack of change in store Ca²⁺ content in healthy myocytes treated with DTT (11), the persistent application of DTT did not alter the frequency of SOICR (Fig. 6) or elicit a change in the release threshold for SOICR (data not shown) suggesting that, in our cell model and healthy myocytes, the basal levels of RyR2 oxidation are negligible or are at least insufficient to alter the activity of RyR2. This is also consistent with isolated RyR2 protein studies where the application of DTT does not alter RyR2 single-channel activity (35,38). Similarly, pretreatment with DTT also had no impact on the effect on the subsequent addition of H₂O₂ (Fig. 6). This strengthens the notion that the basal levels of RyR2 oxidation are negligible in our cell model, because an already low oxidation state of RyR2 would not be meaningfully altered by DTT pretreatment.

In summary, we demonstrate that changes in RyR2 function observed at the single-channel level result in alterations in the release threshold for SOICR, which likely underlies both the increase and decrease in RyR2 function observed in cardiac myocytes during oxidative stress. Our results indicate that oxidation regulates RyR2 via the same mechanism as phosphorylation, methylxanthines, and RyR2 mutations, further supporting the importance of the thresholds for SOICR in regulating the channel.

SUPPORTING MATERIAL

Supporting Materials and Methods and one figure are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(16\)30239-9](http://www.biophysj.org/biophysj/supplemental/S0006-3495(16)30239-9).

AUTHOR CONTRIBUTIONS

H.M.M.W. and P.P.J. designed the research; H.M.M.W., K.J.H., J.J.M., and J.C.M. performed the research; H.M.M.W., J.Z.Z., K.J.H., J.J.M., J.C.M., and P.P.J. analyzed the data and H.M.M.W., J.Z.Z., and P.P.J. wrote the article.

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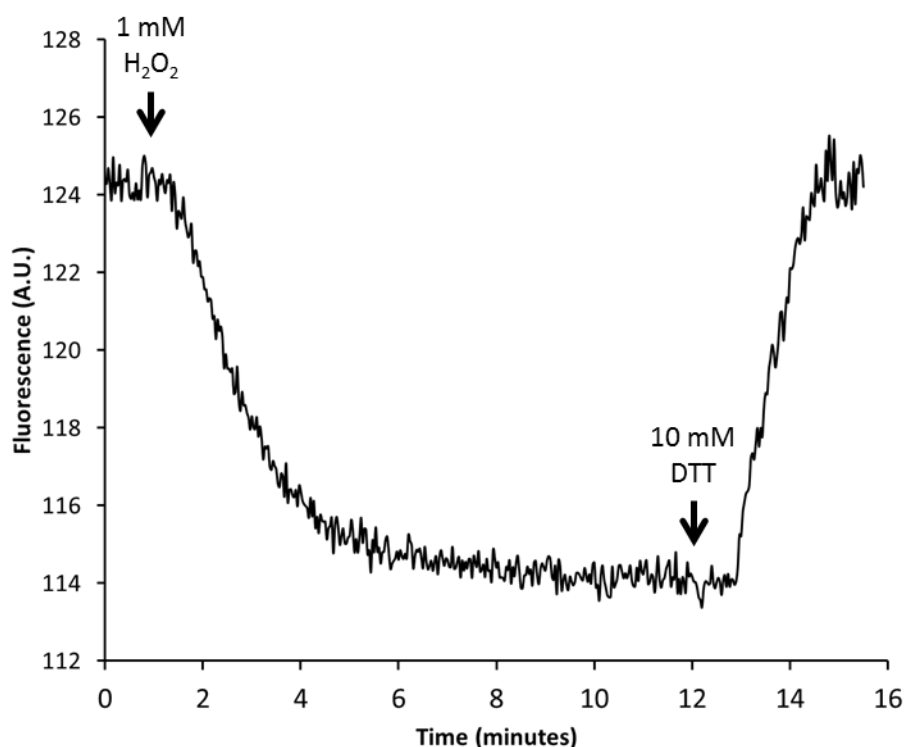
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Supplemental Information

**Oxidation of RyR2 Has a Biphasic Effect on the Threshold for Store
Overload-Induced Calcium Release**

Helen M.M. Waddell, Joe Z. Zhang, Katie J. Hoeksema, Julia J. McLachlan, Janet C. McLay, and Peter P. Jones

Supplementary Material



Supplementary Figure 1. Effect of H₂O₂ and DTT on protein oxidation in HEK293 cells
HEK293 cells stably expressing RyR2 were transfected with the H₂O₂ sensitive fluorescent protein roGFP2-Orp1. The cells were then transiently superfused with KRH containing 1 mM Ca²⁺ followed by KRH containing 1 mM H₂O₂ or 10 mM DTT. Trace is representative of 69 cells.

Supplementary Methods

Stable, inducible HEK293 cells expressing RyR2 were used with the addition of transfection with roGFP2-Orp1 cDNA (1). Transfection took place 24 h before imaging. The cells were perfused continuously at room temperature with KRH containing 1 mM Ca²⁺, 1 mM H₂O₂ or 10 mM DTT. Fluorescence images of HEK293 cells were acquired every 2 s with an exposure time of 100 ms and excitation at 470 nm (40 nm bandwidth) using a CoolLED system (Coherent Scientific Pty. Ltd, Australia). Fluorescence was detected through a long pass dichroic mirror (495 nm) and a long pass emission filter (>515 nm) by a CoolSNAP HQ2 CCD camera (Photometrics, AZ).

Supporting Reference

1. Gutscher, M., M. C. Sobotta, G. H. Wabnitz, S. Ballikaya, A. J. Meyer, Y. Samstag and T. P. Dick. 2009. Proximity-based Protein Thiol Oxidation by H₂O₂-scavenging Peroxidases. *J. Biol. Chem.* 284:31532-31540.