Formation of reactive oxygen species at increased contraction frequency in rat cardiomyocytes

Frank R. Heinzel a, Yukun Luo a, Giuliano Dodoni a, Kerstin Boengler a, Frank Petrat b, Fabio Di Lisa c, Herbert de Groot b, Rainer Schulz a, Gerd Heuscha a,*

* Institut für Pathophysiologie, Universitätsklinikum Essen, Hufelandstraße 55, 45122 Essen, Germany
b Institut für Physiologische Chemie, Universitätsklinikum Essen, Germany
c Dipartimento di Chimica Biologica (F.D.L.), Università di Padova, Italy

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Abstract

Objective: Reactive oxygen species (ROS) play an ambivalent role in cardiomyocytes: low concentrations are involved in cellular signaling, while higher concentrations contribute to cellular injury. We studied ROS formation during increases in contraction frequency in isolated cardiomyocytes.

Methods: Rat ventricular cardiomyocytes were loaded with dichlorodihydrofluorescein and electrically stimulated (37 °C). ROS formation was assessed by the rate of oxidation-dependent fluorescence increase (OxR). Oxygen consumption (V̇O₂) and NAD(P)H autofluorescence were measured in parallel experiments.

Results: Increases in contraction frequency were accompanied by an increase in V̇O₂ and a decrease in NAD(P)H fluorescence. OxR increased to 124±4%, 146±8%, 204±25% and 256±29% of OxR at baseline during 1, 2, 3 and 4 Hz stimulation, and subsequently returned to baseline values with 0.2 Hz. The OxR increase was dose-dependently inhibited by the antioxidant NAC (10 and 100 mM), but unaffected by the NO synthase inhibitor L-NAME (200 μM and 10 mM). The OxR increase was attenuated when myosin ATPase activity was inhibited by butanedione monoxime (BDM; 5 mM).

Conclusion: Increased contraction frequency induces ROS formation in rat cardiomyocytes.

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Keywords: Myocytes; Oxygen radicals; Redox signaling; Contractile function

1. Introduction

Regulation of the intracellular redox state has emerged as an important determinant of cardiomyocyte homeostasis and contractile function [1–3]. Reactive oxygen species (ROS) are involved in long-term changes of the cardiac cellular phenotype, as observed in hypertrophy, heart failure and apoptosis [4], but they also mediate transient changes in protein phosphorylation and activity, as they occur in ischemic preconditioning [5–8].

The redox potential of the cell is determined by the ratio of reducing to oxidizing equivalents [9]. ROS, which are naturally arising from the mitochondrial respiratory chain [10], are neutralized to a great extent by antioxidants, such as vitamins, glutathione, and also NAD(P)H [11]. In hepatocytes, mitochondrial NAD(P)H plays a major role in the elimination of ROS [12]. With increases in contractile activity, mitochondrial electron flux and oxygen consumption (VO₂), a transient decrease of NADH has been reported in in situ hearts [13], isolated trabeculae [14], as well as in single cardiomyocytes, indicating fluctuations of the cellular redox potential with increases in contractile activity which may be associated with an increased formation of ROS.
ROS formation in contracting cardiomyocytes during changes in contractile activity, however, has not been studied so far. In the present study, we therefore measured ROS formation in isolated contracting cardiomyocytes during increases in contraction frequency. We found that an increase in contraction frequency reversibly increases ROS formation.

2. Materials and methods

2.1. Materials

Intracellular ROS were detected using the reactive oxidant-sensitive fluorescent probe 5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein (DCFH) diacetate [16] (Invitrogen-Molecular Probes, Leiden, The Netherlands), prepared from a stock in DMSO. The concentration of DMSO in the final solution did not exceed 0.1%. All other chemicals were obtained from Sigma Aldrich, Taufkirchen, Germany.

2.2. Cell isolation

All animal experiments were approved by the local authorities and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Ventricular cardiomyocytes were isolated by modification of a procedure described previously [17]. Hearts were removed from young Wistar rats (3–6 months) in ether anesthesia and placed in high potassium Tyrode’s solution (4 °C) containing (in mM): NaCl 137, KCl 27, Hepes 11.8, MgCl2 0.5, CaCl2 1.0, glucose 10; pH adjusted to 7.4 with NaOH. Hearts were then retrogradely perfused at 37 °C with Tyrode’s solution (4 °C) containing (in mM): NaCl 137, KCl 27, Hepes 11.8, MgCl2 0.5, CaCl2 1.0, glucose 10; pH adjusted to 7.4 with NaOH. Hearts were then retrogradely perfused at 37 °C with Tyrode’s solution (as above, but 5.4 mM KCl) for 2 min, followed by Ca2+-free Hepes buffer solution for 15 min containing (in mM): NaCl 130, KCl 5.4, Hepes 6, MgSO4 1.2, KH2PO4 1.2, glucose 20; pH adjusted to 7.2. Perfusion was then switched to recirculating Ca2+-free buffer with collagenase (type A, 1.2 mg/mL, Roche, Mannheim, Germany) and protease (type XIV, 1.0 mg/mL) for 15 min. Enzymes were washed out with 0.05 mM Ca2+-buffer solution (contents as in Ca2+-free buffer above) for 15 min. The left ventricle was then minced into 1–2 mm3 pieces and cardiomyocytes were dispersed by gentle agitation. The suspension was filtered and cardiomyocytes settled after 5 min. The cell bath was changed after each recording. Cell volume measurements in calcein-loaded cardiomyocytes were performed using a confocal microscope (as described in [19]) in control conditions (CTRL) and after 1 h incubation with 100 mM NAC to rule out osmotically induced changes in cell volume (35.2±7.2 pL in the presence of NAC vs. 31.3±8.1 pL in CTRL, nanimals=3, with 66 cells/group, p=NS). To reduce contraction amplitude, we used butanedione monoxime (BDM, 5 mM), which at this concentration inhibits myosin-ATPase and cross-bridge cycling with only minor effects on cellular Ca2+-handling [20,21]. BDM was washed in by a rapid perfusion system during 1 Hz stimulation to compare the effect of BDM on cell shortening in the same cell, followed by a stepwise increase in stimulation frequency as above.

2.4. Protocols

Cardiomyocytes in the cell bath were superfused with Tyrode’s solution (37 °C, 1.0 mM CaCl2, equilibrated to room air). The antioxidant and glutathione precursor N-acetylcysteine (NAC, 10 mM or 100 mM, preincubation for 60 min) or the NO-synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 200 μM or 10 mM) was added as required by the protocol. pH-adjustment to 7.4 in the presence of NAC required more NaOH and NaCl was reduced accordingly to achieve a final [Na] of 137 mM. After a baseline recording at rest, cardiomyocytes were stimulated at 1 Hz for 5 min. Stimulation frequency was then increased to 2, 3 and 4 Hz in 1 min intervals (Fig. 1A) and finally reduced to 0.2 Hz for 5 min. The cell bath was changed after each recording. Cell volume measurements in calcein-loaded cardiomyocytes were performed using a confocal microscope (as described in [19]) in control conditions (CTRL) and after 1 h incubation with 100 mM NAC to rule out osmotically induced changes in cell volume (35.2±7.2 pL in the presence of NAC vs. 31.3±8.1 pL in CTRL, nanimals=3, with 66 cells/group, p=NS).
2.5. Measurements in a cell-free system

To test for DCFH oxidation during stimulation in a cell-free system, DCFH diacetate was hydrolyzed and added to Tyrode’s solution in a concentration of 20 μM. DCF fluorescence (wavelengths: excit. 485 nm, emiss. 535 nm) was measured in a cuvette of a fluorescence spectrophotometer (Cary Eclipse, Varian Inc., Palo Alto, CA, USA) equipped with platinum stimulation electrodes and a stirrer. Length of the electrodes was adjusted to obtain the same ratio of platinum surface to immersion solution as in the cardiomyocyte bath.

After a 1 min baseline measurement, stimulation was initiated with the same setting and following the same stimulation frequency sequence as described for OxR measurements in cardiomyocytes. Measurements were repeated in the presence of 10 mM NAC.

2.6. Measurement of cellular V·O₂

Cardiomyocytes were transferred into a custom-made air tight cell bath with AgCl-electrodes for electrical field stimulation. Mounting the cell bath on an inverted microscope confirmed that >90% of the cardiomyocytes were contracting to stimuli (biphasic square pulses of 5 ms duration, 10–20 V to reach 1.5× rheobase). The bath was placed in an incubator (37°C) and gently agitated to prevent O₂-gradients. Oxygen partial pressure (pO₂) and temperature were continuously monitored (Licox O₂-sensor, GMS, Kiel, Germany). VO₂ was quantified from the rate of pO₂ change at different stimulation frequencies according to the same protocol as for ROS measurements and calculated by the formula: VO₂ = βw(ΔpO₂/Δt)/(n_totalp_vib.), with βw = 1.34 μM×Torr⁻¹ (solubility of O₂ in water at 37 °C), n_total = number of cardiomyocytes per mL, p_vib. = fraction of viable cells (0.8% Trypan blue staining).

2.7. Measurement of NAD(P)H

Cardiomyocytes in the cell bath mounted on the inverted microscope were excited at 365 nm (100 W xenon light source, intensity attenuated to 0.75%) with pulses of 5 ms duration at 500 ms intervals using a shutter device (Uniblitz, Vincent Associates, Rochester, NY, USA). Autofluorescence was detected at 485 nm. Cardiomyocytes were stimulated at 1 Hz until steady state contraction (at least 2 min) and autofluorescence was measured (F_steady-state). Then stimulation frequency was increased to 4 Hz and decreased to 0.2 Hz following the same protocol as for ROS measurements in one group, while in another group (control) stimulation was continued at 1 Hz. NAD(P)H fluorescence was averaged over the last 30 s of each frequency step up to 4 Hz and after 5 min of 0.2 Hz stimulation. Fluorescence during stimulation was
normalized to \( F_{\text{steady-state}} \). To verify that the fluorescence signal was sensitive to changes in [NAD(P)H], we exposed cardiomyocytes to 5 mM sodium cyanide (NaCN) to achieve a complete reduction of intracellular NAD(P)\(^+\) to NAD(P)H \[22\]. Cardiomyocytes were also exposed to Tyrode's solution containing the ionophore carbonyl cyanide chlorophenyl hydrazone (CCCP, 10 \( \mu \)M), which induces breakdown of the mitochondrial proton gradient and oxidation of mitochondrial NADH to NAD\(^+\), resulting in the loss of mitochondrial NADH-related fluorescence \[23\]. NaCN increased the fluorescence signal to 148±14%, and CCCP lead to a rapid decrease to 33±2% of \( F_{\text{steady-state}} \) (\( n_{\text{cells}} \geq 15 \) for both substances).

2.8. Experiments in isolated mitochondria

We examined potential direct effects of BDM on mitochondrial respiration. Mitochondria were isolated by standard procedures of differential centrifugation \[24\]. Hearts were quickly excised from anesthetized rats and atria were cut off. The myocardium was minced and washed several times in an isolation buffer containing (in mM): sucrose 250, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, ethylene glycol tetraacetic acid (EGTA) 1, and bovine serum albumin 0.5%, pH adjusted to 7.4 with NaOH. The tissue was homogenized (Ultra Turrax, IKA-Labortechnik, Staufen, Germany), centrifuged and washed twice in isolation buffer (4 °C) without BSA. Mitochondrial protein concentration was assessed using the DC protein assay kit (Bio-Rad, Hercules, CA, USA), and mitochondria were resuspended in incubation buffer (in mM): KCl 125, tris-hydroxymethylaminomethane (Tris)-3-morpholinopropanesulfonic acid (MOPS) 1, Pi–Tris 1.2, MgCl\(_2\) 1.2, EGTA–Tris 0.02, glutamate 5, malate 2.5, supplemented with 1 mM \( L \)-arginine before the BDM experiments. Oxygen consumption was determined polarographically using a Clark oxygen electrode (782 Oxygen Meter, Strathkelvin, Glasgow, UK) \[25\]. State 3 respiration was initiated by the addition of 200 \( \mu \)M ADP. Functionality of the mitochondrial preparations was validated by an increase in \( V_{\text{O}_2} \) from 9.1±1.0 to 40.7±3.8 \( \mu \)mol \( \text{O}_2 \times \text{s}^{-1} \times \text{g protein}^{-1} \) upon switching from state 4 to state 3 (\( n_{\text{animals}} = 3 \), \( n_{\text{animals}} = 6 \), 19 cells, and \( n_{\text{animals}} = 3 \), 8 cells) respectively. NAC largely attenuated DCFH oxidation. Curves represent the average of 3 measurements.

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**Fig. 2. Rate of DCFH-oxidation during increased stimulation frequency in control cells and in the presence of NAC or L-NAME.**

A: The antioxidant and glutathione precursor NAC led to a dose-dependent attenuation of ROS formation (\( N = 13 \) animals, 42 cells in total for Control and \( n_{\text{animals}} = 5 \) with \( \geq 18 \) cells for each concentration of NAC). B: Inhibition of NO synthase with 200 \( \mu \)M (\( n_{\text{animals}} = 19 \) cells) or 10 mM (\( n_{\text{animals}} = 8 \) cells) L-NAME did not prevent ROS formation with increased contraction frequency. \( ^{*}p<0.05 \) vs. OxRbase, \( ^{†}p<0.05 \) vs. Control. C: DCF fluorescence in the cell-free system containing 20 \( \mu \)M deesterified DCFH. Initiation of stimulation induced DCFH oxidation, not considerably affected by increases in stimulation frequency. 10 mM NAC largely attenuated DCFH oxidation. Curves represent the average of 3 measurements.
p<0.05). All mitochondrial experiments were performed at 25 °C.

2.9. Statistical analysis

Data are shown as mean±S.E.M. n denotes the number of measurements statistical analysis was based on. One-way ANOVA for repeated measurements was performed for frequency-induced changes in \( L/L_0 \) and \( \dot{V}O_2 \). Two-way ANOVA for repeated measurements was used to evaluate the effect and interaction of stimulation frequency, NAC, L-NAME and BDM with regard to changes in OxR. Fisher’s least-significant difference test was employed when significant overall effects were detected. Student’s t-test was used for respiratory rate measurements in mitochondria. \( p<0.05 \) was considered significant.

3. Results

3.1. Formation of ROS at increased contraction frequency

As exemplified in Fig. 1, in contracting cardiomyocytes OxR amounted to 124±4%, 146±8%, 204±25% and 256±29% of OxR\(_{base}\) following stimulation at 1, 2, 3 and 4 Hz, respectively (\( n\_animals=13, 42 \) cells in total), reflecting an increased formation of ROS at higher stimulation frequency. Switching from 4 to 0.2 Hz stimulation was followed by a drop of OxR back to resting values (103±10%). In unstimulated cardiomyocytes, despite some variation, OxR did not deviate significantly from OxR\(_{base}\) at any of the corresponding time points (121±1%, 116±5, 126±4%, 128±6% and 120±5% of OxR\(_{base}\), \( n\_animals=6, 15 \) cells in total), and was significantly different from OxR in stimulated cardiomyocytes (\( p<0.05 \)).

The stimulation-induced increase in ROS was attenuated in the presence of the antioxidant NAC in a dose-dependent manner, whereas inhibition of NOS with L-NAME had no effect on the increase in OxR at higher stimulation frequency (Fig. 2A,B).

In Fig. 2C, the electrical stimulation protocol was performed in a cell-free system containing deesterified DCFH. The different experimental conditions at baseline (e.g. no cellular sources of ROS formation) preclude quantitative comparison of the increase in oxidation rate. However, in contrast to the observations in stimulated cardiomyocytes, in the cell-free system DCFH oxidation increased immediately with initiation of electrical stimulation at 1 Hz and was much less affected by changes in stimulation frequency. Also, under these conditions 10 mM NAC almost completely attenuated the stimulation-induced DCFH oxidation (Fig. 2C).

Fig. 3. ROS formation at reduced contraction amplitude. BDM (5 mM) decreased the contraction amplitude to about 40% of control (A). In the presence of BDM, ROS formation at increased contraction frequency was significantly attenuated (B, \( n\_animals=4, 13 \) cells). \*\( p<0.05 \) vs. OxR\(_{base}\), †\( p<0.05 \) vs. Control.
3.2. ROS formation and energy turnover

In untreated cardiomyocytes, contraction amplitude modestly increased from 6.0±0.4% at 1 Hz to 7.3±0.4% at 4 Hz (p<0.05, Fig. 3A). Inhibition of myosin ATPase with 5 mM BDM decreased contraction amplitude to about 40% of control values at all frequencies, and ROS formation was attenuated particularly at high stimulation frequencies (Fig. 3).

To exclude direct effects of BDM on mitochondrial respiration, we examined the effects of BDM on VO₂ in respiring isolated mitochondria in the presence of ADP. BDM did not affect mitochondrial VO₂ (31.7±4.4 vs. 30.7±4.3 μmol O₂ s⁻¹ g protein⁻¹, before and after addition of 5 mM BDM resp., n=3).

Fig. 4A confirms a rise in VO₂ with increased stimulation frequency and recovery during subsequent 0.2 Hz stimulation (N=5 animals with a total of 19 experiments). NAD(P)H fluorescence decreased during increases in stimulation frequency, while in cardiomyocytes continuously stimulated at 1 Hz, NAD(P)H fluorescence remained unchanged, with a tendency towards an increase (Fig. 4B, C).

To compare the changes in ROS formation during increased contraction frequency with exogenously induced oxidative stress, we applied H₂O₂ to resting cardiomyocytes [26]. Within approx. 3 min, OxR increased to a maximum of 833±218% of OxRbase with 100 μM, and to 46±19 times and 105±13 times OxRbase with 1 and 5 mM H₂O₂, respectively (n_cells≥6 from ≥4 animals for each concentration). During the observation period, the cardiomyocytes remained rod-shaped.

3.3. ROS formation as a result of triggered afterdepolarisations

Fig. 5 shows a recording from a cardiomyocyte at the end of the frequency stair protocol (as in Fig. 1). After switching from 4 Hz to 0.2 Hz, the elevated OxR declined. Following a regular contraction at 0.2 Hz stimulation, the cardiomyocyte displayed a short episode of high frequency aftercontractions. The rapid boost of contractile activity was associated with a pronounced increase in ROS formation. While such triggered contractile oscillations were only observed in a fraction of cardiomyocytes (4 out of 84 cells), they were invariably accompanied by a burst of ROS.

4. Discussion

A decrease of the cellular redox potential in cardiomyocytes, equivalent to oxidative stress, has been shown to result from a variety of external stimuli, such as toxins, cytokines, neurohormones, ischemia or mechanical stress [27,28]. However, our results provide first evidence for an increased formation of ROS in cardiomyocytes with an increase in contraction frequency.
In skeletal muscle, formation of ROS during exercise is a well-established phenomenon [29]. ROS formation in skeletal muscle is detectable even before signs of muscular fatigue are evident [30,31] and has been shown to originate in skeletal myocytes [30,32].

We hypothesized that ROS formation also occurs in cardiomyocytes contracting with increased frequency and that ROS formation is related to $\dot{V}O_2$ and changes in the cellular redox state.

DCFH, like most of the currently available redox indicators, can be oxidized by a variety of reagents, including superoxide anions and derived species, i.e. hydrogen peroxide (in the presence of cellular peroxidases [33]) and hydroxyl radicals [34], but also by nitric oxide (NO) [35] or peroxynitrite [35,33]. The increase in OxR was dose-dependently inhibited by the antioxidant NAC. The fact that addition of L-NAME did not prevent increases in OxR with increased contraction frequency suggests that the observed changes in OxR are not the consequence of NO or peroxynitrite formation.

We added BDM to contracting cardiomyocytes to reduce $\dot{V}O_2$ by inhibition of myosin ATPase. While at the low concentration used BDM is mainly targeted at myosin ATPase with little effect on cytosolic Ca$_2^+$ [20,21], unspecific phosphatase-like activity has to be taken into account [36]. We performed measurements on isolated mitochondria to rule out direct effects on mitochondrial respiration at the concentration used in this study.

In stimulated cardiomyocytes BDM reduced the contraction amplitude, accompanied by a pronounced decrease in ROS formation at high contraction frequencies (3 and 4 Hz, Fig. 3), suggesting that the formation of ROS during increased contraction frequency was related to cytosolic ATP turnover and $V_2$. Parallel experiments confirmed that an increased contraction frequency was accompanied by an increase in $V_2$ (Fig. 4A) and coincided with a transient decrease of the NAD(P)H concentration (Fig. 4B, C). A decrease in intracellular NAD(P)H with increased contraction frequency has been observed in some [13–15] but not in all [37–39] previous studies on cardiac muscle, which may be related to the fact that tissue-averaged [38,39] or steady state [37] measurements may have missed transient deviations from metabolic equilibrium, as shown for intracellular [NADH] in isolated rat ventricular trabeculae [14,40] and single cardiomyocytes [15].

NAD(P)H-dependent changes in autofluorescence largely reflect changes in the mitochondrial redox state [23], and although the origin of increased ROS formation was not identified, mitochondria are a likely source as they are the main site of ROS formation within the cell [10]. The accumulation of ROS within the mitochondria may result from an increased production or a decrease in reducing equivalents such as NADH. Two mechanisms have been proposed to trigger changes in mitochondrial metabolism and redox potential during increased contraction frequency in cardiomyocytes: an increased mitochondrial Ca$^{2+}$ uptake induced by an increased time-averaged cytosolic [Ca$^{2+}$] [41] or a localized increase in cytosolic ADP near the mitochondrial membrane [42]. While the pronounced inhibitory effect of low-dose BDM may favour the latter mechanism, intracellular [Ca$^{2+}$] was not measured in this study. Nevertheless, our findings are in agreement with a time-dependent decrease in the mitochondrial redox potential with increased stimulation frequency as a basis for increased intracellular ROS formation.

ROS formation with higher contraction frequency was rapidly reversible after returning to low stimulation frequencies (Fig. 1) and small when compared to ROS formation induced by externally applied H$_2$O$_2$ as a model of cell damaging oxidative stress [26]. However, Zorov et al. have shown that localized ROS accumulation may be amplified by reversible mitochondrial permeability transition in cardiomyocytes [43]. In this context it is noteworthy that after the completion of exhaustive physical activity, oxidative intracellular damage, such as lipid and protein oxidation has been detected in myocardial tissue.
from young [44,45] and senescent rats [46] (but see also [9]).

Various signaling pathways have been shown to depend on intracellular ROS [1,8]. In the heart, ROS formation during cardiac exercise seems to be an important trigger of myocardial ischemic preconditioning [5] and may also have profound effects on intracellular Ca\(^{2+}\)-handling [47].

Bursts of ROS were consistently detected in some cardiomyocytes in association with triggered contractile oscillations following the rapid pacing protocol (Fig. 5). With respect to the observed short sequences of spontaneous activity associated with ROS formation, however, the temporal resolution to detect changes in OxR was limited. While triggered aftercontractions seemed to precede increases in OxR, it cannot be excluded that intracellular ROS formation may have preceded Ca\(^{2+}\) release resulting in these aftercontractions, as it has been described following different external stimuli [48,3].

We have shown that in a cell-free system DCFH oxidation increased during electrical stimulation, which may indicate ROS generation by electrolysis but may also be due to direct oxidation of DCFH at the platinum wires (Fig. 2C). Several observations indicate that this phenomenon was not related to the OxR increase in stimulated cardiomyocytes. Firstly, DCFH oxidation increased with initiation of stimulation without considerable change at increased frequencies, which is in contrast to the pronounced OxR increase observed in the cardiomyocytes at higher frequencies. Secondly, the lower dose of NAC (10 mM) was sufficient to largely attenuate cardiomyocytes at higher frequencies. Secondly, the lower dose of NAC (10 mM) was sufficient to largely attenuate OxR increase in cardiomyocytes could be reduced by decreasing contraction amplitude with BDM.

4.1. Limitations of the study

We have studied ROS formation in isolated cardiomyocytes, as we expected contractile activity-dependent ROS formation to be small (as compared to toxic ROS concentrations, see Results), and measurements using a fluorescent probe allowed us to identify ROS formation within individual cardiomyocytes, and with a higher sensitivity than currently available in vivo methods. While stimulation frequencies in vitro remained below the physiologic range of the rat in vivo (350 to 550 bpm [49]), we were able to induce a significant frequency-dependent increase in cellular VO\(_2\) as would be expected in the exercise-stressed rat. In the present study, we used HEPES-buffered solution equilibrated to room air to allow for a constant pH in the heated cell bath independent of pCO\(_2\) partial pressures. However, we cannot exclude that a higher availability of physically dissolved O\(_2\) in solution (as compared to tissue pO\(_2\)) may have exaggerated ROS formation with increased stimulation frequency.

In summary, we demonstrate intracellular ROS formation in response to increases in contraction frequency, associated with increased oxygen consumption. ROS formation in cardiomyocytes induced by increased contractile activity may be part of intracellular signaling cascades and warrants further study.

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