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Localization of dichlorofluorescin in cardiac myocytes: implications for assessment of oxidative stress

LUTHER M. SWIFT AND NARINE SARVAZYAN
Department of Physiology, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

Swift, Luther M., and Narine Sarvazyan. Localization of dichlorofluorescin in cardiac myocytes: implications for assessment of oxidative stress. *Am. J. Physiol. Heart Circ. Physiol.* 278: H982–H990, 2000.—Localization and staining features of the oxidant-sensitive fluorescent probe 2′7′-dichlorofluorescin (DCFH) were evaluated in isolated cardiac muscle cells. Cardiomyocytes rapidly accumulated the probe and retained steady levels of DCFH and its highly fluorescent oxidized product dichlorofluorescein (DCF) in probe-free medium for 1.5 h. DCF was associated with mitochondria and was released by the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone but not by saponin, which permeabilizes the plasma membrane. A mitochondrial distribution of DCF was also suggested by experiments with the mitochondrial marker MitoTracker Red, in which quenching was observed between DCF and MitoTracker Red in live cells. Isolated cardiac mitochondria rapidly accumulated DCF, and high micromolar concentrations of the probe inhibited ADP-stimulated respiration rate. The study provides an information base essential for the interpretation and design of experiments with DCF as a marker of oxidative stress in cardiac muscle and reveals preferential localization of the probe in mitochondria.

confocal microscopy; hydrogen peroxide; heart; fluorescent indicators

AN INCREASING NUMBER of studies have used the fluorescent marker dichlorofluorescein (DCF) and its derivatives to study the role of reactive oxygen species (ROS) in various pathophysiological and physiological phenomena (4, 11, 12, 25). To successfully employ such fluorescent markers and to correctly interpret the acquired data, one needs to know what types of ROS react with the dye, in which cellular compartment the probe localizes, and how long it can be retained inside the cells in its reduced and oxidized forms. The first question has been addressed by several studies both in vivo and in vitro. It was shown that dichlorofluorescin (DCFH) can be rapidly oxidized to a highly fluorescent compound called dehydrodichlorofluorescin or DCF by various ROS as well as by peroxynitrite (3, 13, 15, 30). However, no systematic studies have addressed the localization and retention of DCFH and its oxidation product DCF. According to the general scheme suggested over a decade ago (1), the esterified form of DCFH (dichlorofluorescin diacetate or DCFH-DA) rapidly penetrates cell membranes and becomes deacetylated by intracellular esterases. Nonfluorescent DCFH is then trapped in the cytosol and, upon oxidation to DCF, serves as a sensitive cytosolic marker for oxidative stress. Later, however, the ability of cells to retain DCF and DCFH was challenged by findings in cultured aortic endothelial cells, which are incapable of retaining DCFH and DCF (24). Controversy also exists regarding the cellular distribution of the probe, and it becomes more and more evident that intracellular localization and other features of the probe depend on the particular tissue or cell type (3, 7, 24, 25). This study characterizes DCF staining in isolated cardiac myocytes from adult rats; specifically, it evaluates the rate of accumulation and retention of the probe and examines its preferential localization.

MATERIALS AND METHODS

Preparation of cardiomyocytes. Rat cardiac myocytes were isolated as described previously (25). Briefly, 2-mo-old Sprague-Dawley rats (200–300 g) were injected intraperitoneally with 500 U/kg heparin sodium. After 20–25 min, the rats were anesthetized with pentobarbital sodium (45 mg/kg), and the excised hearts were perfused for 10 min with Joklik's modified minimum essential medium supplemented with 1.25 mM $CaCl_2$. This was followed by a 5-min perfusion with a nominally calcium-free medium supplemented with 20 mM creatine and 60 mM taurine and a 5-min perfusion with the same medium containing 0.5–1 mg/ml of type II collagenase and 0.1% BSA. The ventricles were then minced and vigorously shaken in the same medium containing 1% BSA. After two washes in collagenase-free medium, the $CaCl_2$ concentration of the medium was gradually increased to 1.25 mM. With this method, a yield of $5–7 \times 10^6$ calcium-tolerant cells per heart was routinely obtained. The percentage of myocytes retaining rod-shaped morphology ranged from 65 to 75%, whereas the percentage of cells impermeable to Trypan blue was ~80%.

Human glomerular endothelial cells. Human glomerular endothelial cells were obtained from Cell Systems Corporations (Kirkland, WA). Cells were grown in T-75 culture flasks using CS-C medium with CS-C RocketFuel supplement in 5% CO_2 atmosphere at 37°C. Cells were subcultured on glass coverslips at an initial density of $\sim 5 \times 10^3$ cells/cm^2 and were used after 2 wk of culture.

Cell loading with fluorescent probes. A 10 mM stock solution of DCFH-DA was prepared in ethanol on a daily basis. Myocytes were loaded with DCFH by a 30-min incubation with 10 μM DCFH-DA at room temperature in the dark unless otherwise noted. Fluo-3 was used to compare DCF distribution with the staining pattern of another cytosolic probe (21). Fluo-3 was loaded by incubating the myocytes

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with 2 μM fluo-3-AM [the acetoxymethyl ester (AM) form of the probe] at room temperature for 20 min. For comparative experiments (DCF vs. fluo-3), myocytes were loaded with DCFH-DA identically with the fluo-3 protocol (2 μM final concentration from a 1 mM stock solution in DMSO). The cells were stained with MitoTracker Red (MTR) by a 10-min incubation with 5 μM MTR at room temperature.

Fluorescence measurements. Levels of DCF in cell lysates or in solutions were measured using 488 nm excitation/530 nm emission settings with 4-nm slit widths (DMX-1000 spectrofluorometer; Spectronics Instruments, Urbana, IL). When used in suspension, myocytes (2.5×10^5 cells/ml) were loaded with the probe by preincubation in a Tyrode solution supplemented with 20 mM HEPES, pH 7.4, 10 mM glucose, and 10 μM DCFH-DA. At designated times, myocytes were washed two times with dye-free Tyrode and disrupted in a sonication buffer of 50 mM potassium phosphate buffer, 0.1 mM EDTA, and 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. The level of nonenhanced fluorescence was used to indicate the basal level of oxidized probe (DCF) in the myocytes. Addition of 25 μg/ml horseradish peroxidase and 1 mM H_2O_2 leads to a complete conversion of DCFH to DCF and was used to assess the total dye amount (DCFH + DCF). To assess the interaction of DCF and MTR probes, several concentrations of MTR were added to 1 μM DCF in 50 mM phosphate buffer, pH 7.0, and emission spectra were acquired while exciting at 488 nm. To address the effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) in cells suspensions, myocytes were loaded with DCFH-DA, exposed to 100 μM CCCP for 5 min, and centrifuged to separate supernatants from cell pellets (2 min at 70 *g*). Total dye (DCFH + DCF) levels in supernatants and sonicated pellets were then measured.

Image acquisition and analysis. Cells were observed through a Plan-Apo X60 oil-immersion objective mounted on an inverted Olympus microscope with an Olympus LSM GB200 confocal imaging system attached. Excitation of dyes was achieved using the 488-nm line of a 15-mW argon ion laser attenuated to 1% intensity. The emitted light was collected using a 515-nm longpass filter, and a 510 ± 20 nm bandpass was added in dual-staining experiments. With these settings, cell autofluorescence was below detection levels. All images were collected at room temperature. Mean fluorescence intensity was calculated by averaging area intensities from a number of outlined cells. For each condition described, four to seven images of different cells were collected, and experiments were repeated at least three times. Representative myocyte images in Figs. 2–6 and 8 were saved as TIFF files and were pseudocolored using National Institutes of Health Image software.

Plasma membrane permeabilization by saponin. After attachment to laminin-treated glass coverslips and loading with DCFH-DA, cells were exposed to 2 mM H_2O_2 for 1 min to obtain bright images of DCF-stained myocytes. Alternatively, repetitive laser scanning was used to convert DCFH to DCF by photooxidation. The bathing medium was then changed to a cytosol-like high-potassium buffer (in mM: 120 potassium aspartate, 25 KCl, 3 MgATP, 0.5 $MgCl_2$, 0.2 EGTA, 20 K-HEPES, and 5 phosphocreatine, pH 7.3) to avoid myocyte collapse after plasma membrane disruption, and the cells were permeabilized by a 1-min treatment with 0.01% saponin. The same buffers were used in experiments with cell suspensions, when DCFH/DCF and fluo-3 release into the medium was assessed.

Isolation of rat heart mitochondria. Mitochondria were isolated as described previously (18). After brief retrograde perfusion to remove blood, the ventricles were minced and homogenized in ice-cold isolation buffer (300 mM mannitol, 1 mM EGTA, 1 mg/ml BSA, and 10 mM HEPES, pH 7.4). Cell debris was removed by two 5-min centrifugations at 1,000 *g*. Supernatant was collected and centrifuged for 5 min at 8,000 *g*. The mitochondrial pellet was washed with isolation buffer and centrifuged again (5 min at 8,000 *g*). The mitochondria were then washed two times with BSA-free isolation buffer, resuspended in 2 ml of respiration medium (210 mM mannitol, 60 mM Tris·HCl, 10 mM KCl, 10 mM KH_2PO_4, and 0.5 mM EGTA, pH 7.4), and kept on ice. Protein content was determined by a Lowry assay kit (Sigma Chemical) using BSA as the protein standard.

Mitochondrial respiration. Respiration experiments were carried out immediately after isolation of the mitochondrial fraction. Oxygen consumption was measured at 30°C with an oxygen electrode (MI-730; Microelectrodes, Bedford, NH) in 2 ml of respiration medium. Rotenone (5 μM) was added to block electron transport proximal to succinate entry into the respiratory chain. ADP-stimulated respiration (*state III*) was measured in the presence of 0.3 mM ADP (17).

Materials. Collagenase II was obtained from Worthington (Freehold, NJ). Culture medium for human glomerular endothelial cells was purchased from Cell Systems Corporations. BSA, HEPES, saponin, and other chemicals were purchased from Sigma (St. Louis, MO). Fluorescent indicators were obtained from Molecular Probes (Eugene, OR).

RESULTS

Kinetics of DCF accumulation and retention in suspensions of cardiomyocytes. The amount of the probe (DCFH + DCF) increased in a time-dependent manner and reached a steady state within 10 min after DCFH-DA addition (Fig. 1*A*). Thereafter, the amount of fluorescent probe was constant for loading times up to 60 min (Fig. 1*A* and data not shown). To evaluate retention of the probe, loaded cells were incubated in probe-free media for up to 1.5 h, and intracellular DCFH + DCF levels were assessed every 30 min. The levels of DCF + DCFH remained constant for at least 90 min of incubation in probe-free medium (Fig. 1*B*).

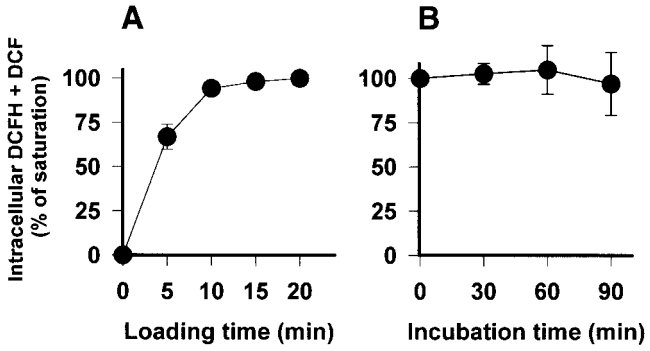

Fig. 1. Accumulation and retention of dichlorofluorescein (DCF) and dichlorofluorescin (DCFH) by isolated cardiomyocytes in suspensions. *A*: cells were incubated for indicated periods of time with 10 μM DCFH diacetate (DCFH-DA), washed by probe-free medium, and assayed for DCF + DCFH content as described in MATERIALS AND METHODS. *B*: cells were preloaded with 10 μM DCFH-DA for 30 min, washed, and incubated in probe-free medium. At indicated times, aliquots were removed to assess cell viability and probe levels. DCFH + DCF content in rod-shaped cells is presented as percentage of initial values. Values are means ± SE from 4 different preparations.

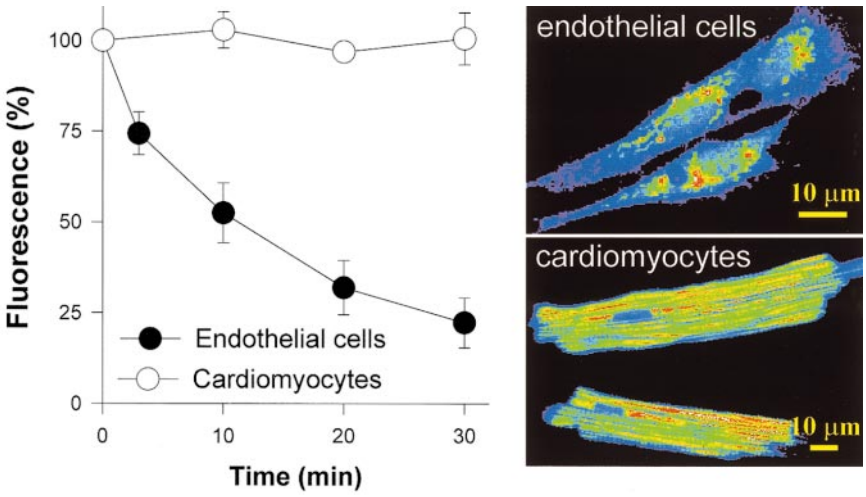

Fig. 2. Retention of DCF by cardiomyocytes and human glomerular endothelial cells. Monolayers of attached cells were preloaded with DCFH-DA and exposed to 2 mM H_2O_2 for 1 min. Intensity of DCF fluorescence was quantified after medium was changed to H_2O_2-free buffer. Average values for representative experiment are shown (mean ± SD, 5 cells). Experiment was repeated three times with similar results.

The baseline DCF levels in quiescent (nonstimulated) myocytes remained <3% of the total probe amount (data not shown).

DCFH retention and oxidation: Endothelial cells vs. cardiomyocytes. The ability of cardiomyocytes to retain DCFH/DCF sharply contrasts with rapid leakage of the dye from endothelial cells reported earlier (24). Therefore, the abilities of myocytes and human glomerular endothelial cells to retain the dye were compared in the same experimental settings. Specifically, both types of cells were attached to glass coverslips, loaded with 10 μM DCFH-DA, washed with dye-free Tyrode buffer, and treated with 2 mM H_2O_2 for 1 min. Buffer was then replaced, and DCF levels were monitored for another hour. Cardiomyocytes treated with H_2O_2 showed the same level of DCF fluorescence after 1 h as newly treated cells. In contrast, endothelial cells rapidly leaked the probe (Fig. 2).

Intracellular distribution of DCFH. The DCF staining pattern was readily revealed by treatment of DCFH-loaded cells with H_2O_2 or by repeated scanning with the laser beam, leading to photooxidation of DCFH to DCF. The intracellular pattern of staining was different from the expected uniform distribution of cytosolic probes such as fluo-3 (Fig. 3*A*). It suggested the presence of DCF either inside or in close proximity to the mitochondria (Fig. 3*B*), because the staining pattern closely resembled one with the mitochondrial marker MTR (Fig. 3*C*) or rhodamine 123 (25). Mitochondrial compartmentalization of cytosolic probes is a well-known phenomenon (21) that can be reduced by decreasing the time or altering the temperature of dye loading (2). However, neither shortening the incubation time to 5 min nor lowering DCFH-DA concentration to 2 μM substantially affected the distribution of DCF. Another potential explanation for the preferential mitochondrial localization is active transport of the probe out of the cells, although this is unlikely considering the retention data (Fig. 1*B*). However, addition of 50 μM verapamil to the loading solution, which reportedly

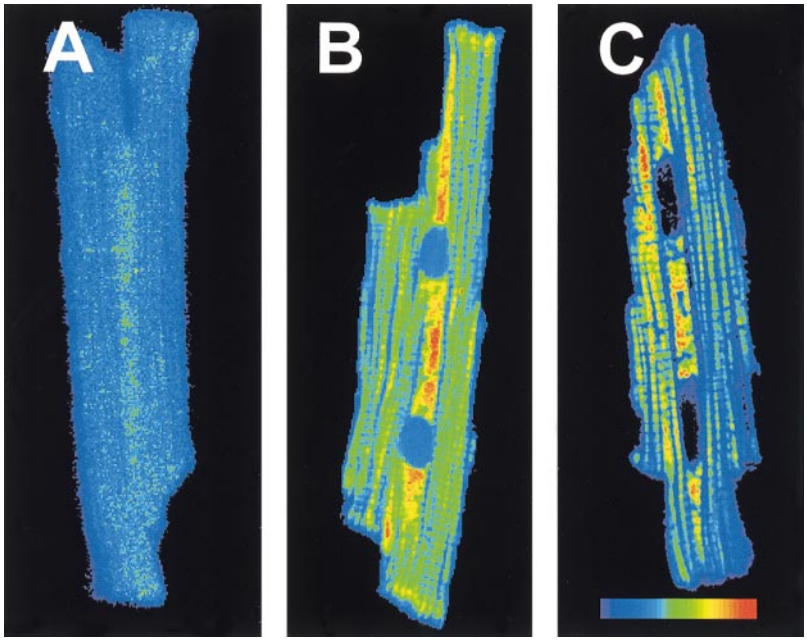

Fig. 3. Representative myocyte staining with characteristic cytosolic and mitochondrial pattern. *A*: cell loaded with calcium-sensitive probe fluo-3. *B*: typical DCF staining pattern. *C*: mitochondrial staining with MitoTracker Red (MTR). Cardiac mitochondria can be seen in long rows between rows of myofibrils. Dye-free areas correspond to the nuclei (myocytes are mostly binuclear cells). Color scale shows false color referring to increasing fluorescence values, from lowest (purple) to highest (red).

prevents cells from extruding the AM form of the dyes before they can be hydrolyzed (8, 20), did not change the distribution of DCF.

Effects of saponin on DCF-stained myocytes. Permeabilization of cells with saponin to release cytosolic components is a widely used technique in different cell systems (29), including myocytes. To avoid irreversible cell contraction after perforation of sarcolemma, such treatment is performed in a cytosol-like buffer containing high potassium and ATP (details in MATERIALS AND METHODS). Indeed, application of 0.01% saponin to fluo-3-loaded myocytes led to a rapid loss of fluorescence (although mitochondria can become increasingly bright due to the Ca^{2+} uptake by these organelles and inclusion of ruthenium red prevents this effect). In contrast, DCF staining was only slightly affected by saponin addition. Specifically, saponin "clarified" the DCF staining pattern by releasing a small amount of cytosolic DCF (see below).

The results of the imaging experiments were confirmed by fluorometer measurements of cell suspensions. Specifically, myocytes were loaded identically with fluo-3-AM and DCFH-DA, washed by probe-free Tyrode, and transferred to cytosol-like buffer. Cells were then incubated with 0.01% saponin for 10 min, followed by centrifugation and assessment of DCF and fluo-3 content in cell pellets and supernatants (peroxidase/H_2O_2 and 1 mM $CaCl_2$ were added to convert DCFH to DCF and saturate the fluo-3 signal). Saponin treatment released only 24.4 ± 5.4% of initial DCF/DCFH content compared with 66.9 ± 7.9% loss of the fluo-3 fluorescence ($n = 3$).

Effects of CCCP on DCF-stained myocytes. Additional evidence that DCF accumulates primarily in the mitochondria is that application of a well-known protonophore, CCCP, that disrupts the mitochondrial membrane potential led to a rapid decrease of the intensity of DCF staining (Fig. 4). We confirmed the specificity of CCCP treatment by comparing its effect on cells stained with mitochondrial dye (MTR) and cytosolic dye (fluo-3). CCCP application diminished MTR staining similar to DCF. In contrast, protonophore application to fluo-3-loaded cells led to a transient elevation of the fluorescent signal, presumably due to Ca^{2+} release from depolarized mitochondria into the cytosol (28). However, we saw no indication of decreased fluo-3 signal in CCCP-treated myocytes.

To confirm that CCCP-induced changes in DCF staining intensity were not artifacts of changes in intracellular pH or dye redistribution, we measured release of the probe in the surrounding medium. Specifically, a 5-min addition of suspensions of DCF-stained cells to 100 μM CCCP (myocytes were loaded with 10 μM DCFH-DA, oxidized by brief exposure to H_2O_2, washed, and exposed to CCCP) did not have an immediate impact on cell viability but caused a fall of total DCF/DCFH by 59.2 ± 3.8% and increased the probe's concentration in the medium by 451 ± 18 nM ($n = 3$).

DCF colocalization with MTR. Concentration-dependent quenching of DCF fluorescence occurred in vitro when MTR was added to a solution containing DCF

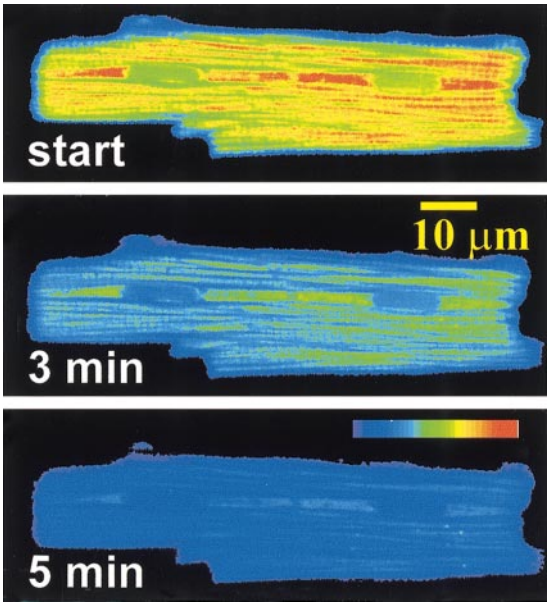

Fig. 4. Protonophore-induced release of the mitochondrial pool of DCF. Cells loaded with 10 μM DCFH-DA were exposed to 2 mM H_2O_2 for 1 min to reveal DCF staining. Application of 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) rapidly released DCF staining. Experiment was repeated 5 times with similar results, and a representative myocyte is shown.

(Fig. 5*A*). To further illustrate this phenomenon, data were presented in a Stern-Volmer relationship: the ratio of DCF fluorescence in the absence and in the presence of MTR was plotted as a function of increasing quencher (MTR) concentrations (Fig. 5*B*), and the Stern-Volmer quenching constant ($6.9 \pm 0.2 \times 10^4\ M^{-1}$) was obtained. Similar quenching effects were observed in vivo (Fig. 5, *C* and *D*) when the addition of MTR to DCF-stained cells led to a rapid "redistribution" of DCF from a distinct mitochondrial pattern to a more homogeneous distribution (with quenching occurring faster if higher concentrations of MTR were added). When the excitation spectrum of one probe overlaps the emission spectrum of another, quenching of fluorescence may occur via a fluorescence resonance energy transfer (FRET) mechanism (9, 10). DCF emission peaks at 525 nm, and the MTR excitation spectrum overlaps significantly (MTR excitation is maximal at 578 nm). Therefore, the observed quenching likely is due to FRET between DCF and MTR within myocytes and indicates their colocalization in the same intracellular compartment.

DCF effects on mitochondria. Because the above data suggest mitochondria as a primary site for DCF accumulation, we addressed the ability of isolated mitochondria to accumulate DCF and to release the dye after disruption of the organelle's membrane potential. The mitochondria obtained were of good quality, with oxidation and phosphorylation tightly coupled and comparable to values obtained in other studies (6, 18). Isolated mitochondria avidly accumulated DCF and, similar to the live cells, released the probe after exposure to CCCP. Specifically, DCF-loaded mitochondria incubated with 50 μM CCCP for 10 min contained only

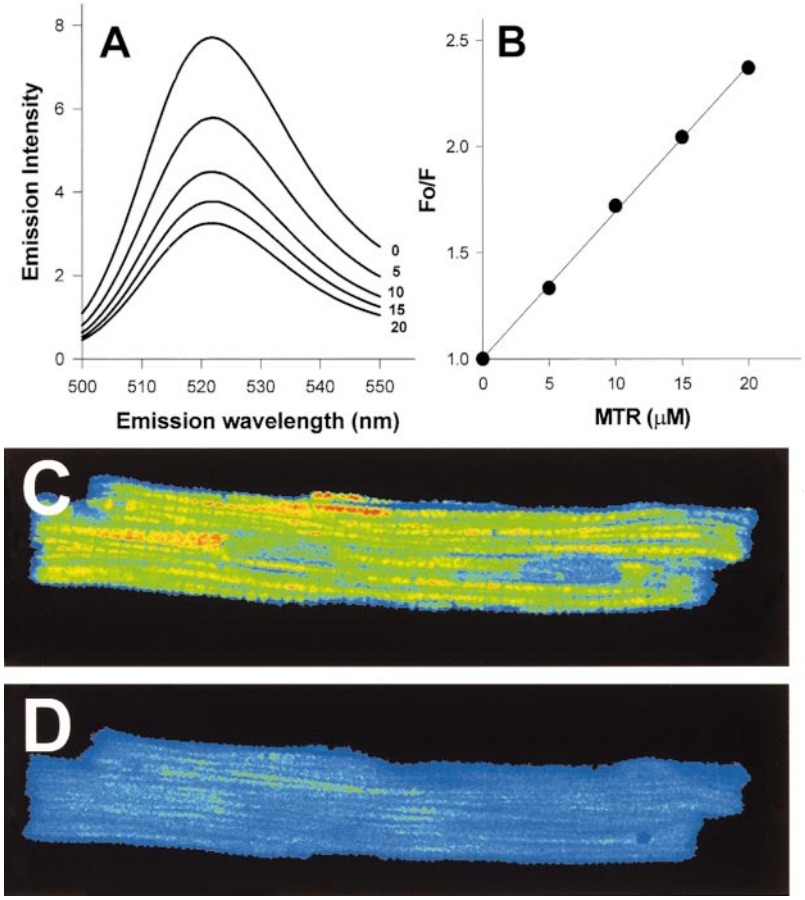

Fig. 5. Quenching of DCF staining by MTR in vitro and in situ. *A*: addition of MTR in μM concentrations (indicated at *right* of each emission curve) diminished intensity of DCF fluorescence (1 μM DCF in 50 mM phosphate buffer, pH 7.0) in a concentration-dependent manner. *B*: Stern-Volmer plot: ratio of DCF fluorescence in the absence (F_o) and in the presence (F) of MTR as a function of MTR concentration. *C*: cardiomyocyte loaded with 10 μM DCFH-DA and laser scanned to reveal DCF staining (image acquired using 515- to 530-nm emission range). *D*: same cell after addition of 5 μM MTR (using the same acquisition settings).

28.4 ± 9.5% (n = 3) of the probe compared with mitochondria subjected to the same washing procedures but without CCCP.

Avid accumulation of DCF by the mitochondria was also visualized when DCF was added to saponin-permeated myocytes. DCF by itself is not cell permeable; therefore, in the absence of saponin, black shadows of myocytes on a bright DCF background were seen. For one such experiment, representative traces of mean fluorescence intensity for random regions of interest are shown (Fig. 6*A*). Figure 6*B* displays the observation field 2 min after DCF addition (presence of 10 μM DCF in the incubation medium gives a purple color to the background). This time point was chosen to illustrate extracellular and intracellular DCF levels within the same image, since longer exposure leads to a further increase in DCF signal from the mitochondria (>30-fold accumulation), exceeding the dynamic range of the system to capture mitochondrial and the medium's DCF levels concurrently. After changing to a

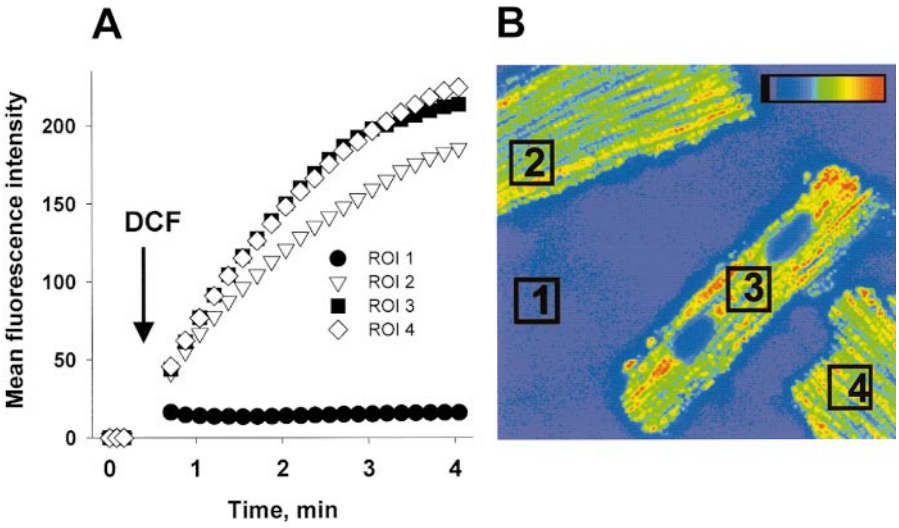

Fig. 6. DCF accumulation by saponin-permeated myocytes. Myocytes were placed in intracellular-like buffer and treated with 0.01% saponin. *A*: 10 μM DCF was then added, and mean fluorescence intensity of specified regions of interest (ROI) was measured (ROI 1 corresponds to the level of DCF in the medium). *B*: image taken 2 min after DCF addition. Longer incubations led to exceedingly high DCF signals from the intracellular ROI. Experiment was repeated 3 times with similar results.

probe-free medium, we observed that mitochondrial DCF levels of saponin-permeated cells remained quite stable, but fluorescence was immediately (within tens of seconds) diminished by the application of 100 μM CCCP.

Another set of experiments addressed DCF effects on mitochondrial respiration. High concentrations of DCF (100 μM) added to mitochondrial suspensions led to a significant inhibition of ADP-dependent respiration when succinate was used as a substrate (Fig. 7).

Localization of DCFH. The above experiments suggest that DCF accumulates mostly in mitochondria. However, the question about intracellular localization of its reduced precursor, nonfluorescent DCFH, still remains. Two alternatives can be suggested. The first alternative is that DCFH is oxidized to DCF in the cytosol, and DCF then enters the mitochondria. The second alternative is that DCFH accumulates in mitochondria where it is converted to DCF. We addressed DCFH localization by the following two experiments. The first experiment used DCFH prepared from DCFH-DA by mild hydrolysis (24). Saponin-permeated cardiomyocytes were placed in cytosol-like buffer containing either 10 μM DCF or 10 μM DCFH. Cells in DCF-containing buffer rapidly acquired fluorescence signals due to the accumulation of DCF in the mitochondria (similar to Fig. 6). In contrast, DCFH accumulation in saponin-treated cells was not visible. However, when the medium was replaced with probe-free buffer and cells were subjected to photooxidation to convert accumulated DCFH to DCF, similar levels of fluorescence intensity were reached compared with the cells in DCF buffer (as assessed by average fluorescence intensity for 6 randomly chosen cells from each treatment group: 114.7 ± 22.6 vs. 113.8 ± 6.3). This experiment suggests that mitochondria can accumulate DCFH to the same extent as DCF. In the second set of experiments, intact myocytes were loaded with cell-permeable DCFH-DA, washed by probe-free medium, and divided into two groups. The first group was treated with saponin (to release the cytosolic part of the probe) and was scanned with the laser beam to convert the remaining DCFH to fluorescent DCF (Fig. 8). The second group was not subjected to saponin but was scanned in the same way as the first group. The fluorescent intensities of cells from both groups rose to similar levels and had similar mitochondrial staining patterns. The fact that the release of cytosolic components before oxidation did not significantly affect mean fluorescence intensity after oxidation strongly suggests that the reduced form of the dye is mostly present in the mitochondria of cardiac myocytes.

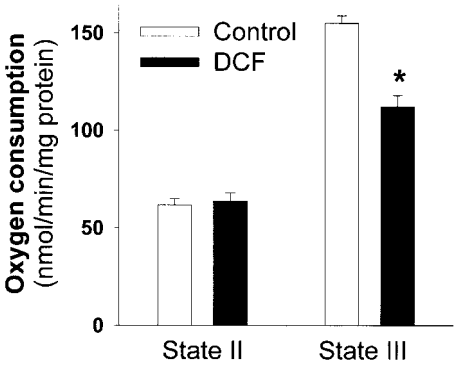

Fig. 7. Effect of DCF on mitochondrial respiration. Respiratory rate of isolated cardiac mitochondria in the absence and presence of 100 μM DCF. Respiration was measured in 2 ml buffer at 30°C in the presence of 5 μM rotenone in the basal state (*state II*) and in the ADP-stimulated state (*state III*). Values are means ± SE, $*P < 0.01$ for *state III*, $n = 6$ preparations.

DISCUSSION

ROS have been shown to be of major importance in heart ischemia-reperfusion injury, left ventricular hypertrophy, induction of preconditioning, cardiac arrhythmias, and drug cardiotoxicity (4, 5, 11, 12, 19, 25). Therefore, development and characterization of suitable fluorescent markers for oxidative stress, employable simultaneously with other fluorescent indicators (like calcium, pH, or cAMP probes), are extremely important. The present study evaluates DCFH as a fluorescent probe for intracellular oxidation in cardiac tissue. Two names, 2 7 -dichlorodihydrofluorescein and dichrolofluorescin (DCFH) have been used in the literature for this oxidant-sensitive probe. Cells and tissues are usually loaded with the esterified form, DCFH-DA. DCFH-DA is a stable lipid-soluble compound that can easily penetrate cell membranes and, subsequently, become deacetylated by intracellular esterases, forming a nonfluorescent product, DCFH. Although a small amount of DCFH-DA can penetrate cellular organelles (especially if high concentrations of DCFH-DA are used for loading), DCFH is currently considered to be a cytosolic probe (3, 8, 13, 30). It is a small negatively charged water-soluble molecule that can be oxidized to a highly fluorescent compound, DCF, by various ROS (7, 15, 30), peroxynitrite (3, 7, 13), intracellular oxidases, and peroxidases (15). Although several studies have aimed to establish the specificity of DCFH oxidation, it was concluded that it is nonspecific and that the appearance of a DCF-derived signal reflects an increase in overall ROS formation radicals (15, 30). In our previous study, this probe was used successfully to detect low levels of oxidative stress in situ when live cardiomyocytes were treated with the anticancer antibiotic doxorubicin (25). However, the confocal images obtained in that study posed the question of whether the augmentation of DCF fluorescence in close proximity to the mitochondria reflected an increase in ROS formation by these organelles or was an artifact of DCF staining. Mitochondrial localization is a well-known phenomenon for so-called cytosolic probes, including many Ca^{2+} indicators. However, when the origin of an increase in Ca-sensitive fluorescence is questionable, inhibitors of mitochondrial calcium transport are available to resolve these issues. Unfortunately, no such inhibitors exist to correctly interpret increases in DCF fluorescence, making it important to understand where this probe predominantly resides. The present study addresses this question and further characterizes the

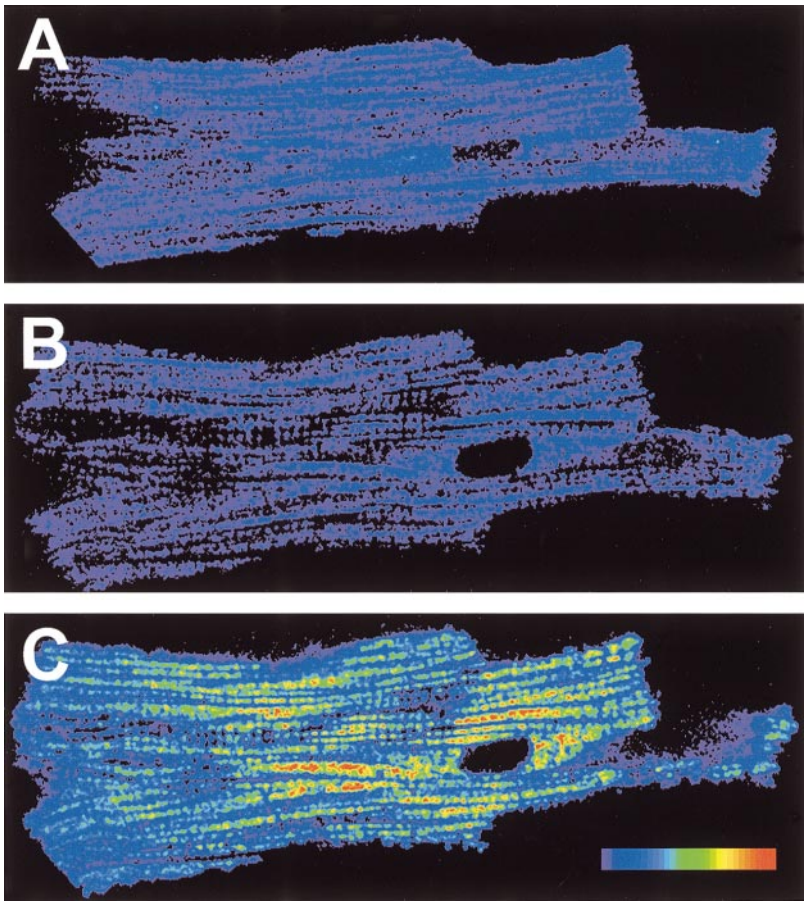

Fig. 8. Intracellular localization of DCFH. *A*: DCFH-loaded myocytes after small amount of DCFH was oxidized by low-intensity laser exposure to reveal its staining pattern. *B*: same cell after 1 min incubation with 0.01% saponin. A slight widening of the cells, typical for saponin treatment, is seen as well as release of the probe's cytosolic component. *C*: subsequent oxidation by laser reveals high levels of oxidized probe in cardiac mitochondria, suggesting that the major portion of DCFH is not released by the saponin treatment.

probe's behavior in cardiomyocytes isolated from adult rats.

Several factors make DCFH an appealing dye to monitor oxidative stress in cardiac tissue: *1*) steady intracellular levels of DCFH can be achieved rapidly by myocyte incubation with the diacetate form of the probe (Fig. 1*A*); *2*) intracellular levels of the probe remain constant for at least 1 h after cell transfer to a probe-free medium (Fig. 1*B*); *3*) the baseline oxidation levels of the probe in quiescent (nonstimulated) cells are very low (<3% of total probe amount); *4*) oxidation of DCFH to DCF can be induced by extracellular application of H_2O_2 in a concentration-dependent manner (data not shown); and *5*) the achieved levels of DCF stay constant for at least 1 h. Excellent retention of the probe by cardiomyocytes is in sharp contrast to leakage of DCF and DCFH from bovine aortic endothelial cells (24) and human glomerular endothelial cells (Fig. 2). These findings stress that experiments with DCFH/DCF require knowledge of probe behavior in the specific cell or tissue type to be used. The reason for this specificity may be accumulation of the probe in cell organelles, as discussed below.

When DCFH-loaded cells were incubated with H_2O_2, treated with doxorubicin (25), or exposed to laser illumination (resulting in photooxidation of DCFH to DCF), a distinct intracellular staining pattern was observed (Fig. 3). It was quite different from the pattern observed with many cytosolic probes, such as fluo-3, a calcium indicator (Fig. 3*A*), and was identical to the mitochondrial staining pattern of rhodamine 123 (25) or MTR (Fig. 3*C*). Neither shortening of the loading time to 5 min, lowering dye concentration to 2 μM, nor altering the loading temperature significantly changed the staining pattern. Moreover, addition of verapamil to the loading solution, which prevents extrusion of the AM form of dyes from the cytosol before they can be hydrolyzed (8, 20), did not have any effect on the dye distribution.

The observed effect cannot be explained by the pH sensitivity of DCF, which (similar to other fluorescein-based probes) has a higher quantum yield at more alkaline pH (23). The fluorescence intensity of 1 μM DCF in solution increased <10% when the pH was raised from 6.5 to 8.0, which are estimates of the lowest cytosolic and mitochondrial pH values, respectively. Thus pH sensitivity cannot account for the bright mitochondrial staining observed in DCF-stained cells.

Another possibility is that, contrary to common belief, DCF is not a cytosolic probe but preferentially stains the mitochondria in cardiac myocytes. Indeed, the DCF staining pattern was not changed by sarcolemmal permeabilization with saponin, which releases cytosolic dyes. The observed quenching of DCF by the mitochondrial probe MTR (Fig. 5) can occur only if the two fluorophores are separated by <100 Å (9), which

requires colocalization of DCF and MTR in the mitochondria. Moreover, our experiments with mitochondria isolated from rat hearts revealed that they rapidly accumulate the probe. Addition of CCCP, a well-know protonophore, led to the rapid release of DCF either from mitochondria or from isolated cells (Fig. 4), suggesting that the probe's binding could be affected by either the mitochondrial membrane potential, the proton gradient, or both. Mitochondria have several carriers to support anion transport into the organelle. Therefore, because DCF is an organic anion, it could inhibit mitochondrial respiration by competing with another anion transport system. This was tested in the experiments with isolated mitochondria in which DCF significantly reduced succinate-linked mitochondrial respiration (Fig. 7). These data indicate that DCF may limit the availability of succinate, implicating the dicarboxylate transporter as a means of mitochondrial transport of DCF. This is similar to fluorescein, which has been shown to compete with this anion carrier in rat isolated proximal tubular cells (16, 17). The fact that high micromolar concentrations of DCF can impact mitochondrial function also calls for caution when one selects the concentration of DCFH-DA to be employed (22).

Low mitochondrial potentials (2) were used to explain unusually high retention rates of rhodamine 123 in cardiac muscle cells (27) compared with other cell types. It cannot be excluded that such specific properties are also responsible for the observed ability of cardiomyocytes to accumulate and retain DCF and DCFH. However, a closer examination of the confocal images of endothelial cells loaded with DCFH-DA also reveals mitochondria-like structures, which may be less apparent in this and other cell types due to their low mitochondria content. The large quantity of mitochondria in ventricular myocytes, which encompassed 30% of total cell volume in contrast to the cytosol, which occupies only 10% of total cell volume (26), can be a reason for the cardiomyocyte prominent DCF staining pattern and the probe's retention. Indeed our experiments with saponin-permeated myocytes, which demonstrated a high rate of DCF accumulation by the mitochondria, also revealed steady levels of mitochondrial DCF staining after removal of extracellular DCF. Addition of CCCP to such permeated cells immediately (within tens of seconds) diminished DCF fluorescence. On the other hand, when DCF-stained intact myocytes were treated with CCCP to disrupt the mitochondrial potential, the probe was found in the extracellular space within several minutes (Fig. 4). Together, these observations suggest that, once released from the mitochondria to the cytosol, DCF readily escapes through the sarcolemma, similar to its ability to penetrate the plasma membrane of endothelial cells (30). Based on our data, we therefore hypothesize that cells with fewer mitochondria retain the probe to a lesser extent; that is, DCF retention is proportional to the mitochondrial volume.

The fact that DCFH localizes predominantly in cardiac mitochondria makes it an excellent marker for ROS produced by these cellular organelles. However, this does not exclude the possibility that DCFH also reports ROS formed extracellularly or in the cytosol. For example, H_2O_2, an ultimate product of superoxide dismutation, is highly permeable and can oxidize mitochondrial DCFH, regardless of its initial location. The question remains whether intracellularly formed H_2O_2 can exceed the antioxidant capacity of the cytosol and reach the mitochondria during anoxia-reoxygenation (12), drug administration (25), or other experiments that simulate pathophysiological conditions (4, 7, 11). Although cardiac muscle is relatively low in antioxidant enzyme activities (14), they still would be expected to diminish the ability of DCFH to report ROS formed outside the mitochondria.

It is concluded that DCFH and DCF localize mainly in the mitochondria and not in the cytosol of cardiac myocytes. Consequently, the distinct mitochondrial pattern of DCF staining in cardiomyocytes subjected to an oxidative stress cannot be attributed solely to ROS formation in these organelles but is also a result of the probe's localization. Nevertheless, due to the cytosol's scavenging capacity, DCFH is likely to be more affected by the ROS formed in the mitochondria.

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Address for reprint requests and other correspondence: N. Sarvazyan, Dept. of Physiology, Texas Tech University Health Sciences Center, 3601 4th St., Lubbock, TX 79430 (E-mail: phynas@ttuhsc.edu).

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