Intracellular Signaling by Reactive Oxygen Species during Hypoxia in Cardiomyocytes*

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Cardiomyocytes suppress contraction and O2 consumption during hypoxia. Cytochrome oxidase undergoes a decrease in V \textsubscript{max} during hypoxia, which could alter mitochondrial redox and increase generation of reactive oxygen species (ROS). We therefore tested whether ROS generated by mitochondria act as second messengers in the signaling pathway linking the detection of O2 with the functional response. Contracting cardiomyocytes were superfused under controlled O2 conditions while fluorescence imaging of 2,7-dichlorofluorescein (DCF) was used to assess ROS generation. Compared with normoxia (PO2 ~ 107 torr, 15% O2), graded increases in DCF fluorescence were seen during hypoxia, with responses at PO2 = 7 torr > 20 torr > 35 torr. The antioxidants 2-mercaptopropionyl glycerine and 1,10-phenanthroline attenuated these increases and abolished the inhibition of contraction. Superfusion of normoxic cells with H2O2 (25 μM) for >60 min mimicked the effects of hypoxia by eliciting decreases in contraction that were reversible after washout of H2O2. To test the role of cytochrome oxidase, sodium azide (0.75–2 μM) was added during normoxia to reduce the V \textsubscript{max} of the enzyme. Azide produced graded increases in ROS signaling, accompanied by graded decreases in contraction that were reversible. These results demonstrate that mitochondria respond to graded hypoxia by increasing the generation of ROS and suggest that cytochrome oxidase may contribute to this O2 sensing.

Alterations in oxygen tension (PO2) elicit a variety of functional responses in different cell types, including gene expression, altered metabolic function, altered ion channel activation, and release of neurotransmitters (1). In spontaneously contracting embryonic cardiomyocytes, we previously found significant decreases in contractile activity during prolonged moderate hypoxia (PO2 = 20 torr for >2 h) (2). This inhibition was not associated with a depletion of ATP or phosphocreatine stores, and was reversible when normoxic conditions were restored. Similar findings of decreased contractile function during hypoxia (48 h at 1% O2) have also been seen in rat cardiac myocytes (3), which suggests that this response is not unique to embryonic cells. An ability to respond to changes in oxygen tension within the physiological range implies the existence of a cellular O2 sensor linked to a signal transduction pathway. When activated by hypoxia, the sensor presumably would initiate a signaling cascade which ultimately leads to the functional response (e.g. diminished contractile activity). However, the O2 sensing mechanism and the subsequent signal transduction pathways involved in the cardiomyocyte responses to hypoxia are not known.

A number of different potential mechanisms of cellular O2 sensing have been identified (1). Mitochondria are responsible for most of the O2 consumption by the cell and would seem to be well suited because their local PO2 responds to changes in the ratio of O2 supply to demand. However, the low apparent K \textsubscript{m} of cytochrome oxidase for O2 (4–6) would appear to render these organelles incapable of detecting changes until very low O2 concentrations are reached. Nevertheless, our studies of hypoxic cardiomyocytes (2) and of normal rat hepatocytes (7, 8) have implicated mitochondria as a likely site of O2 sensing underlying their metabolic and functional responses to hypoxia. In this regard, we found that cytochrome oxidase undergoes a ~50% decrease in V \textsubscript{max} during exposure to prolonged moderate hypoxia (9). This change was manifested by decreases in N,N,N\textsubscript{t}tetramethyl-p-phenylenediamine-ascorbate respiration during hypoxia (2), and also by increases in [NADP(H)] autofluorescence (10). Collectively, these findings cast a new light on the possible role of mitochondria in the cellular responses to hypoxia in cardiomyocytes.

The presence of a cellular O2 sensor implies the existence of a signaling pathway linking it to the targeted response. If mitochondria function as that sensor, what signaling system could be activated by a decrease in the V \textsubscript{max} of cytochrome oxidase? We hypothesized that a decrease in V \textsubscript{max} of the oxidase should increase the reduction state of mitochondrial electron carriers upstream of cytochrome aa\textsubscript{3}. This should increase the lifetime of reduced electron carriers such as ubiquinone, which would increase the generation of superoxide via univalent electron transfer to O2 in the mitochondria (11). Trace levels of reactive oxygen species (ROS) such as superoxide or H2O2 could then potentially act as signaling elements by activating subsequent steps in a signal transduction cascade. Indeed, numerous studies have implicated ROS as participants in a variety of intracellular signaling sequences including members of the stress- and mitogen-activated protein kinases (12, 13), the nuclear transcription factors c-Jun and NF-xB (14), and other signaling systems (15). The mechanisms responsible for intracellular oxidant generation involved in the activation of those pathways are not fully clear, but could involve mitochondrial sources during hypoxia. The present study therefore sought to test the hypothesis that ROS are generated by mitochondria during hypoxia in cardiomyocytes, and that ROS signaling is involved in coupling the O2 sensor to the contractile response to hypoxia.

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§ The abbreviations used are: ROS, reactive oxygen species; DCF, 2,7-dichlorofluorescein; DCFH, reduced DCF; TTFA, thenoyltrifluoroacetone.
**MATERIALS AND METHODS**

**Primary Cell Isolation**—Embryonic chick cardiomyocytes were isolated using a method (2) modified from Barry et al. (16). Briefly, hearts of 10–11-day-old chick embryos were removed and placed in Hank’s balanced salt solution lacking magnesium and calcium (Life Technologies, Inc.). Ventricle tissue was minced and the cells were dissociated using four to six cycles of trypsin (0.025%, Life Technologies, Inc.) degradation at 37 °C with gentle agitation. Trypsin digestion was halted after 8 min by transferring the cells to a trypsin inhibitor solution. After filtering (100-μm mesh), the cells were centrifuged for 5 min at 1200 rpm at 4 °C and resuspended in nutritive medium. Cells then were placed in a Petri dish in a humidified incubator (5% CO\textsubscript{2}, 95% air at 37 °C) for 45 min to promote early adherence of fibroblasts. Nonadherent cells then were counted with a hemacytometer, and their viability was measured using trypan blue (0.4%). Approximately 1 × 10\textsuperscript{6} cells in nutritive medium (54% Barry’s solution (in mM: NaCl (116), KCl (1.3), NaHCO\textsubscript{3} (22), MgSO\textsubscript{4} (0.8), NaH\textsubscript{2}PO\textsubscript{4} (1.0), CaCl\textsubscript{2} (0.87), glucose (5.6)), 40% M199 with Earle’s salts (Life Technologies, Inc.), 6% heat inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 mg/ml)) were plated onto glass coverslips (25 mm). Cell yield averaged 5–6 × 10\textsuperscript{5} cells per embryo. Cells were maintained in a humidified incubator for 2–3 days, at which point spontaneous synchronous contractions of the monolayer were noted. Experiments were performed on spontaneously contracting cells at day 3 or 4 after isolation. Rat hepatocytes were isolated using the methodology described previously (18).

**Perfusion System**—Spontaneously contracting myocytes on glass coverslips were placed in a stainless steel flow-through chamber (1-ml volume, Penn Century Co., Philadelphia). The chamber was sealed using silicone rubber gaskets to minimize any O\textsubscript{2} exchange between the chamber wall and the perfusate, and mounted on a heated (37 °C) platform (Warner Instruments) on an inverted microscope. A water-jacketed glass equilibration column (37 °C) mounted above the microscope stage was used to equilibrate the perfusate to known O\textsubscript{2} tensions (P\textsubscript{O\textsubscript{2}}). The perfusate consisted of a buffered salt solution (BSS) (in mM: NaCl (117), KCl (4.0), NaHCO\textsubscript{3} (18), MgSO\textsubscript{4} (0.8), NaH\textsubscript{2}PO\textsubscript{4} (1.0), CaCl\textsubscript{2} (0.87), glucose (5.6)). The gas used to control the P\textsubscript{O\textsubscript{2}} and P\textsubscript{CO\textsubscript{2}} of the perfusate was supplied by a precision mass flow controller. Stainless steel or low O\textsubscript{2} solubility polymer tubing was used to connect the equilibration column to the flow-through chamber in an effort to minimize any ambient O\textsubscript{2} transfer into the perfusate. In previous studies, the P\textsubscript{O\textsubscript{2}} in the chamber was confirmed under conditions identical to those of the experiments using an optical phosphorescence quenching method (17, 18) (Oxyspot, Medical Systems Inc.).

**Fluorescence and Light Microscopy**—An inverted microscope was equipped for epifluorescent illumination and included a xenon light source (75 W), a 12-bit digital cooled CCD camera (Princeton Instruments), a shutter and filter wheel (Sutter), and appropriate excitation and emission filter cubes. The microscope also was equipped with Hoffman-modified phase illumination to accentuate surface topology, facilitating the measurement of contractile motion (see below). Fluorescent cell images were obtained using a 40× oil immersion objective (Nikon Plan Fluor). Data were acquired and analyzed using Metamorph software (Universal Imaging).

**Measurement of Reactive Oxygen Species**—ROS generation in cells was assessed using the probe 2,7-dichlorofluorescein (DCF) (Molecular Probes). The membrane-permeable diacetate form of the dye (reduced DCF (DCFH-diacetate)) was added to the perfusate at a final concentration of 5 μM. Within the cell, esterases cleave the acetate groups on DCFH-diacetate, thus trapping the reduced probe (DCFH) intracellularly (19). ROS in the cells oxidize DCFH, yielding the fluorescent product DCF (20). Our previous studies of the behavior of DCFH in cardiomyocytes revealed that the probe is readily oxidized by H\textsubscript{2}O\textsubscript{2} or hydroxyl radical, but is relatively insensitive to superoxide (21). Fluorescence was measured using an excitation wavelength of 480 nm, dichroic 505-nm long pass, and emitter bandpass of 535 nm (Chroma Technology) using neutral density filters to attenuate the excitation light. Fluorescence intensity was assessed in clusters of several (<10) cells identified as regions of interest, and background was identified as an area without cells or with minimal cellular fluorescence. Intensity values are reported as percent of initial values, after subtracting background.

**Contractile Motion of Cells**—Cell motion was recorded during low intensity visible light illumination using Hoffman modulation optics to accentuate the cellular topological changes during contraction. Images were recorded at video rates on magnetic tape using a high resolution video camera (Hamamatsu). During later analysis, sequential video frames were digitized, and pixels were assigned an intensity value ranging from 0 to 255. For each pixel, the absolute change in intensity was summed over ~250 frames. These summed changes in intensity were summed for all pixels, providing a single measure of motion in the field that consistently described the motion that already was evident by inspection. This analysis was carried out using a series of macro instructions in the Metamorph imaging software.

**RESULTS**

**Effects of Hypoxia on Intracellular Oxidant Signaling and Contractile Function in Cardiomyocytes**—Cultured embryonic cardiomyocytes on coverslips were placed in a flow-through chamber on an inverted microscope and perfused with buffered salt solution. Striated levels of hypoxia were produced by equilibrating perfusate with different gas mixtures. Duration of hypoxia was 2 h, beginning 30 min after normoxic base-line measurements. Recovery to normoxia was initiated at 150 min. a, effect of hypoxia on contractile motion in the same contracting cardiomyocytes. Progressive decreases in contraction developed during hypoxia, which were reversible after return to normoxia. Values are means ± S.E., n = 3 independent studies in each group.

**Reagents**—In different experiments the cells were treated with the electron transport inhibitors thenoyltrifluoroacetone (TTFA), antimycin A, and rotenone (Sigma). The antioxidants 2-mercaptopyrropropionyl glycine and 1,10-phenanthroline were also obtained from Sigma.

![Fig. 1](http://www.jbc.org/)
motion was assessed by periodically recording motion at video rates under low-light illumination conditions for 1 min. Graded decreases in contractile motion were seen during hypoxia, which were reversible when normoxic conditions were restored. These results were consistent with our previous studies (2). As seen previously, the decrease in contraction did not develop immediately but required 1–2 h to reach a stable level of inhibition. Likewise, full recovery of motion required 2–3 h after restoration of normoxia.

To confirm that oxidant signaling was responsible for these changes, the study was repeated (3% O₂, PO₂; 20 torr) in the presence of the thiol reducing agent 2-mercaptopropionyl glycine (300 μM) and the metal chelator 1,10-phenanthroline (10 μM). In the presence of these antioxidants, the increases in cell fluorescence were significantly attenuated (Fig. 2).

Source of ROS Generation—To determine the source of oxidants during hypoxia, cells loaded with DCFH were incubated at PO₂; 20 torr (3% O₂) while DCF fluorescence images were acquired. The mitochondrial electron transport inhibitors rotenone (1 μg/ml) and TTFA (10 μM) subsequently were added to block electron supply to ubiquinol, thereby limiting the formation of ubisemiquinone. This produced a rapid decrease in DCF fluorescence during hypoxia (Fig. 3a). In another experiment, cardiomyocytes loaded with DCFH and perfused under hypoxic conditions were given antimycin A, which inhibits the oxidation of cytochrome b₅₆₆, thereby increasing the lifetime of ubisemiquinone (11). This produced a further increase in DCFH oxidation, as detected from fluorescence measurements (Fig. 3b).

Role of ROS in Signal Transduction—To test whether oxidant signaling was involved in the suppression of contraction seen during prolonged moderate hypoxia, the antioxidants 2-mercaptopropionyl glycine (300 μM) and 1,10-phenanthroline (10 μM) were added to the perfusate during hypoxia (3% O₂). As shown in Fig. 4, antioxidant treatment abolished the decreases in contractile motion seen during hypoxia, thus implicating ROS in the functional response to hypoxia.

Conceivably, superoxide generated by the mitochondrial electron transport system could activate subsequent signaling steps by oxidizing a substrate directly. Alternatively, dismutation of superoxide by superoxide dismutase could yield H₂O₂, which could function as the active intermediate in this response. To test this possibility, contractile motion was studied in cardiomyocytes perfused with different concentrations of H₂O₂ under normoxic conditions. Fig. 5 shows the effect of 120 min perfusion with H₂O₂ (25 μM) on contractile motion. Within 1 h, significant decreases in contractile motion were detected. Washout of H₂O₂ was associated with recovery of contractile motion, although this required several hours to reach baseline levels. Interestingly, lower concentrations of H₂O₂ (<10 μM) did not significantly affect contractile motion during perfusion for 1 h, while higher concentrations of H₂O₂ (>50 μM) elicited a marked decrease or cessation of contractile activity which did not return during recovery (data not shown).
Role of Cytochrome Oxidase in the Generation of ROS by Mitochondria—Previous studies of cytochrome oxidase revealed a decrease in the $V_{\text{max}}$ of the enzyme during hypoxia (9). Such a change could alter the mitochondrial redox state and accelerate ROS generation by mitochondria during hypoxia. If so, then inhibitors of cytochrome oxidase that reduce the $V_{\text{max}}$ of the enzyme during normoxia should mimic that effect. To test this we used sodium azide, a noncompetitive inhibitor of the oxidase (22) to partially inhibit the oxidase during normoxia. Fig. 6a shows the effect of different concentrations of azide on DCF fluorescence in cultured cardiomyocytes maintained at $P_{O_2} \sim 107$ torr. Low concentrations of azide (0.75–1 mM) produced graded increases in fluorescence that decreased after washout of the inhibitor. Greater increases in fluorescence were observed with higher concentrations of azide (2–5 mM), which also were reversible after removal of azide. Fig. 6b shows the effects of azide on contractile motion of cardiomyocytes. Dose-dependent progressive decreases in contractile motion were observed during 2 h of azide treatment, which were reversible after removal of the inhibitor. However, rapid and irreversible decreases in contraction were seen with high concentrations of azide (5 mM), which were suggestive of cellular injury.

Relationship between Cytochrome Oxidase $V_{\text{max}}$ and $O_2$-dependent ROS Generation during Hypoxia—To test whether $O_2$-dependent decreases in cytochrome oxidase $V_{\text{max}}$ were responsible for the increase in ROS generation during hypoxia, primary rat hepatocytes were loaded with 2,7-dichlorofluorescin and studied during normoxia (1 h) followed by hypoxia (3% $O_2$, 2 h) and reoxygenation (1 h) (Fig. 7). In previous studies, hepatocytes were shown to require 1–2 h of hypoxia in order to elicit the decrease in $V_{\text{max}}$ of the oxidase (8), whereas the oxidase in cardiomyocytes responded within 10 min of hypoxia (23). Therefore, ROS generation should correlate temporally with the changes in $V_{\text{max}}$ of the oxidase among different cell types. In accordance with this hypothesis, hepatocytes showed minimal increase in DCF fluorescence during the first hour of hypoxia, but demonstrate a more rapid increase at 1.5–2 h. Upon reoxygenation, cellular fluorescence decreased toward baseline levels. Control cells maintained under normoxic conditions for the same period showed minimal increases in DCF fluorescence.

DISCUSSION

Many cell types respond to changes in the local $O_2$ concentration by activating functional or adaptive responses. This suggests the existence of an $O_2$ sensor coupled to a signal transduction system, which ultimately triggers the functional response. Although a variety of $O_2$ sensing mechanisms are known to exist (1), our previous studies suggested that mitochondria may act in that role in embryonic cardiomyocytes (2, 24). The present study aimed to test the hypothesis that ROS generated by mitochondria act as second messengers in the contractile response to physiological levels of hypoxia in those cells. Our data show that mitochondrial ROS generation increases as the concentration of $O_2$ decreases during hypoxia.
Compared with normoxia (15% O<sub>2</sub>), graded increases in DCFH oxidation were observed at 5%, 3%, and 1% O<sub>2</sub>, with the highest levels of oxidant signaling seen at the lowest O<sub>2</sub> concentrations. Administration of antioxidants attenuated this oxidant signaling, and also abolished the decrease in contractile function seen during prolonged hypoxia. Dose-dependent inhibition of cytochrome oxidase with azide produced graded increases in ROS generation during normoxia and elicited reversible decreases in contraction. Exogenous administration of H<sub>2</sub>O<sub>2</sub> produced reversible decreases in contraction during normoxia that mimicked the response to hypoxia. Collectively, these observations suggest that PO<sub>2</sub>-dependent mitochondrial ROS generation participates in the signal transduction cascade linking the O<sub>2</sub> sensor with the contractile response via the sequence of events: hypoxia → decreased cytochrome oxidase V<sub>max</sub> → increased mitochondrial redox → increased mitochondrial superoxide generation → increased H<sub>2</sub>O<sub>2</sub> generation → subsequent signaling steps → decreased contraction.

**ROS as Second Messengers of Signal Transduction**—The data suggest that ROS released from mitochondria participate as second messengers in the signaling pathway linking the O<sub>2</sub> sensor to the decrease in contractile function of cardiomycocytes. We propose that the decreases in contractile function and O<sub>2</sub> consumption are a consequence of decreased ATP utilization (24), possibly arising from a signaling sequence that inhibits activation of the actin-myosin ATPase system. ROS would appear to act at an early step in this sequence, since the contractile response to hypoxia or exogenous of H<sub>2</sub>O<sub>2</sub> required 1–2 h to develop, and recovery from these activators required 2–3 h for completion. The low levels of ROS generation during hypoxia do not appear to be toxic, as judged by the ability of the cells to recover contractile function after restoration of normoxia, and the absence of any loss of cell viability despite prolonged hypoxia noted previously (2). Our study did not investigate steps subsequent to H<sub>2</sub>O<sub>2</sub> generation, but a growing number of studies indicate an important role of ROS in a variety of intracellular signaling pathways (14, 25, 26). For example, Guyton et al. (12) have shown that H<sub>2</sub>O<sub>2</sub> can cause activation of mitogen-activated protein kinase (ERK-2, p44); Lander et al. (27) have described a mechanism allowing nitric oxide to regulate the G protein p21<sup>ras</sup>, and ROS appear to participate in the activation of stress-activated protein kinases (JNKs/SAPKs) (13). Future studies will be required to clarify the later steps in the signal transduction cascade responsible for the inhibition of contraction.

**What Is the Source of Oxidant Signals during Hypoxia?**—Mitochondria have long been recognized as sites where reactive oxygen species are generated in cells. In the electron transport chain, ubisemiquinone (11) appears to be a major site of superoxide generation because of its predisposition for univalent electron transfer to O<sub>2</sub>. Indeed, it has been estimated that superoxide generation accounts for 1–2% of mitochondrial O<sub>2</sub> consumption even under normal conditions (28). Two pieces of evidence support the conclusion that mitochondria were the source of ROS signals in our study. First, electron transport inhibition with rotenone (site I) plus TTFA (site II) attenuated the ROS signal during hypoxia, whereas antimycin A, an inhibitor of complex III, accelerated oxidant production (Fig. 8). Rotenone and TTFA limit the formation of superoxide by attenuating formation of ubisemiquinone, whereas antimycin A augments superoxide generation by increasing the lifetime of that intermediate. Collectively, these results demonstrate that mitochondria function as a source of ROS during hypoxia, generating increasing amounts of oxidants at lower oxygen concentrations. This behavior could allow mitochondria to function as a cellular O<sub>2</sub> sensor at physiological levels of hypoxia.

**Do Other Sources of Oxidant Production Contribute to the Hypoxic Response?**—Many different oxidase systems could conceivably contribute to the generation of ROS in cells. For example, Wolin and colleagues identified a cytosolic NADH oxidoreductase whose ROS generation varies in response to changes in PO<sub>2</sub> over a wide physiological range (29). Using homogenates of cardiac myocytes, they found that addition of NADH (but not NADPH) caused a marked increase in superoxide generation, as detected by lucigenin chemiluminescence (30). Those results suggest that the NADH-dependent oxidase is responsive to cytosolic, rather than mitochondrial, NAD(H) redox. However, their lucigenin chemiluminescence signal increased with PO<sub>2</sub>, and was greatest during 21% O<sub>2</sub> incubation. During hypoxia their chemiluminescence signal decreased, becoming undetectable at PO<sub>2</sub> = 8–10 torr. By contrast, using DCFH we found increases in mitochondrial oxidant generation at low PO<sub>2</sub> values, with the highest levels at 1% O<sub>2</sub> (PO<sub>2</sub> = 7 torr). Because the decrease in contractile motion during hypoxia was abolished with antioxidants, and the ROS generation detected with DCFH increased under hypoxia, we conclude that the functional response to hypoxia in our study must have involved an increase in ROS production from mitochondria, rather than decreased ROS generation at low PO<sub>2</sub> from NAD(P)H oxidases.

**Why Does Mitochondrial Oxidant Generation Increase during**
Hypoxia?—The factors that control the rate of ROS production by mitochondria are not fully understood, but likely include (a) the reduction state of the mitochondrial electron transport system and (b) availability of O$_2$. Factors that increase the reduction state of the electron carriers appear to increase the generation of superoxide, even when [O$_2$] is low. For example, accelerated mitochondrial ROS generation has been shown to occur during ischemic conditions when electron carriers are highly reduced and the cells are nearly anoxic (21, 31). Likewise, increases in mitochondrial redox at a given [O$_2$] appear to increase ROS generation (32). For example, both azide and antimycin A produced increases in reduction of ubiquinone during normoxia, which led to the increases in DCF fluorescence. Thus, mitochondrial superoxide generation appears to respond to changes in redox state at a given availability of O$_2$.

What Mechanisms Contribute to Changes in Mitochondrial Redox during Moderate Hypoxia?—In our study, the increases in ROS detected during hypoxia suggest that mitochondrial reduction must have increased as PO$_2$ was lowered. Two models could conceivably explain this change. First, we previously reported that $V_{max}$ of cytochrome oxidase decreases by ~50% during hypoxia in cardiac myocytes (2), normal rat hepatocytes (8, 10), rat liver mitochondria (7), liver submitochondrial particles (10), and in isolated bovine heart cytochrome oxidase (9). We now suggest that the increase in mitochondrial reduction caused by the decrease in cytochrome oxidase $V_{max}$ elicits an increase in mitochondrial superoxide generation.

A second mechanism tending to increase mitochondrial reduction during hypoxia was described by Wilson and co-workers who measured cytochrome c reduction as PO$_2$ was reduced from 150 torr toward zero (17, 33). According to their model, small increases in cytochrome c reduction occur at physiological levels of hypoxia, which tend to maintain a constant electron flux (and thus O$_2$ consumption rate) until critically low levels of [O$_2$] are reached. Although the increases in cytochrome c reduction they reported were small during physiological hypoxia, they may nevertheless have been sufficient to augment mitochondrial superoxide generation.

To distinguish the relative importance of these two mechanisms, we compared the ROS response in cardiac myocytes to that in normal rat hepatocytes. In their studies of PO$_2$-dependent cytochrome c reduction, Wilson and co-workers observed increases in reduction within seconds after the start of hypoxia in a wide variety of cell types (17, 34, 35). We previously noted that cytochrome oxidase differs among cell types with respect to the duration of hypoxia required to elicit a decrease in its $V_{max}$. The oxidase in embryonic cardiac myocytes responded to hypoxia within 10 min (2), whereas rat hepatocytes required incubation under hypoxia for 1 to 2 h to elicit a change in $V_{max}$ (8, 10). If the decrease in $V_{max}$ were responsible for the increase in ROS signal during hypoxia, then increased DCF fluorescence should not occur in hepatocytes until they had been hypoxic for >1–2 h. In contrast, if the PO$_2$-dependent increases in mitochondrial redox described by Wilson and colleagues were responsible, the increases in ROS production would occur immediately in both cell types. As shown in Fig. 7, hepatocytes loaded with DCFH and superfused at 3% O$_2$ showed an increase in fluorescence that did not begin until 1–2 h after the start of hypoxia, and which decreased after return of normoxia. By contrast, increases in DCF fluorescence were evident after 15 min of hypoxia in the cardiomyocytes. We conclude that changes in the $V_{max}$ of cytochrome oxidase during hypoxia, rather than O$_2$-dependent changes in mitochondrial reduction state (17), must have been responsible for the increase in ROS signaling during hypoxia.

Modulation of Cytochrome Oxidase $V_{max}$ by Azide or Hypoxia—As a noncompetitive inhibitor of cytochrome oxidase (22), azide has effects that depend on its concentration. At high concentrations (>2 mM) it is possible that azide could throttle mitochondrial respiration by inhibiting cytochrome oxidase. However, lower concentrations (< 2 mM) should mimic the effects of hypoxia by partially inhibiting cytochrome oxidase. This should lower the $V_{max}$ of the oxidase via non-competitive inhibition during normoxia. Indeed, the dose-dependent increase in reduction of electron carriers caused a graded increase in ROS generation detected by DCFH, which mediated a progressive and reversible suppression of contractile activity.

However, previous studies of the effects of O$_2$ on cytochrome oxidase $V_{max}$ suggested that the oxidase can function in either of two kinetic states, rather than over continuous spectrum (9). Therefore, when hypoxia induces a step decrease in cytochrome oxidase $V_{max}$ a step increase in superoxide generation should be seen. In this respect, the graded increases in oxidant signal seen at lower PO$_2$ levels would seem paradoxical, because mitochondrial redox should remain constant while O$_2$ availability for superoxide generation would decrease. These observations suggest that additional factors may influence the rate of superoxide generation at different levels of moderate hypoxia. Further studies will be required to fully delineate the mechanisms controlling superoxide generation in the intact cell at graded levels of hypoxia.
