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# THE USE OF FLUORESCENT PROBES TO ASSESS OXIDATIVE PROCESSES IN ISOLATED-PERFUSED RAT HEART TISSUE

JAMES P. KEHRER and THOMAS PARAIDATHATHU

*Division of Pharmacology and Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, TX 78712-1074, U.S.A.*

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The formation of reactive oxygen species (ROS) in intact heart tissue has been assessed by direct ESR measurements, and indirectly by the formation of characteristic tissue products and the protective effects of various antioxidants. The development of lipid soluble esters of compounds which can be trapped intracellularly after hydrolysis, and which fluoresce after oxidation, has provided a new tool to investigate ROS *in vitro*. The utility of 2′,7′-dichlorofluorescin diacetate (DCFDA) in isolated-perfused rat heart tissue was investigated in the present study. DCFDA and its deacetylated form were incubated with various levels of hydrogen peroxide or *t*-butylhydroperoxide (*t*BOOH). Conversion of the diacetate form to a fluorescent product required 4–5 h with hydrogen peroxide and up to 24 h with *t*BOOH. In contrast, the deacetylated form fluoresced at 80% of maximum levels 1 h after the addition of 100 mM *t*BOOH. DCFDA was loaded into heart tissue by infusing for 10 min at a final concentration of 10 $\mu$M in Krebs-Henseleit bicarbonate buffer. After a 10 min washout period, analysis of freeze-clamped heart tissue revealed that the trapped material was readily converted to a fluorescent product by *t*BOOH, indicating hydrolysis had occurred. Fluorescence of material trapped in heart tissue was approximately 24% of the maximum achieved after oxidation with 100 mM *t*BOOH. This value decreased to 18 and 13% when the loading and washout periods were from 0 to 20 or 10 to 30 min of hypoxia, respectively. Similar results were obtained with the less readily oxidized dicarboxy derivative of DCFDA. Infusion of 500 $\mu$M *t*BOOH increased the oxidation of DCFDA in heart tissue from 24 to 31%. These data demonstrate that DCFDA can be loaded into heart tissue and is capable of reflecting relative changes in the oxidative state of this organ.

KEY WORDS: Fluorescent probes, oxidative, heart.

## INTRODUCTION

Numerous electron spin resonance studies have reported that reactive oxygen species (ROS) are generated both in oxygen deficient and reperfused tissues.[1] Although a number of these studies have been criticized for deficiencies which may have lead to artifactual measurements of free radicals, the preponderance of evidence suggests ROS are produced in heart tissue under a variety of conditions. However, the mere presence of ROS does not mean that they interact with tissues in any detrimental fashion. Several investigators have studied this question by looking for changes in tissue molecules which would reflect oxidative injury.[2] The results of these studies have generally revealed changes consistent with oxidative processes during oxygen (or energy) deprivation, and also at times after oxygen is returned to this tissue. However, the techniques used to assess oxidative changes are not ideal, and are all prone to alternative explanations. Our findings that changes consistent with an oxidative stress occur in rat and rabbit heart tissue during hypoxia, but are not exacerbated by

reoxygenation,[3,4] are consistent with a role for ROS in the overall process of hypoxia/reperfusion damage. However, it is also possible that these changes are simply secondary events associated with a diminished capacity of the heart to maintain proper redox status. This latter deficiency (which is probably related to a decreased energy status) and not increases in ROS production may, therefore, be the critical determinant of the extent of oxidative changes measured in hypoxic tissue.

Most support of a role for ROS in reperfusion injury has relied on the protection achieved with various antioxidants. However, there are an equal number of studies which fail to show such protection[2] and, as experimental approaches to measuring tissue injury following reperfusion have been refined, the role for ROS in this injury seems to have diminshed.[5] This has been particularly true for studies on heart necrosis where early work using tetrazolium stains to define zones of infarct have been found to be prone to artifactual results.[2] Superoxide dismutase (and possibly other antioxidants) causes tissue which is necrotic when examined by histology to retain its ability to reduce tetrazolium salts.[6] Nevertheless, most researchers now agree that ROS are an important factor in myocardial stunning (defined as the mechanical dysfunction that persists upon reperfusion despite the absence of permanent damage)[7] and are likely to be a factor in reperfusion-induced arrhythmias.[8]

A relatively new approach to studying the formation of ROS in biological systems involves using one of several compounds that, when oxidized, exhibit intense fluorescence.[9-12] Such compounds appear to be quite useful in cell culture and other *in vitro* systems. The purpose of the present study was to assess their utility in an intact organ such as the isolated-perfused rat heart.



## MATERIALS AND METHODS

### *Animals and Perfusion Apparatus*

Male rats (Sprague-Dawley, 180 to 250 g) were obtained from Harlan Sprague Dawley (Houston, TX). The animals (unfasted) were anesthetized with ether and dosed with 150 IU heparin through the inferior vena cava. Hearts were rapidly removed and placed into ice-cold Krebs–Henseleit bicarbonate solution containing 2.5 mM $CaCl_2$, 115 mM NaCl, 25 mM $NaHCO_3$, 5.9 mM KCl, 1.18 mM $MgCl_2 \cdot 6H_2O$, 1.23 mM $NaH_2PO_4$, 1.2 mM $Na_2SO_4$ and 10 mM glucose. Hearts were retrogradely perfused with the same solution in a non-recirculating apparatus with a constant pressure of 80 cm water as previously described.[4] The perfusion medium was equilibrated at 36°C (coronary effluent temperature) with 95% $O_2$, 5% $CO_2$, and all hearts were allowed to stabilize for 30 min with this medium before treatments. Hypoxic perfusions (without any ischemia) were performed by equilibrating the perfusion medium with 95% $N_2$, 5% $CO_2$. Equilibration and warming of the medium was achieved with an oxygenator device containing 10 m of gas-permeable silicon tubing (wall thickness 0.024 cm).

Samples of the coronary effluent were collected at 1 min intervals for determination of coronary flow rates, content of fluorescent material, and lactate dehydrogenase (LDH) release. LDH activity, at 30°C, was determined spectrophotometrically at 340 nm in 100 mM triethanolamine HCl buffer, pH 7.6, containing 0.15 mM NADH, 1 mM EDTA, and 1.5 mM pyruvate.

*Chemicals*

$NAD^+$, pyruvate, glucose, and *t*-butyl hydroperoxide (*t*BOOH) were obtained from Sigma Chemical Co. (St. Louis, MO). 2′,7′-Dichlorofluorescin diacetate (DCFDA) and its deacetylated and oxidized analog 2′,7′-dichlorofluorescein (DCF) were obtained from Eastman Kodak (Rochester, NY). 5-(and 6)-Carboxy-2′,7′dichlorofluorescin diacetate (DCDCFDA) was obtained from Molecular Probes (Eugene, OR). All other chemicals were reagent or spectrophotometric grade.

*Assay Procedure*

DCFDA was dissolved in dimethyl sulfoxide and DCDCFDA in dimethyl formamide. These compounds were infused into the perfusion medium immediately prior to the heart at a final concentration of 10 $\mu$M. Final vehicle concentrations in the perfusion medium were $\approx$0.25%. Infusions took place for 10 min and hearts were then perfused for an additional 10 min with oxygenated or hypoxic Krebs–Henseleit medium to allow deacetylation inside heart cells and to washout residual material. Coronary effluent was collected for 1 min intervals throughout the entire infusion and washout periods.

Hearts were freeze-clamped in liquid nitrogen at the end of each experimental period. Frozen heart tissue was pulverized under liquid nitrogen with a mortar and pestle. The tissue powder was homogenized in 8 ml of Krebs–Henseleit buffer, pH 7.4, for 5 s at 60% maximum using a Tekmar Tissuemizer. The homogenate was then centrifuged for 30 s at maximum speed (14 000 $\times$ *g*) in a microfuge. The supernatant was placed on ice and then the fluorescence determined using an Hitachi F2000 spectrofluorometer. Excitation and emission wavelengths for DCF and 5-(and 6)-carboxy-2′7′dichlorofluorescein (DCDCF) were, respectively, 490–525 nm and 504–529 nm.

2′,7′-Dichlorofluorescin (DCFH) and 5-(and 6)-carboxy2′,7′ dichlorofluorescin (DCDCFH) are not commercially available since they are rapidly oxidized to their fluorescent forms. Preparing the deacetylated species by alkaline hydrolysis of the ester linkage according to the method described previously[9] did not yield material which could be oxidized to a fluorescent form. This may have been due to the breakdown of parent material as a similar effect was observed when attempts were made to oxidize DCFDA in samples treated with perchloric acid. As an alternative, 4 ml of 10 $\mu$M DCFDA or DCDCFDA solutions were mixed with 20 $\mu$l of the 5000 $\times$ *g* supernatant fraction of a 1 : 5 (w/v) rat liver homogenate. After 10 min, 1 mM (final concentration) sodium azide was added to inhibit catalase and this solution was then immediately used to assess the ability of $H_2O_2$ and tBOOH to oxidize DCFH or DCDCFH.

Data were analyzed by one-way analysis of variance. Where significance was indicated, *post hoc* analyses were performed using Student Newman Keul's test. A *p* value less than 0.05 was considered significant.

## RESULTS

The diacetate forms of the fluorescin derivatives used in this study were relatively resistant to oxidation (Figure 1). Four hours were required to reach maximal fluorescence when DCFDA was oxidized with $H_2O_2$ and 24 h was needed with *t*BOOH.

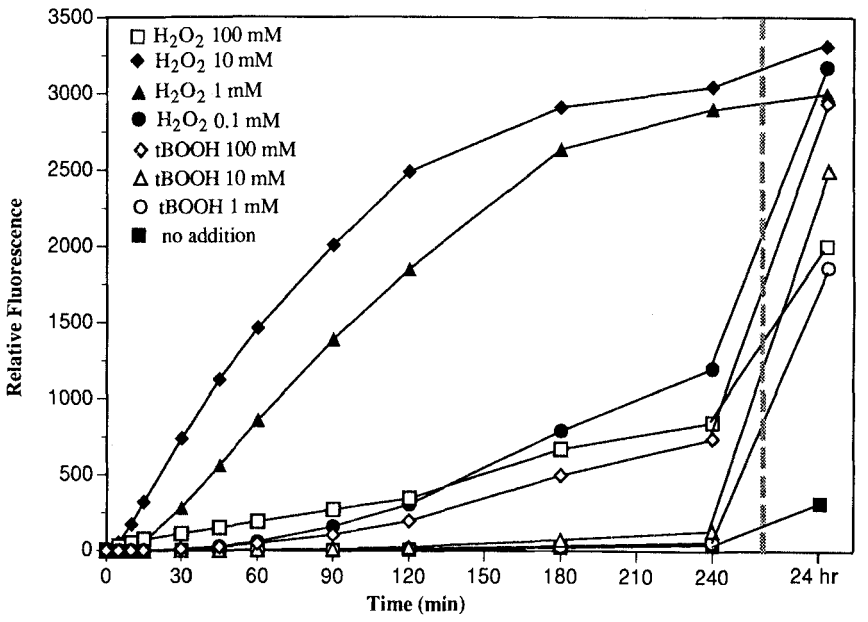


FIGURE 1 Time-dependent changes in the fluorescence of DCFDA incubated with different concentrations of $H_2O_2$ or *t*BOOH. DCFDA was dissolved in DMSO and added to Krebs–Henseleit buffer (pH 7.4) at a final concentration of 10 $\mu$M. Fluoresence measurements were taken with a Hitachi F2000 spectrofluorometer at the 400 V sensitivity setting. Maximum fluoresence under these conditions is around 3000 relative fluorescence units.

A similar pattern was seen with DCDCFDA except maximum fluorescence required 5 h after 1 or 10 mM $H_2O_2$ (data not shown). The $H_2O_2$- and *t*BOOH-dependent increases in fluorescence observed in this experiment are likely caused by the oxidation of these compounds following oxidative cleavage of the diacetate moiety. The lower maximum fluorescence in the presence of 100 mM $H_2O_2$ is probably due to oxidative destruction of the parent material.

In contrast to results with the diacetate forms, the hydrolyzed forms were rapidly oxidized with *t*BOOH and exhibited intense fluorescence after 30 to 60 min (Table I). DCFH was oxidized more rapidly than DCDCFH. $H_2O_2$ was a somewhat less effective oxidant, perhaps again due to oxidative destruction of the compounds. The gradual increase in fluorescence observed without adding any oxidant to DCFH or DCDCFH is likely due to autooxidation subsequent to hydrolysis.

The *t*BOOH-mediated oxidation of the DCFH and DCDCFH produced by hydrolysis with liver enzymes was not dependent on the continued presence of liver enzymes. Addition of 100 mM *t*BOOH to trypsin-treated (1 mg/ml/10 min) or heat-denatured (100°C/10 min) samples resulted in 100 and 76 percent of the fluorescence obtained in control samples containing active liver enzymes.

The infusion of DCDCFDA or DCFDA into heart tissue at a final concentration of 10 $\mu$M for 10 min had no effect on LDH release or coronary flow (data not shown). Heart rates with both probes decreased about 20% during the 10 min washout period. As illustrated by the small changes in fluorescence in the coronary effluent from both

TABLE I
DCFH and DCDCFH fluorescence after exposure to oxidants[1]

| Time (min) | $H_2O_2$ (mM) | | | | | tBOOH (mM) | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 0.1 | 1 | 10 | 100 | 1 | 10 | 100 |
| **DCFH** | | | | | | | | |
| 0 | 17 | 22 | 26 | 43 | 104 | 20 | 22 | 30 |
| 5 | 22 | 57 | 145 | 334 | 598 | 30 | 62 | 451 |
| 10 | 32 | 101 | 276 | 674 | 739 | 47 | 207 | 1306 |
| 15 | 44 | 146 | 415 | 937 | 867 | 68 | 440 | 1845 |
| 30 | 86 | 247 | 732 | 1318 | 1062 | 146 | 1070 | 2495 |
| 60 | 205 | 471 | 1323 | 1802 | 1371 | 395 | 2189 | 3055 |
| **DCDCFH** | | | | | | | | |
| 0 | 1.1 | 1.6 | 2.1 | 6.0 | 20 | 1.4 | 1.7 | 4.8 |
| 10 | 2.9 | 17 | 75 | 330 | 282 | 6.0 | 72 | 410 |
| 15 | 3.8 | 34 | 146 | 528 | 394 | 15 | 233 | 842 |
| 30 | 7.9 | 71 | 294 | 779 | 468 | 56 | 642 | 1358 |

[1]Data are expressed as relative fluorescence units. The deacetylated forms were prepared by enzymatic hydrolysis (see Methods) and were present at a final concentration of 10 $\mu$M. The maximum possible fluorescence under these conditions was approximately 3000 for DCFH and 2300 for DCDCFH.

oxygenated and hypoxic perfusions, little oxidation of DCFDA occurred extracellularly or in the collecting tubes prior to analysis (Table II). There appeared to be slightly less oxidation in the hypoxic effluent, but in both cases fluorescence was less than 1 percent of the maximum achievable. Furthermore, the material in the effluent was less than 3 percent oxidized 1 h after adding 100 mM *t*BOOH indicating that little hydrolysis of the ester linkage occurred extracellularly. In contrast, the material in freeze-clamped heart tissue was 80% oxidized by *t*BOOH within 1 h (Table II) suggesting complete hydrolysis of the ester linkage.

The oxidation of DCFDA was assessed in normoxic and hypoxic isolated perfused rat heart tissue. Compared to the maximum possible fluorescence obtained after the addition of 100 mM *t*BOOH, approximately twenty four percent of the DCFH trapped in oxygenated heart tissue was oxidized at the end of the perfusion period (Table III). Heart tissue infused with DCFDA during the first 10 min of hypoxia and analyzed after a subsequent 10 min hypoxic washout period exhibited only 18% oxidation of the trapped material. When the infusion of DCFDA occurred from 10 to 20 min of hypoxia, only 13% oxidation was observed after the washout period. Similar results were obtained in heart tissue infused with DCDCFDA with oxygenated exhibiting 19% and hypoxic (first 10 min) 8% oxidation of this probe (data not shown).

The infusion of 500 $\mu$M, but not 100 $\mu$M *t*BOOH, for 10 min followed by a 2 min washout period, increased the oxidation of DCFDA in heart tissue (Table III). In a preliminary study, 38 mM *t*BOOH produced complete oxidation of the probe, but was rapidly toxic to the heart (causing cessation of contractions with 5 min) and was therefore not used further. 500 $\mu$M *t*BOOH slowed the heart rate only slightly and 100 $\mu$M *t*BOOH had no effect on heart rate during the 10 min infusion. 500 $\mu$M *t*BOOH was incapable of increasing cardiac fluorescence with DCDCFDA above oxygenated control levels. This may be due to the poorer oxidizability of this probe

TABLE II
DCFDA fluorescence in perfused heart

| Time | Effluent | |
|---|---|---|
| | Oxygenated[1] | Hypoxia[1] |
| 1 | 0.4 | 0.3 |
| 2 | 1.0 | 1.1 |
| 3 | 1.8 | 1.3 |
| 4 | 2.5 | 1.5 |
| 5 | 3.1 | 1.8 |
| 6 | 3.9 | 3.0 |
| 7 | 4.7 | 2.7 |
| 8 | 4.9 | 2.9 |
| 9 | 5.3 | 3.5 |
| 10 | 5.6 | 3.6 |

| Time | Relative fluorescence in heart[2] | | |
|---|---|---|---|
| | O | 1 h | 24 h |
| Oxygenated | 297 ± 17 | 1077 ± 55 | 1243 ± 42 |
| Hypoxia | 152 ± 21 | 728 ± 49 | 910 ± 58 |

[1]Data are expressed as relative fluorescence in the effluent. Maximum fluorescence in the effluent rapidly increased with infusion to approximately 3000 fluorescence units with 10 $\mu$M DCFDA.

[2]Relative fluorescence was measured immediately after processing freeze-clamped heart tissue and 1 and 24 h after addition of 100 mM *t*BOOH to the same sample. Data are expressed as mean ± S.E. $n = 3$.

TABLE III
Cardiac fluorescence following infusion of DCFDA[1]

| Treatment | % DCFDA oxidized |
|---|---|
| Oxygen | 23.7 ± 1.6 (5) |
| Hypoxia–DCFDA 0 min | 17.6 ± 1.8 (4)* |
| Hypoxia–DCFDA 10 min | 12.8 ± 0.2 (3)* |
| *t*BOOH 100 $\mu$M | 24.5 ± 0.7 (3) |
| *t*BOOH 500 $\mu$M | 30.8 ± 0.9 (3)* |

[1]All hearts were subjected to a 30 min equilibration period with oxygenated media. DCFDA was infused at a concentration of 10 $\mu$M for 10 min during oxygenated perfusion (oxygen), beginning at the initiation of hypoxia (hypoxia–0 min), or 10 min after hypoxia was initated (hypoxia–10 min). Hearts were freeze-clamped after 10 min (oxygen and hypoxia) or 2 min (*t*BOOH) washout period. Data are expressed as the mean ± SE. Maximum fluorescence values for oxygenated and hypoxic hearts are shown in Table II.

*Significantly different from oxygenated hearts ($p < 0.05$).

as shown by the slower time course of oxidation *in vitro* and the diminished level of oxidation in heart tissue.

## DISCUSSION

Numerous cellular changes have been observed during hypoxia including activation of a plasma membrane bound phospholipase, decreased and increased[9] membrane fluidity, increased permeability of the plasma membrane, formation and rupture of surface blebs and decreased cytosolic pH. The relative importance of these changes in cell death has not been established and understanding the mechanism by which oxygen deprivation damages heart tissue remains an important area of research in order to develop effective countermeasures. Recently, the use of various new technologies, in particular fluorescent probes for various ions and intracellular processes, have begun to reveal in more detail some of the biochemical events which occur in response to oxygen deprivation.[10] The availability of compounds which fluoresce upon oxidation has provided a new tool to explore the role of ROS in various conditions. Previous studies have shown the utility of these probes in cell culture systems.[11-14] The data presented in the present study indicate that these probes may have some utility in detecting relative changes in ROS production in an intact organ.

DCFDA and DCDCFDA were hydrolyzed and trapped in myocytes following infusion via the isolated perfused heart. Both oxygenated and hypoxic hearts exhibited relatively high levels of trapped fluorescent material. The reasons for this is not known, and several possible artifacts may exist. Autoxidation is a potential contributor but, as shown in Table I, even at a DCFDA concentration producing twice the maximal fluorescence observed in heart tissue, autoxidation is responsible for only about 50 fluorescence units during a 20 min period corresponding to the infusion and washout times. Hypoxia will diminish autoxidation, but this is not sufficient to explain the 145 fluorescence unit difference between oxygenated and hypoxic hearts.

Another potential cause of fluorescence is the production of radicals during pulverization of freeze-clamped tissue which could oxidize trapped DCFH. Much lower levels of basal and maximal fluorescence were found if heart tissue was analyzed without freeze-clamping. However, fluorescence increased if homogenates were frozen and thawed without pulverization (unpublished data). Since the same treatment of DCFH alone did not increase fluorescence, it would appear that the oxidation of this probe is a function of biological processes, and not an artifact of the preparation procedure. The increased fluorescence in frozen then thawed samples may arise from rupturing a subcellular structure containing significant amounts of fluorescent material (perhaps mitochondria) which is otherwise lost during centrifugation steps.

Only minimal formation of ROS has been reported in isolated cells loaded with DCFDA.[12,13] This may be a consequence of the low respiratory activity (and thus ROS production) in cultured cells. In contrast, the large level of fluorescence seen in intact heart tissue loaded with DCFDA is suggestive of extensive ROS production. This may be a result of the highly active oxidative phosphorylation in this tissue required to supply the energy for cardiac contractile activity. Hypoxia (which will inhibit oxidative phosphorylation) decreased the formation of fluorescent material in heart, supporting this concept. Complete ablation of fluorescence was not achieved in hypoxic hearts since (1) some autooxidation undoubtedly occurred, (2) it took 10 min to reach minimal oxygen concentrations in the perfusate, and (3) even during maximal

hypoxia the medium contained approximately 10 $\mu$M oxygen. This level of oxygen, and the presence of glucose in the medium, provided sufficient energy to maintain cardiac contractile activity at a greatly slowed rate throughout the hypoxic period and may have resulted in the generation of sufficient ROS to explain the production of fluorescent material in hypoxic heart tissue.

Changes induced by ROS, either directly or subsequently to impaired cellular defense systems, remain an attractive hypothetical mechanism to explain hypoxic and/or ischemic tissue damage.[5,6,15,16] The data presented here showing an increase in the formation of fluorescent product(s) following infusion of nonfluorescent precursor molecules indicate that ROS are generated in aerobic and hypoxic heart tissue. However, in contrast to other studies which indicated an increased production of ROS under conditions of oxygen deprivation,[1,13,17,18] these data show that hypoxia decreases the production of ROS. This conclusion is supported by studies showing that hearts subjected to total global ischemia have no detectable inactivation of glyceraldehyde-3-phosphate dehydrogenase[19] [an enzyme susceptible to oxidative inactivation] and that chemiluminescence decreased during ischemia,[20] although conflicting data to this latter finding are available.[21] Together, these results suggest that the oxidative changes observed in our previous studies on hypoxic rat and rabbit heart tissue[3,4] are caused by an inability of an energy deficient tissue to maintain a normal redox balance.

In summary, the data presented here indicate that under oxygenated perfusion conditions sufficient ROS escape endogenous defense systems to react with exogenous materials infused into isolated heart tissue. It is difficult to draw quantitative conclusions about ROS production from these data because of confounding factors such as tissue anti- and prooxidant systems. However, hypoxia appears qualitatively to decrease the production of ROS while the direct oxidant *t*BOOH was able to increase intramyocardial oxidative stress. This effect of *t*BOOH was not evident at concentrations below 500 $\mu$M. Although even higher levels of *t*BOOH were needed to directly oxidize DCFH *in vitro*, it is possible that enzymatic reactions *in situ* facilitate the oxidation of the probe. The infusion of *t*BOOH has been shown previously to oxidize cardiac glutathione[22] in isolated-perfused tissue. The data presented here provide additional evidence that at high levels of *t*BOOH endogenous defense systems are unable to prevent interactions with cellular molecules.



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