Mitochondria-derived ROS bursts disturb Ca\(^{2+}\) cycling and induce abnormal automaticity in guinea pig cardiomyocytes: a theoretical study

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\(^{1}\)Division of Cardiovascular Disease, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama; \(^{2}\)Cardiac Rhythm Management Laboratory, University of Alabama at Birmingham, Birmingham, Alabama; and \(^{3}\)Department of Biomedical Engineering, University of Alabama at Birmingham, Birmingham, Alabama; and \(^{4}\)Division of Cardiology, Department of Medicine, The Johns Hopkins University, Baltimore, Maryland

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Li Q, Su D, O’Rourke B, Pogwizd SM, Zhou L. Mitochondria-derived ROS bursts disturb Ca\(^{2+}\) cycling and induce abnormal automaticity in guinea pig cardiomyocytes: a theoretical study. Am J Physiol Heart Circ Physiol 308: H623–H636, 2015. First published December 24, 2014; doi:10.1152/ajpheart.00493.2014.—Mitochondria-derived ROS bursts disturb Ca\(^{2+}\) cycling and induce abnormal automaticity in guinea pig cardiomyocytes: a theoretical study. As an important universal signaling ion, cardiac Ca\(^{2+}\) is precisely regulated by a complicated system comprising of multiple sarcosomal and intracellular ion channels and transporters that activate and inactivate in a coordinated fashion during the cardiac cycle (6). Of those channels, the sarcoplasmic reticulum (SR) Ca\(^{2+}\) release, known as ryanodine receptors (RyRs), are the primary Ca\(^{2+}\) release sites. Structural studies have shown that tetrameric RyRs have 89 cysteine residues, of which 21 are susceptible to oxidation by free radicals (61). Oxidation of RyR cysteine residues forms disulfide bonds, leading to reversible activation of channel activity. Gen et al. (25) have shown that extracellular H\(_2\)O\(_2\) activates SR Ca\(^{2+}\) release and causes Ca\(^{2+}\) overload in rat ventricular myocytes. Similarly, exogenous H\(_2\)O\(_2\) can directly modify the gating of sheep cardiac RyRs and increase the channel open probability (21). In addition to RyRs, reactive oxygen species (ROS) also affect SR Ca\(^{2+}\) uptake proteins such as SR Ca\(^{2+}\)-ATPase (SERCA). It has been shown that H\(_2\)O\(_2\) and superoxide (a.k.a. O\(_2^-\)) inhibit SERCA activity by directly oxidizing its thiol groups (42, 68) or indirectly interfering with its ATP binding sites (60). Although the effects of exogenous ROS on SR Ca\(^{2+}\) handling proteins are evident (4, 29, 59), study of how endogenous ROS influence Ca\(^{2+}\) homeostasis has begun only recently (49, 62, 63, 66), partially due to the difficulty of inducing controllable endogenous ROS production in the cell in the experimental setting.

There is convincing evidence that mitochondria, the major sites of intracellular ROS production, and SR are in close proximity and physically tethered via mitofusin proteins (12, 19). This unique mitochondria-SR tethering forms a close coupling of Ca\(^{2+}\) signaling between SR release sites and nearby mitochondria, facilitating rapid mitochondrial Ca\(^{2+}\) uptake that stimulates oxidative phosphorylation (11). This coupling is essential for matching energy supply with demand in response to increased workload (39, 52). We speculate that this mitochondria-SR tethering is also involved in the interorganellar redox signaling, allowing dynamic modulation of SR Ca\(^{2+}\) handling by mitochondria-derived ROS (mdROS). In supporting this notion, recent studies have shown that mitochondria-SR coupling forms a close coupling of Ca\(^{2+}\) release (RIRR) are closely correlated to enhanced Ca\(^{2+}\) sparks in resting guinea pig cardiomyocytes (62, 66). In addition, others have reported that mdROS cause significant changes in global Ca\(^{2+}\) transients and regional Ca\(^{2+}\) sparks in smooth muscle cells (13), skeletal muscles (46), and cerebral arteries.

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Given these advancements, how mdROS may influence Ca²⁺ cycling and electrical activity in paced cardiomyocytes has not been fully elucidated. We hypothesize that the pathological mdROS can diffuse to the proximal SR and dynamically alter SR Ca²⁺ handling (e.g., RyRs and SERCA), disrupting Ca²⁺ cycling and causing erratic action potential (AP) generation.

To test the hypothesis, we developed a novel guinea pig cardiomyocyte model that integrates excitation-contraction coupling, Ca²⁺ handling, and mitochondrial energetics, as well as the modulations of RyRs/SERCA by the surrounding mdROS. The simulations show that mdROS bursts rapidly induce cytosolic Ca²⁺ overload by stimulating RyRs and inhibiting SERCA. The elevating [Ca²⁺], activates the Na⁺/Ca²⁺ exchanger (NCX) and Ca²⁺-sensitive nonspecific cationic channels (nSCa) that underlie transient inward currents (Iₜ), further exacerbating Ca²⁺ dysregulation and leading to APs triggered by the abnormal automaticity.

METHODS

Model Development

In this study, a multiscale guinea pig cardiomyocyte model was developed to examine the effect of mitochondrial oxidative stress on Ca²⁺ regulation and cellular electrophysiological behaviors. This model was based on our recently published excitation-contraction coupling, mitochondrial energetics and ROS-induced ROS release (ECME-RIRR) model (37, 67) and incorporated new model components including local control Ca²⁺-induced Ca²⁺-release (CICR), mitochondria-SR microdomain (MSM), and mdROS modulations of RyRs and SERCA. In a recent study we showed that the mdROS-mediated Ca²⁺ sparks can be suppressed by TMPyP (a O²⁻ dismutase mimetic) or 4’-Cl diazepam (the peripheral benzodiazepine receptor ligand that blocks mitochondrial O²⁻ release) (66), suggesting that mitochondria-derived O²⁻ (mdO₂⁻) plays a major role in the dynamic modulation of SR Ca²⁺ release during RIRR. Thus only the direct effect of mdO₂⁻ on SR Ca²⁺ handling proteins was considered in the present model development. For simplification, we assumed that all mitochondria in the cardiomyocyte depolarize/oscillate synchronously (1). It is worth noting that such a synchronization may only occur in pathological, rather than physiological, conditions (2). Consequently, the mitochondria were lumped as a giant mitochondrion and mitochondria-SR subspaces were modeled as a single compartment. The schemes of the new whole cell model and the MSM model are shown in Supporting Information Fig. S1 and Fig. S2, respectively (see ENDNOTE).

**mdO₂⁻ diffusion.** Studies have shown that under pathophysiological conditions, mitochondrial O₂⁻ production increases significantly and upon reaching a threshold triggers the opening of the inner membrane anionic channels (1, 3, 65) and/or mitochondrial permeability transition pore, leading to RIRR (69). We hypothesize that the pathological mdO₂⁻ could diffuse from the perimitochondrial space to the proximal SR, dynamically modulating the redox-sensitive Ca²⁺ channels. Assuming that MSM is homogenous, the concentration profiles of mdO₂⁻ in the microdomain ([O₂⁻]ᵦ)=D_mdo2_iD_mdo2_t=\frac{\partial^2 [O_2^-]_{\text{MSM}}(x,t)}{\partial x^2} = \frac{\partial^2 [O_2^-]_{\text{MSM}}(x,t)}{\partial x^2} + \nu_{\text{cyto, MSM}} \cdot f([O_2^-]_{\text{MSM}}(x,t)) \tag{1}

where \(D_{\text{mdO}_2^-}\) is O₂⁻ diffusion coefficient, \(x\) is the distance from the mitochondrion, \(\nu_{\text{cyto, MSM}}\) is cytosol and MSM effective volume ratio, and \(f([O_2^-]_{\text{MSM}}(x,t))\) is O₂⁻ scavenging rate.

The numerical simulation of Eq. 1 was performed with a finite difference method, whereby the spatial component at \(x\) was approximated by the following expression (Supporting Information Fig. S3A):

\[D_{\text{mdO}_2^-}\frac{\partial^2 [O_2^-]_{\text{MSM}}(x,t)}{\partial x^2} = D_{\text{mdO}_2^-}\frac{\partial^2 [O_2^-]_{\text{MSM}}(x,t)}{\partial x^2} - \frac{\partial [O_2^-]_{\text{MSM}}(x,t)}{\partial x} [([O_2^-]_{\text{MSM}}(x+\Delta x,t) - [([O_2^-]_{\text{MSM}}(x,t) - \frac{\partial [O_2^-]_{\text{MSM}}(x,t)}{\partial x}](\Delta x,t))] \tag{2}

where \(\Delta x\) is the spatial step size. To reduce computation time, the MSM was discretized as two subcompartments, with one (MSM_SR) adjacent to the peri-SR space and another (MSM_mito) adjacent to the perimitochondrial space (Supporting Information Fig. S3B). Assuming nonflux boundary conditions (i.e., \(\frac{\partial [O_2^-]_{\text{MSM}}(0,t)}{\partial x} = 0\)) and the concentration of mdO₂⁻ in the peri-SR space ([O₂⁻]ᵦ=\text{SR}) can be approximated to equal \([O_2^-]_{\text{MSM,SR}}\) and \([O_2^-]_{\text{MSM,mito}}\) respectively. Consequently, the concentration profile of \([O_2^-]_{\text{SR}}\) can be described by the following equation (the detailed derivation of the equation can be found in Supporting Information S1):

\[
\frac{d [O_2^-]_{\text{SR}}(t)}{dt} = D_{\text{O}_2^-} \cdot [O_2^-]_{\text{P,mito}}(t) - [O_2^-]_{\text{SR}}(t) \cdot \chi^2 + \nu_{\text{cyto, MSM}} \cdot f([O_2^-]_{\text{MSM}}(t)) \tag{3}
\]

The values of newly added model parameters (e.g., \(D_{\text{O}_2^-}\) and \(X\)) were taken from the literature and listed in the Supporting Information (Table S7.16). The effect of diffusion coefficient on mdO₂⁻ concentration in the peri-SR space is plotted in Fig. 1A, which shows that the larger the diffusion coefficient, the higher the SR mdO₂⁻ concentration.

**Local Ca²⁺ control.** To account for the local interaction of L-type Ca²⁺ channels (LCCs) and RyRs in the dyadic microdomain that controls CICR, we incorporated the coupled 40-state LCC-RyR model developed by Gauthier et al. (28) into the ECME-RIRR model (67) (see Supporting Information S2 for details). Since in the ECME-RIRR model the SR is separated into two compartments (i.e., NSR and JSR) that is different from the Gauthier et al. model, several model equations and parameters were modified (see Supporting Information S3 for details) to better fit the simulated current-voltage (I-V) relationship and L-type Ca²⁺ current (\(I_{\text{Ca,L}}\)) trace during steady-state AP with experimental data (Supporting Information Fig. S4). It is worthy to note that the values of the peak current of \(I_{\text{Ca,L}}\) were different among experiments, which might be caused by the variance of channel density (24). In our model, the number of LCC (i.e., the number of Ca²⁺ release unit ([NCa,RH])) was set to 300,000, which is within the range of experimental measurements [from ~276,000 (7) to ~500,000 (27)]. Other model parameters were the same as those used in the previous model (24). The modified ECME-RIRR model showed a ~37% decrease of SR Ca²⁺ content during a normal AP cycle, which is comparable to that reported in the previous studies (~35%) (5, 24).

**ROS and RyR Ca²⁺ release.** Experimental studies have shown that ROS exponentially increase RyR open probability (\(P_{\text{O,RyR}}\)), with the enhancement effect determined by both the ROS concentration and the state of the channel: when \(P_{\text{O,RyR}}\) is low, the enhancing effect of ROS is dramatic, but when \(P_{\text{O,RyR}}\) is already high, the stimulatory effect of ROS is much less significant. Consequently, the RyR open probability in the presence of ROS (\(P_{\text{O,sys,ROS}}\)) can be described by an exponential equation, which is a function of \([O_2^-]_{\text{SR}}\) and \(P_{\text{O,RyR}}\).
where $c_{\text{ryr}}$ is ROS enhancement coefficient and $k_{\text{ryr}}$ is effective factor. The values of these parameters (0.20 and 19.55 mM, respectively) were obtained by the least-square curve fitting using experimental data from the literature (8, 44, 45) (Fig. 1B). The enhancement of ROS on $P_{O_2}$ was incorporated into the RyR Ca$^{2+}$ release formula (Supporting Information S4).

Unlike RyRs, SERCA Ca$^{2+}$ uptake ($J_{\text{up}}$; Supporting Information Eq. S6.E47) is exponentially inhibited by ROS and the effect can be described by:

$$J_{\text{up,ROS}} = J_{\text{up}} \left[ e_{\text{SERCA}} \cdot \exp \left( -k_{\text{SERCA}} \left[ O_2 \right]_{\text{SR}} \right) \right]$$  \hspace{1cm} (5)

where $e_{\text{SERCA}}$ is the inhibition coefficient and $k_{\text{SERCA}}$ is the effective factor of ROS inhibition. These values were obtained using the least-square curve fitting method. The experimental data used for parameter optimization is from Refs. 60, 60, and the fitting result is shown in Fig. 1C. Equation 5 ($J_{\text{up,ROS}}$) was then added back to ECME-RIRR model to replace $J_{\text{up}}$ in Eq. S6.E47 (Supporting Information).

**Simulation Protocol**

The model formulations and parameters of other processes (e.g., ion currents and metabolic reactions) were the same as those in the ECME-RIRR model (67) and CICR model (24) unless otherwise indicated (see Supporting Information S6 and S7). The whole cell model was coded in C (Visual Studio; Microsoft, Redmond, WA). The nonlinear ordinary differential equations were integrated numerically with CVODE as described previously (67).

Before examining the effect of $mdO_2$ on SR Ca$^{2+}$ handling and cellular electrophysiology, the behavior of a paced (0.5 Hz) cardiomyocyte was simulated under physiological conditions (i.e., $mdO_2$ production was at physiological level). The obtained steady-state values were then fed into the model as initial conditions (Supporting Information Table S7.17) for all runs in the subsequent simulations. Mitochondrial depolarization (and associated $mdO_2$ burst) was induced by increasing the fraction of $O_2$ production from the electron transport chain (a.k.a. shunt) from 2% to 10% as described previously (67). To systematically examine the effect of $mdO_2$ on...
Ca\(^{2+}\) handling and AP generation, the mdO\(_2\) burst was induced at different time during the AP cycle (e.g., phase 2 or 4). In some subset simulations, the extent of mdO\(_2\) production or the distance between mitochondria and SR was altered to examine its effect on the mdROS-mediated Ca\(^{2+}\) transient and AP alterations. APD\(_{90}\) was defined as the interval between the time of the maximum upstroke velocity of the AP, [dV/dt]\(_{\text{max}}\), and 90% repolarization.

**RESULTS**

**Model Validation**

After parameters were optimized, the new/modified model modules were incorporated into the ECME-RIRR model. We first simulated the dynamics of mitochondrial energetics under stress conditions. Increasing shunt from 2 to 10% triggered sustained mitochondrial oscillations including membrane potential (ΔΨ\(_{\text{m}}\)) and mdO\(_2\) production (Supporting Information Fig. S5), as well as NADH oxidation and reduced glutathione depletion (data not shown) in a paced cardiomyocyte (0.5 Hz). These simulations were consistent with our previous experimental and computational studies (1, 17, 65, 67), suggesting that the addition of new model components (e.g., mdO\(_2\)-modulation of RyRs and local Ca\(^{2+}\) control) did not influence the dynamics of the existing model subsystems. Simulations also showed that [O\(_2\)\(^{-}\)]\(_{\text{m,mito}}\) and [O\(_2\)\(^{-}\)]\(_{\text{SR}}\) bursts occurred concurrently with ΔΨ\(_{\text{m}}\) depolarization during each oscillation cycle (see Fig. 1D for enlargement). Thus, for better visualization, ΔΨ\(_{\text{m}}\) depolarization was plotted to represent the O\(_2\) burst in some figures.

The model was further validated by simulating mdROS-mediated abnormal APs and comparing the results with experimental data. Due to the lack of experimental studies on the direct effect of mdROS on APs in guinea pig cardiomyocytes, the comparisons were made between model simulations and data obtained from isolated rabbit cardiomyocytes exposed to external oxidative stress. A mdO\(_2\) burst induced before AP firing led to an early afterdepolarization (EAD; Fig. 2A) similar to that observed in a cardiomyocyte subject to H\(_2\)O\(_2\) perfusion (Fig. 2A, inset) (59), including the ~2.5-fold APD\(_{90}\) prolongation (Fig. 2B). The mdO\(_2\) burst also caused significant increase in Ca\(^{2+}\) transient (1.63-fold higher than that of normal AP cycles) and reduction in SR Ca\(^{2+}\) storage (~69%; Fig. 2C). The mdO\(_2\) burst-induced [Ca\(^{2+}\)]\(_{\text{i}}\) elevation was comparable to that reported in a recent experimental study (57). In addition, a mdO\(_2\) burst induced during phase 4 of the AP cycle elicited a delayed afterdepolarization (DADs) that was also observed in the H\(_2\)O\(_2\) perfusion experiments (Fig. 3A, inset) (59). These validations, although semiquantitatively, demonstrated the utility and capability of our model in examining the interaction between mitochondrial energetics and cellular electrophysiology.

**Effect of mdO\(_2\) on Ca\(^{2+}\) Handling Channels**

We next examined the ionic mechanisms underlying the mdO\(_2\)-induced EAD or DAD described above. When a mdO\(_2\) burst was induced before AP firing, the activated \(I_{\text{rel}}\) (Fig. 2D, black line, solid arrow) and inhibited \(I_{\text{up}}\) (Fig. 2D, gray line) caused not only [Ca\(^{2+}\)]\(_{\text{i}}\) elevation but also [Ca\(^{2+}\)]\(_{\text{SR}}\) reduction (Fig. 2C, solid arrow). The accumulated cytosolic Ca\(^{2+}\) subsequently enhanced the Na\(^+\)/Ca\(^{2+}\) exchanger current (\(I_{\text{NaCa}}\)) in the forward mode (Fig. 2E, black curve, solid arrow) and the Ca\(^{2+}\)-sensitive nonspecific cationic current (\(I_{\text{NSCa}}\)) (Fig. 2F, grey curve), eliciting \(I_{\text{h}}\) that caused the reduction of AP repolarization reserve (Fig. 2A, arrow). Intriguingly, the transient reversal of AP repolarization reactivated the \(I_{\text{Cal}}\) (Fig. 2F, arrow), which triggered a second CICR (Fig. 2D, dashed arrow), causing a further [Ca\(^{2+}\)]\(_{\text{i}}\) elevation and [Ca\(^{2+}\)]\(_{\text{SR}}\) depletion (Fig. 2C, dashed arrow). The resultant Ca\(^{2+}\) overload caused further \(I_{\text{NCX}}\) and \(I_{\text{NSCa}}\) activation (Fig. 2E, dashed arrow), which eventually elicited an EAD (Fig. 2A).

In the case of DAD (Fig. 3A, inset), the mdO\(_2\)-activated \(I_{\text{rel}}\) (Fig. 3B) evoked extracellular Ca\(^{2+}\) transient, which was slightly smaller than that of normal AP cycles (Fig. 3C). The enhanced RyR Ca\(^{2+}\) release also caused a significant SR Ca\(^{2+}\) depletion (Fig. 3C, arrow). Consequently, both \(I_{\text{NSCa}}\) and \(I_{\text{NSCa}}\) were enhanced (Fig. 3, D and E, arrow), which induced \(I_{\text{h}}\) that underlay the DAD. However, \(I_{\text{Cal}}\) was not reactivated since the membrane potential was low (Fig. 3F).

**Effect of Timing of mdROS Burst Induction on AP Morphology**

Simulations shown in Figs. 2 and 3 suggest that the pattern of mdO\(_2\)-induced AP abnormality is dependent on the timing of mdO\(_2\) burst induction during a AP cycle or the time interval between the firing of AP and the burst of mdO\(_2\) (\(I_{\text{AP-mdROS}}\)). To better understand the ionic mechanisms underlying the mdO\(_2\)-induced AP abnormality, we analyzed the correlation between \(I_{\text{AP-mdROS}}\) and the morphology of mdO\(_2\)-mediated AP.

Figure 4Ai shows the control AP (i.e., mdO\(_2\) production was at physiological level). The mdO\(_2\) burst induced before AP firing (e.g., \(I_{\text{AP-mdROS}} = -160\) ms) caused AP\(_{90}\) prolongation (from 167 to 274 ms) (Fig. 4Aii). A mdO\(_2\) burst began to elicit EADs when \(I_{\text{AP-mdROS}}\) gradually increased. The mdROS burst induced at \(I_{\text{AP-mdROS}} = -130\) ms caused a single EAD (Fig. 4Aiii), and a delayed mdO\(_2\) burst (e.g., \(I_{\text{AP-mdROS}} = 117\) ms) elicited multiple EADs (Fig. 4Aiv). The multiple EADs degraded into a single EAD (Fig. 4Aiv) and then AP prolongation (data not shown) when the occurrence of mdO\(_2\) burst was further postponed to phase 3 of AP. Finally, when mdO\(_2\) burst was induced after the AP was fully repolarized (e.g., \(I_{\text{AP-mdROS}} = 355\) ms), a DAD (Fig. 4Avi) ensued.

The dynamics of [Ca\(^{2+}\)]\(_{\text{i}}\) and Ca\(^{2+}\) currents were then analyzed to explore the ionic mechanism accounting for the variance of the mdO\(_2\) burst-induced APs. When \(I_{\text{AP-mdROS}} = -160\) ms (Fig. 4Aii), a mdO\(_2\) burst caused a moderate [Ca\(^{2+}\)]\(_{\text{i}}\) increase (Fig. 4Bii), which activated \(I_{\text{NSCa}}\) (Fig. 4Cii) in the forward mode and enhanced \(I_{\text{NSCa}}\) (Fig. 4Dii). The resultant \(I_{\text{h}}\) caused a small AP depolarization and prolongation of APD\(_{90}\) (Fig. 4Aii) and \(I_{\text{Cal}}\) (Fig. 4Eii) but could not reverse AP repolarization.

When \(I_{\text{AP-mdROS}} = -130\) ms, the mdO\(_2\) burst gradually raised [Ca\(^{2+}\)]\(_{\text{i}}\) (Fig. 4Biii), which augmented \(I_{\text{NSCa}}\) and \(I_{\text{NSCa}}\) (Fig. 4, Ciii and Diii) and evoked \(I_{\text{h}}\), which reduced AP repolarization reserve, reactivated \(I_{\text{Cal}}\) (Fig. 4Eiii), and elicited an EAD (Fig. 4Aiii). The reactivated \(I_{\text{Cal}}\) subsequently triggered CICR and led to further increases of [Ca\(^{2+}\)]\(_{\text{i}}\) and augmentation of \(I_{\text{h}}\). Further postponing the induction of mdROS (e.g., \(I_{\text{AP-mdROS}} = 117\) ms; Fig. 4Aiv) led to a larger Ca\(^{2+}\) transient, which enhanced \(I_{\text{NSCa}}\) and \(I_{\text{NSCa}}\) (Fig. 4, Biv-Div). The
Fig. 2. Model-simulated mdO$_2$– burst-induced early afterdepolarization (EAD) in a “beating” (0.5 Hz) cardiomyocyte. A: mitochondria-derived reactive oxygen species (mdROS) burst-elicited EAD (inset: EAD observed in a rabbit cardiomyocyte subjected to ROS perfusion; Ref. 59). B: comparison of APD$_{90}$ between model simulation (guinea pig) and experimental data (rabbit) under control and oxidative stress conditions (where APD$_{90}$ is the interval between the time of the maximum upstroke velocity of the action potential (AP), [dV/dt]$_{max}$, and 90% repolarization). C–F: model simulated intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) and SR Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{SR}$) (C), RyR Ca$^{2+}$ release ($J_{rel}$), and SERCA Ca$^{2+}$ uptake ($J_{up}$) (D); Na$^+$/Ca$^{2+}$ exchanger current ($I_{NaCa}$) and Ca$^{2+}$-sensitive nonspecific cationic current ($I_{nsCa}$) (E); and $I_{CaL}$ (F) before and after a mdROS burst. The solid arrow in A represents the AP reversal; in C, D, and E, the [Ca$^{2+}$]$_i$ elevation, SR Ca$^{2+}$ release, and activations of $I_{NaCa}$ and $I_{nsCa}$ caused by the mdO$_2$– burst, respectively; in F, the L-type Ca$^{2+}$ current ($I_{CaL}$) reactivation. The dashed arrow in C, D, and E represents the second [Ca$^{2+}$]$_i$ elevation, SR Ca$^{2+}$ release, and activations of $I_{NaCa}$ and $I_{nsCa}$ caused by $I_{CaL}$ reactivation, respectively. The dashed lines represent $\Delta \Psi_m$ and its depolarization represents the acute mdO$_2$– burst.
resultant large $I_{\text{CaL}}$ activated $I_{\text{CaL}}$, repetitively (Fig. 4Eiv), generating an EAD with multiple phases (Fig. 4Eiv).

When the mdROS was induced in phase 3 (e.g., $t_{\text{AP-mdROS}} = 136$ ms) of the AP cycle, the mdO$_2^-$ burst-induced Ca$^{2+}$ elevation (Fig. 4Bv) and $I_{\text{NaCa}}$ and $I_{\text{inCa}}$ enhancements (Fig. 4Cv and Dv) could only trigger a single EAD (Fig. 4Av). As the cell membrane voltage was low when $I_{\text{CaL}}$ was reactivated, the amplitude of reactivated $I_{\text{CaL}}$ was larger (Fig. 4Ev, arrow) than that evoked in the previous cases (Fig. 4, Eiii and Eiv). This is consistent with the experimental result showing that a more repolarized AP produced larger EAD amplitude (59). Further delay of the mdROS burst in phase 3 diminished mdO$_2^-$ burst-induced Ca$^{2+}$ elevation and induced APD prolongation (data not shown).
When the mdO$_2^-$ burst was induced in phase 4 of the AP cycle (Fig. 4 Avi), the mdO$_2^-$ burst-induced SR Ca$^{2+}$ release generated a second Ca$^{2+}$ transient (Fig. 4 Bvi). In this case, the gradual AP depolarization induced by $I_{\text{NaCa}}$ and $I_{\text{nsCa}}$ activations (Fig. 4, Cvi and Dvi) slightly depolarized the membrane potential and evoked a DAD (Fig. 4 Avi). However, $I_{\text{Cal}}$ was not activated (Fig. 4 Evi). The relationship between $t_{\text{AP-mdROS}}$ and mdO$_2^-$ burst-induced AP abnormality is summarized in Fig. 4 F, with zones 1–4 representing single EAD, multiple EADs, APD prolongation, and DADs, respectively.
Effect of mdO$_2$\textsuperscript{−} Mediated RyR Activation or SERCA Inhibition Alone on Ca$^{2+}$ Cycling and AP

We next examined how mdO$_2$\textsuperscript{−} could affect Ca$^{2+}$ transients and AP via modulating solely the activity of RyRs or SERCA. A mdO$_2$\textsuperscript{−} burst induced during AP firing, when modeled to activate RyR Ca$^{2+}$ release solely, caused a substantial [Ca$^{2+}$]$_{SR}$ reduction (~65%) (Fig. 5A) and a significant [Ca$^{2+}$]$_i$ elevation (~1.7-fold in amplitude and ~2.3-fold in duration) (Fig. 5B). The elevated Ca$^{2+}$, as we expected, caused an EAD (Fig. 5B) similarly to that shown in Fig. 2A in which the mdO$_2$\textsuperscript{−} burst affected both RyRs and SERCA. The mdO$_2$\textsuperscript{−} burst-induced RyR activation itself, however, could not elicit multiple EADs regardless the timing of mdROS burst induction during the AP cycle (data not shown).

The effects of mdO$_2$\textsuperscript{−} burst on Ca$^{2+}$ transients and AP were much less evident when it was modeled to modulate SERCA only. Particularly, the mdO$_2$\textsuperscript{−} burst-inhibited SERCA Ca$^{2+}$ uptake ($J_{up}$, from ~0.144 to ~0.097 mM/s) led to a small [Ca$^{2+}$]$_{SR}$ reduction (Fig. 5C) and a modest [Ca$^{2+}$]$_i$ increase (Fig. 5D). In this case, the mdO$_2$\textsuperscript{−} burst could only cause APD prolongation (from 167 to 207 ms in Fig. 5D), implying that the mdO$_2$\textsuperscript{−} mediated SERCA inhibition had a smaller effect on AP than mdO$_2$\textsuperscript{−} mediated RyR activation in our model.

Effect of Shunt or Mitochondria-SR Distance on AP Morphology

In addition to mdO$_2$\textsuperscript{−} induction timing, the pattern of mdROS-mediated abnormal AP was also affected by the concentration of [O$_2$\textsuperscript{−}]$_{SR}$, which was determined by mdO$_2$\textsuperscript{−} production rate (shunt) and mitochondria-SR distance ($X$) (or diffusion coefficient). Specifically, increasing shunt enhanced mdO$_2$\textsuperscript{−} burst-induced Ca$^{2+}$ elevation (Fig. 6A), promoting earlier occurrence of EAD, and potentially shifting APD prolongation to EAD and single EAD to multiple EADs (Fig. 6B). Reducing $X$ had the similar effect: the shorter the distance, the stronger the effects of mdO$_2$\textsuperscript{−} on Ca$^{2+}$ and AP (Fig. 6, C and D). It is worth noting that the Ca$^{2+}$ transient became “non-physiologically” large when O$_2$\textsuperscript{−} production was very high (e.g., shunt = 20%) or mitochondria-SR distance was very short (e.g., $X$ = 25 nm) (Fig. 6, A and C). Under those extreme conditions, mdO$_2$\textsuperscript{−} caused dramatic SR Ca$^{2+}$ depletions compared with those under normal conditions (76–85 vs. ~37%).
In the case when mdO₂⁻ burst was induced in phase 4 of the AP cycle, increasing shunt (from 10 to 30%) or reducing X (from 75 to 25 nm) significantly exaggerated Ca²⁺ elevation (Fig. 6E). The resultant large Iₙ depolarized the membrane potential to the threshold for Iₙa activation, leading to triggered activities (Fig. 6F). Increasing mdO₂⁻ diffusion coefficient (Dₒ₂⁻) similarly enhanced the effects of mdO₂⁻ burst on Ca²⁺ transients and APs (Supporting Information Fig. S6).

**Effect of Blocking Ca²⁺ Handling Channel on mdROS-Induced EADs**

Finally, we examined the effect of blocking individual Ca²⁺ handling channels on the mdROS-induced AP abnormality. Under normal mdO₂⁻ production conditions, blocking Iₙac had minimal effects on both AP and [Ca²⁺], (Fig. 7, Ai and Aii). In the presence of pathological mdO₂⁻ bursts, the block-
ade of \( I_{\text{NaCa}} \) (Fig. 7Aiii) inhibited \( I_i \) in spite of enhanced \( I_{\text{nsCa}} \) activation (Fig. 7Av), which suppressed \( I_{\text{CaL}} \) reactivation (Fig. 7Av). The lack of the subsequent CICR lowered Ca\(^{2+}\) elevation (Fig. 7Aii) and abolished the EAD (Fig. 7Ai). However, since the Ca\(^{2+}\) extrusion via \( I_{\text{NaCa}} \) was blocked, the duration of Ca\(^{2+}\) transient was prolonged (Fig. 7Aii).

Blocking \( I_{\text{nsCa}} \) did not significantly affect AP (Fig. 7Bi) and Ca\(^{2+}\) transient (Fig. 7Bii) no matter whether there was a
pathological mdO$_2^-$ burst. Particularly, blocking $I_{\text{NaCa}}$ slightly shortened the APD (Fig. 7Bi) and shifted the second Ca$^{2+}$ transient to the left by ~10 ms (Fig. 7Bii). However, blocking $I_{\text{NaCa}}$ slightly reduced $I_b$ (Fig. 7Biv), shifted the $I_{\text{CaL}}$ reactivation to a more polarized AP, and therefore increased the amplitude of the reactivated $I_{\text{CaL}}$ (Fig. 7Bv).

Experimental studies have demonstrated that blocking LCC eliminates the oxidative stress-induced EADs and causes remarkable APD prolongation (59). Our simulations showed the similar results: the mdO$_2^-$-induced EAD was suppressed but APD remained prolonged when $I_{\text{CaL}}$ was blocked (Fig. 7Ci, dashed green curve). The prolonged APD was mainly caused by the mdO$_2^-$-induced RyR activation and SERCA inhibition, which resulted in a relatively small but prolonged [Ca$^{2+}$]$_i$ elevation (Fig. 7Cii). However, blockade of $I_{\text{CaL}}$ (Fig. 7Cy) hindered the subsequent CICR and prevented the further Ca$^{2+}$ elevation. Consequently, $I_{\text{NaCa}}$ and $I_{\text{NaCa}}$ enhancements were suppressed (Fig. 7, Ciii and Civ) and the $I_b$ was too small to elicit an EAD. The outcomes of $I_{\text{CaL}}$ blockade in the absence of mdROS bursts were shortened APD and abolished AP plateau (Fig. 7Ci) as well as diminished Ca$^{2+}$ transient (Fig. 7Cii), which were consistent with previous studies (16, 23).

**DISCUSSION**

In this study, a novel multiscale guinea pig cardiomyocyte model was developed, which incorporates mitochondrial energetics, excitation-contraction coupling, local Ca$^{2+}$ control, and RIRR, as well as mdROS-mediated modulations of RyR and SERCA activities. This model, for the first time, provides a computational framework to quantitatively examine how mdROS may influence cardiomyocyte Ca$^{2+}$ regulation and electrophysiological behaviors under oxidative stress. Our main findings are 1) mdO$_2^-$-induced AP abnormality involves alterations of multiple Ca$^{2+}$ handling channels in a coordinated fashion, including RyRs, SERCA, $I_{\text{NaCa}}$, $I_{\text{NaCa}}$, and $I_{\text{CaL}}$; 2) the variance of mdO$_2^-$-burst-induced AP normality largely depends on the time interval between its induction and AP firing; and 3) the mdO$_2^-$-induced AP abnormality is also influenced by mdROS dosage and/or mitochondria-SR distance.

It has been shown that the excessive O$_2^-$ produced by a stressed mitochondria can diffuse to its immediate neighbors and activate their inner membrane anion channels, triggering RIRR in a self-amplifying mode (70). The present work suggests that if mdO$_2^-$ could travel to the proximal SR, they may activate the redox-sensitive RyRs, triggering ROS-induced Ca$^{2+}$ release (RICR). This RICR alone, or together with inhibition of SERCA-mediated SR Ca$^{2+}$ uptake, evoked erratic APs similar to those observed in cardiomyocytes exposed to H$_2$O$_2$ perfusion (59). Importantly, we showed that the mdO$_2^-$-mediated SERCA inhibition had smaller effects on Ca$^{2+}$ cycling and AP generation than the mdO$_2^-$-mediated RyR activation, implying that enhanced RyR Ca$^{2+}$ release might play a major role in the mdROS-induced cardiac arrhythmias. It is worth noting that in our simulations the inhibitory effect of mdO$_2^-$ on SERCA is transient. Sustained SERCA inhibition, such as that induced by continuous H$_2$O$_2$ perfusion, would have a much more prominent effect on Ca$^{2+}$ handling. Intriguingly, while the proarrhythmic effect of mdO$_2^-$ burst-induced SERCA inhibition alone was minor, it significantly exacerbated the effect of RyR activation on AP. Particularly, concurrent SERCA inhibition shifted the RyR activation-induced APD prolongation to a single EAD and the single EAD to multiple EADs. These model simulations endorse the antiarrhythmic effect of increasing SERCA2a gene expression in heart failure treatment (18, 20).

Another important finding is that mdO$_2^-$ burst can elicit various patterns of AP abnormality such as APD prolongation, single EAD, and multiple EADs and DAD, similar to those observed in experimental studies (59). Our analysis suggested that mdO$_2^-$ bursts elicit erratic APs by triggering the following events in a coordinated way: 1) the mdO$_2^-$ burst activates RyR Ca$^{2+}$ release and inhibits SERCA Ca$^{2+}$ uptake, resulting in a [Ca$^{2+}$]$_i$ increase; 2) Ca$^{2+}$ accumulation enhances $I_{\text{NaCa}}$ in the forward mode and activates $I_{\text{NaCa}}$, evoking $I_b$; 3) $I_b$ reduces AP repolarization reserve, reactivating $I_{\text{CaL}}$ that triggers CICR and causes further [Ca$^{2+}$]$_i$ elevation; and 4) the resulting large [Ca$^{2+}$]$_i$ increment augments $I_{\text{NaCa}}$ and $I_{\text{NaCa}}$, eliciting larger $I_b$, which may repetitively reactivate $I_{\text{CaL}}$. Depending on the timing (t$_{\text{AP}, \text{mdROS}}$) of mdROS bursting, mdROS dosage, and mitochondria-SR distance, one or more of these events would not be activated, or be activated at different levels, resulting in various Ca$^{2+}$ transient and AP morphologies (Fig. 4F). This paradigm is strengthened by the Ca$^{2+}$ current inhibition studies, which showed that blocking $I_{\text{CaX}}$ or $I_{\text{CaL}}$ significantly altered the pattern of mdO$_2^-$ burst-induced AP abnormality. These findings are highly significant, as under pathological conditions such as ischemia-reperfusion mitochondrial depolarization and associated ROS bursts may occur asynchronously in various cells, resulting in regional electrical heterogeneity that increases the propensity for arrhythmogenesis.

Some studies suggested that direct ROS activation of $I_{\text{CaL}}$ is a primary cause of oxidative stress-induced EADs (59), while others argued that the effects of ROS on $I_{\text{CaL}}$ are controversial (9, 30, 50, 55). Notably, we showed that $I_{\text{CaL}}$ were indirectly activated during mdROS bursts such as in the cases of single and multiple EADs (Fig. 4, $I_{\text{CaL}}$, and $I_{\text{NaCa}}$). Further analyses revealed that whether or not $I_{\text{CaL}}$ can be reactivated was largely determined by two factors: 1) the amplitude of mdO$_2^-$ burst-induced SR Ca$^{2+}$ release, which must be large enough to enhance $I_{\text{NaCa}}$ and $I_{\text{NaCa}}$ so that the resultant $I_b$ can reverse AP repolarization, and 2) the membrane potential when the reversal occurred, which needs to be in the range where $I_{\text{CaL}}$ can be activated. The latter factor also determined the amplitude of the reactivated $I_{\text{CaL}}$, which was in agreement with previous experimental studies (59). Our simulations also suggested that when mdROS amplitude and duration are sufficiently large, $I_{\text{CaL}}$ can be reactivated repeatedly, producing multiple EADs. These types of substantial Ca$^{2+}$ transients (and EADs) may be seen in real life in more extreme situations such as apoptotic conditions, when multiple aspects of the cellular homeostasis are changed significantly and irreversibly.

Transient inward currents ($I_b$) have been known to trigger EADs or DADs, eliciting Ca$^{2+}$ overload-mediated arrhythmias. However, the contribution of $I_{\text{NaCa}}$ to $I_b$ is controversial and appears to vary with different cell types (26, 28, 31, 32, 34, 47). Ca$^{2+}$-sensitive nonspecific cationic channels (nsCa) belong to the “transient receptor potential” protein family and are expressed in both excitable and nonexcitable mammalian cells (26). In human atrial myocytes $I_{\text{NaCa}}$ was shown to contribute to the genesis of arrhythmias during Ca$^{2+}$ overload (34). How-
ever, the situation in ventricular myocytes is more controversial. Several groups have shown that \( I_{\text{NaCa}} \) could be activated by extracellular oxidative stress in guinea pig ventricular myocytes (31, 32); but in rabbit ventricular myocytes, Pogwizd et al. (48) showed that the contribution of \( I_{\text{NaCa}} \) to \( I_n \) was insignificant in both control and failing cells. This may be due to the low expression of those cationic channels in ventricular myocardiun (34). In our simulations, while blocking \( I_{\text{NaCa}} \) completely suppressed the mdROS burst-induced EAD (Fig. 7Ai), the blockade of \( I_{\text{NaCa}} \) had only minor effects on the morphologies of AP and Ca\(^{2+}\) transients. The results suggested that the contribution of \( I_{\text{NaCa}} \) to the mdROS burst-induced AP abnormality was smaller than that of \( I_{\text{NaCa}} \), which is consistent with the observations of Pogwizd et al. (48). The differences in the contributions of these currents to \( I_n \) under stress conditions are also in agreement with their roles in shaping AP under physiological conditions. One limitation of our study is that \( I_{\text{NaCa}} \) might be activated directly by oxidative stress in guinea pig ventricular myocytes (31, 32) but this effect is not considered in the present model. Nevertheless, our simulations underscore the importance of targeting the appropriate \( I_n \) component in the treatment of oxidative-stress-mediated cardiac arrhythmias.

It is well appreciated that mitochondrial dysfunction inhibits cell contraction. However, our simulations showed that the force of contraction was augmented during mitochondrial depolarization and ROS burst (Supporting Information Fig. S7). This paradox could be attributed to several factors: 1) Kohashi et al. (33) showed that enhanced ROS production could directly inhibit cardiomyocyte contraction; however, this effect is not included in the present model; and 2) it is known that ATP depletion directly inhibits cell contraction; however, the reduction of ATP during mitochondrial depolarization is very small in our simulations (~3%) (Supporting Information Fig. S7). Under more stressed conditions such as higher pacing frequency or sustained mitochondrial dysfunction, the concentration of ATP may decline substantially, inhibiting SERCA and contraction (67).

Model Limitations and Future Directions

One of the major limitations is that the diffusion of mdROS in MSM cannot be validated by experimental study due to the lack of methods to measure ROS in such small domains. In addition, the present model does not consider the effects of mitochondrial-derived H\(_2\)O\(_2\) or other oxidizing species. Our simplification is based on the experimental observations that the mdO\(_2^-\) scavenger significantly suppressed enhanced SR Ca\(^{2+}\) release during RIRR. However, these results cannot completely exclude the effect of H\(_2\)O\(_2\) as O\(_2^-\) can be readily dismutated to generate H\(_2\)O\(_2\). Whether it is O\(_2^-\), H\(_2\)O\(_2\), or both that are responsible for the enhanced Ca\(^{2+}\) release deserves further investigations. Compared with O\(_2^-\), H\(_2\)O\(_2\) is more stable and can diffuse further, thus affecting ion channels not only on the proximal SR membrane but also on the sarcoplasmic membrane. In this context, various studies have shown that H\(_2\)O\(_2\) can directly modulate \( I_{\text{CaL}} \), although the effect is controversial (or dependent on H\(_2\)O\(_2\) concentration) (54–57). Furthermore, ROS can affect \( I_{\text{NaCa}} \) either directly (38) or indirectly by activating CaMKII (22, 53, 54). The ROS-mediated CaMKII activation can also enhance SERCA activity via phospholamban phosphorylation (40), counteracting the effect of ROS-induced SERCA inhibition. Other redox-sensitive ion channels include K\(^+\) channels (K\(_{\text{ir}}\) and K\(_{\text{v}}\)) and the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) (4, 15, 35, 43, 55, 68). It is expected that adding the effects of H\(_2\)O\(_2\) on these sarcomembranial ion channels would exacerbate the influence of mdROS and lead to more complex AP morphologies as observed experimentally (59). Nonetheless, the lack of mdROS-induced \( I_{\text{CaL}} \), CaMKII, and/or \( I_{\text{NaCa}} \) modulations would not impact our main conclusions about the mechanisms underlying mdROS-induced Ca\(^{2+}\) dysregulation and abnormal APs. Indeed, the lack of mdROS-mediated membrane channel remodeling allowed us to focus on the crucial role of mdROS on SR Ca\(^{2+}\) handling and genesis of EADs and DADs under oxidative stress conditions. Nevertheless, the effects of mdROS on these proteins should be incorporated into the model when more experimental data become available.

Moreover, we assumed that all mitochondria in the cardiomyocyte depolarize and release ROS simultaneously so that we can focus on the ionic mechanisms underlying mdROS burst-induced alterations in Ca\(^{2+}\) cycling and cellular electrophysiology. In a real cardiomyocyte, the rate of ROS increase might be slower due to the heterogeneity of mitochondrial energetic state and network ultrastructure. The chronic, sustained mdROS increase may deplete SR Ca\(^{2+}\) loading if it spans over several beats or inhibits RyRs and \( I_{\text{CaL}} \) activities if the dose is too high. Apparently, systematic experimental studies will be needed to gain a more complete understanding of how mdROS may exactly influence intracellular ion handling under certain pathological conditions. However, again these limitations are not expected to alter the mechanisms that underlie the mdROS-induced abnormal Ca\(^{2+}\) cycling and AP generation unraveled here.

Finally, a recent study showed that carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)-induced mitochondrial depolarization increased the frequency and amplitude of Ca\(^{2+}\) waves and induced triggered activities, probably via the mitochondrial Ca\(^{2+}\) efflux mediated by the mitochondrial permeability pore (mPTP) (64). Since the present model did not incorporate mPTP, it cannot be used to examine this mechanism and study the interaction between the ROS-modulated and mPTP Ca\(^{2+}\) efflux-mediated cytosolic Ca\(^{2+}\) dynamics.

In summary, the developed multiscale model is capable of simulating the acute effect of mdROS on Ca\(^{2+}\) cycling, providing a novel tool to examine how alterations in mitochondrial energetics can impact cardiomyocyte electrophysiology and electrical activity. The results highlight the role of mdROS in Ca\(^{2+}\) overload-mediated cardiac arrhythmogenesis and abnormal automaticity. They also underscore the importance of considering mitochondrial targets in designing new antiarrhythmic therapies in the context of sudden cardiac death.

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ENDNOTE

At the request of the authors, readers are herein alerted to the fact that additional materials related to this manuscript may be found at the institutional website of one of the authors, which at the time of publication they indicate is http://www.uab.edu/medicine/cscb/images/paper_pdf/Li_et_al_
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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