Detection of intracellular superoxide formation in endothelial cells and intact tissues using dihydroethidium and an HPLC-based assay

Bruno Fink, Karine Laude, Louise McCann, Abdul Doughan, David G. Harrison, and Sergey Dikalov

Free Radicals in Medicine Core (FRIMCORE), Division of Cardiology, Department of Medicine, Emory University School of Medicine, and Atlanta Veterans Administration Hospital, Atlanta, Georgia 30322

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Fink, Bruno, Karine Laude, Louise McCann, Abdul Doughan, David G. Harrison, and Sergey Dikalov. Detection of intracellular superoxide formation in endothelial cells and intact tissues using dihydroethidium and an HPLC-based assay. Am J Physiol Cell Physiol 287: C895–C902, 2004. First published August 11, 2004; 10.1152/ajpcell.00028.2004.—Recently, it was demonstrated that superoxide oxidizes dihydroethidium to a specific fluorescent product (oxyethidium) that differs from ethidium by the presence of an additional oxygen atom in its molecular structure (Zhao H, Kalvedini S, Zhang H, Joseph J, Nithipatikom K, Vásquez-Vivar J, and Kalyanaraman B. Free Radic Biol Med 34: 1359–1368, 2003). We have adapted this new HPLC-based assay to quantify this product as a tool to estimate intracellular superoxide in intact tissues. Ethidium and oxyethidium were separated using a C-18 column and quantified using fluorescence detection. Initial cell-free experiments with potassium superoxide and xanthine oxidase confirmed the formation of oxyethidium from dihydroethidium. The formation of oxyethidium was inhibited by superoxide dismutase but not catalase and did not occur upon the addition of H2O2, peroxynitrite, or hypochlorous acid. In bovine aortic endothelial cells (BAEC) and murine aortas, the redox cycling drug menadione increased the formation of oxyethidium from dihydroethidium ninefold (0.4 nmol/mg in control vs. 3.6 nmol/mg with 20 μM menadione), and polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) significantly inhibited this effect. Treatment of BAEC with angiotensin II caused a twofold increase in oxyethidium formation, and this effect also was reduced by PEG-SOD (0.5 nmol/mg). In addition, in the aortas of mice with angiotensin II-induced hypertension and DOCA-salt hypertension, the formation of oxyethidium was increased in a manner corresponding to superoxide production estimated on the basis of cytochrome c reduction. Detection of oxyethidium using HPLC represents a new, convenient, quantitative method for the detection of superoxide in intact cells and tissues.

oxyethidium; hypertension; menadione; angiotensin II; endothelium

IT HAS BECOME EVIDENT that mammalian cells produce reactive oxygen species (ROS) and that these can serve as signaling molecules that modulate events such as enzyme phosphorylation, cell growth, hypertrophy, and programmed cell death. When produced in excessive amounts, ROS can contribute to cellular dysfunction (9). Overproduction of ROS has been implicated in diverse diseases such as cancer, hypertension, atherosclerosis, Alzheimer’s disease, lung injury, and aging (20).

The production of ROS is mediated by a variety of mammalian enzymes that are capable of reducing molecular oxygen. While occasional enzymes such as glucose oxidase and xanthine oxidase are capable of performing a two-electron reduction of oxygen to form hydrogen peroxide, the most common scenario is a one-electron reduction, leading to formation of superoxide (O2·−). O2·− can in turn serve as a progenitor for other ROS such as hydrogen peroxide, peroxynitrite, and the hydroxyl radical. In vascular cells, increased generation of O2·− has been suggested to occur in hypertension, hypercholesterolemia, diabetes, and heart failure (3). A major consequence of this is enhanced degradation of nitric oxide, leading to the formation of peroxynitrite. Thus the accurate detection and ability to quantify O2·− are critically important in understanding the pathogenesis of these various cardiovascular disorders and other noncardiovascular diseases.

Methods of detecting O2·− in intact tissues include various chemiluminescent techniques, the use of superoxide dismutase-inhibitable cytochrome c reduction, measurement of aconitase activity, and the use of fluorescent dyes (6). Several of these methods are controversial, others require special equipment, and still others provide only semiquantitative information (13). A particular problem is the measurement of intracellular O2·−, which is not detected by methods such as cytochrome c reduction but is likely important in a variety of pathological conditions. For example, it is likely that the vascular smooth muscle NADPH oxidases largely produce O2·− intracellularly.

Given these considerations, it would be highly desirable to develop a reproducible, easily adaptable method of quantifying intracellular O2·− in intact tissues. Recently, it was reported that dihydroethidium reacts with O2·− to form a specific product with a molecular weight 16 greater than that of ethidium (25), tentatively identified as oxyethidium. Oxyethidium can be readily separated from its parent dihydroethidium and ethidium by performing HPLC, and the resultant peak intensity of oxyethidium should reflect intracellular production of O2·−. In the present study, we demonstrate that dihydroethidium can be used in cultured endothelial cells and intact segments of murine aorta to detect intracellular O2·− using HPLC.

METHODS

Cell culture. BAEC (BioWhittaker, Walkersville, MD) were cultured in medium 199 containing 10% fetal calf serum supplemented with 2 mM L-glutamine, 1% vitamins, 20 g/ml streptomycin, and 20 U/ml penicillin. On the day before the study, the fetal calf serum concentration was reduced to 5%. Confluent BAEC from passages 4 and 5 were used for experiments. On the day of study, the cells were rinsed three times with 3 ml of chilled Krebs-HEPES buffer and then exposed to 25 μM dihydroethidium for 20 min at 37°C in Krebs-HEPES buffer containing 0.1% DMSO. Dihydroethidium was then washed from the cells to avoid...
absorption of any extracellular oxyethidium formed by autoxidation of dihydroethidium. Incubation in Krebs-HEPES buffer was then continued at 37°C for an additional 1 h. HPLC analysis showed that intracellular dihydroethidium was always present in excess and therefore was not a limiting factor in the formation of oxyethidium. The cells were then harvested for HPLC analysis by scraping and were placed in 300 μl of cold methanol, homogenized, and filtered (0.22 μm). When noted, mandelione, angiotensin II, and polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) were added 1 h before dihydroethidium.

**RESULTS**

**HPLC separation of ethidium and oxyethidium: influence of O$_2^-_•$ and other ROS.** It was recently reported that the reaction product of O$_2^-_•$ with dihydroethidium yields a fluorescent product different from ethidium, tentatively identified as oxyethidium. Using HPLC with fluorescence detection, we were able to confirm this finding. Authentic ethidium eluted at 16.2 min (Fig. 1A). Treatment of dihydroethidium with potassium superoxide resulted in the formation of a product that eluted at 14.4 min and could be clearly separated from ethidium using these HPLC conditions (Fig. 1, B and C) with a characteristic fluorescence spectrum identical to that previously described for oxyethidium (25). Formation of oxyethidium was dependent on the amount of potassium superoxide added (Fig. 1, C and D). In contrast, the ethidium peak was not affected by either the time or the concentration of O$_2^-_•$. Formation of the oxyethidium peak was abolished by the addition of PEG-SOD (100 U/ml) (Fig. 1F). Exposure of dihydroethidium to hydrogen peroxide or peroxynitrite caused no formation of oxyethidium from dihydroethidium (Fig. 1, G and H).

Previously, a low yield of dihydroethidium oxidation by O$_2^-_•$ was observed (1). We further studied the time course and yield of the reaction between O$_2^-_•$ and 25 μM dihydroethidium using xanthine oxidase and xanthine to generate O$_2^-_•$. In this reaction, oxyethidium increased in a time-dependent manner (Fig. 2), while the concentration of ethidium did not change. Calibration of oxyethidium formation by O$_2^-_•$ (Fig. 2) showed that accumulation of oxyethidium was 3.6-fold lower than the amount of O$_2^-_•$ produced by xanthine oxidase. This low yield of dihydroethidium oxidation by O$_2^-_•$ was previously attributed to dismutation of O$_2^-_•$ by a free radical intermediate of dihydroethidium formed in the reaction of dihydroethidium with O$_2^-_•$ (1). Our data support this concept and suggest that the formation of oxyethidium is not a one-step reaction but involves an ethidium free radical, which decreases the yield of oxyethidium (Scheme 1).

**Intracellular stability of oxyethidium and ethidium.** Despite extensive use of dihydroethidium oxidation as a marker of oxidative stress, little is known about its stability in a biological system. The recent discovery of oxyethidium as a specific reaction product of O$_2^-_•$ and dihydroethidium raised further questions regarding its in vivo stability. We therefore studied the intracellular metabolism of ethidium and oxyethidium by HPLC analysis of BAEC incubated with either ethidium or oxyethidium. Oxyethidium was prepared by 6-h incubation of 50 μM dihydroethidium with 1 mM hypoxanthine and 5 μM/ml xanthine oxidase, and completion of the reaction was monitored using HPLC. Endothelial cells were exposed to...
oxyethidium for 10–60 min and then analyzed using HPLC. After 10 min, the intracellular level of oxyethidium reached saturation and did not increase further over 60 min (Fig. 3). Of note, incubation of BAEC with oxyethidium did not produce...

Fig. 1. High-performance liquid chromatograms of dihydroethidium (DHE), ethidium, and oxyethidium (Oxy-E) formed by exposure of DHE by various oxidants. A: HPLC tracing of 1 μM ethidium. B: HPLC trace of 25 μM authentic DHE. C: elution profile of the reaction of 2 mg of potassium superoxide (KO₃⁻) to 25 μM DHE in 0.9% NaCl solution. D: same as C but using 4 mg of KO₃⁻. E: incubation of 25 μM DHE in 20 mM Krebs-HEPES buffer (pH 7.4) with 5 μM xanthine oxidase (XO) and 0.5 mM xanthine (X). F: same as D but in the presence of 100 U/ml polyethylene glycol-conjugated superoxide dismutase (PEG-SOD). G: incubation of 25 μM DHE with 10 mM H₂O₂. H: incubation of 25 μM DHE with 10 mM peroxynitrite (ONOO⁻).

HPLC traces in E–H were obtained 30 min after incubation with the respective agents. Evaluation of original HPLC tracings was performed after subtraction of water or methanol background spectra using 32 Karat HPLC software from Beckman Coulter.

Fig. 2. Time course of Oxy-E accumulation in DHE solution incubated with X/XO O₂⁻-generating system. DHE (25 μM) in 20 mM Krebs-HEPES buffer (pH 7.4) was incubated with 5 nM/ml XO and 0.5 mM X during continuous oxygenation without (○) or with (●) PEG-SOD (100 U/ml). Inset: calibration of Oxy-E formation from DHE. DHE in Krebs-HEPES buffer was incubated with XO (5 μM) and increasing concentrations of X (10–100 μM). Amount of O₂⁻ released in X/XO O₂⁻-generating system was measured by electron spin resonance using spin probe cyclic hydroxyamine 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (15).

Fig. 3. Intracellular metabolism of Oxy-E in cultured bovine aortic endothelial cells (BAEC). BAEC were incubated with 5 μM Oxy-E in 20 mM Krebs-HEPES buffer (pH 7.4) containing 0.5% DHE and 0.1% ethidium. Detection of DHE, ethidium, and Oxy-E was performed after harvesting of cultured BAEC as described in METHODS. Data are means ± SE (n = 4 experiments).
Table 1. Stability of ethidium and oxyethidium incubated with superoxide, ascorbate, glutathione, BAEC homogenate, and intact endothelial cells

<table>
<thead>
<tr>
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<th>DHE + O$_2^-$</th>
<th>E + KO$_2$</th>
<th>Oxy-E + Reducing Agents</th>
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<tr>
<td></td>
<td>3 µM</td>
<td>15 µM</td>
<td>30 µM</td>
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<tr>
<td>E</td>
<td>0.96</td>
<td>1.04</td>
<td>1.04</td>
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<tr>
<td>Oxy-E</td>
<td>0.84</td>
<td>5.12</td>
<td>9.98</td>
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Values are micromolar concentrations of ethidium or oxyethidium. Incubation of 25 µM dihydroethidium (DHE) in a xanthine/xanthine oxidase superoxide (O$_2^-$)-generating system evoked accumulation of oxyethidium but not ethidium. Treatment of 22 µM ethidium with 10 mM KO$_2$ did not cause production of oxyethidium. Incubation of 3.8 µM oxyethidium with 0.1 mM ascorbate, 1 mM glutathione, 4 mg/ml bovine aortic endothelial cell (BAEC) homogenate (Hom) in the absence or presence of 0.2 mM NADPH did not cause reduction of oxyethidium to ethidium. Analysis of BAEC loaded with 7.2 µM oxyethidium did not reveal significant intracellular reduction of oxyethidium during 60-min incubation. E, ethidium; Oxy-E, oxyethidium.

In additional experiments, endothelial cells were loaded with oxyethidium for 20 min, carefully washed and incubated for 5–60 min, and then analyzed using HPLC. It was found that the intracellular level of oxyethidium did not change over 60 min (Table 1). Incubation of oxyethidium with 0.1 mM ascorbate, 1 mM glutathione, or 4 mg/ml BAEC homogenate with or without 0.2 mM NADPH did not show significant reduction of oxyethidium. These studies indicate that oxyethidium is a stable product that is not reduced to dihydroethidium and does not lead to the formation of dihydroethidium derivatives such as ethidium (Scheme 1).

In additional experiments, we examined the stability of ethidium in endothelial cells. After the addition of 5 µM ethidium to cells, the intracellular concentration of ethidium continuously increased (Fig. 4). Thirty minutes after the addition of ethidium, the formation of oxyethidium was also observed (Fig. 4). The formation of oxyethidium in cells treated with ethidium was inhibited by 50% when cells were pretreated with PEG-SOD (data not shown). The direct formation of oxyethidium from the reaction of ethidium with O$_2^-$ is unlikely because incubation of ethidium with either xanthine/xanthine oxidase or potassium superoxide did not produce oxyethidium (Table 1). These data indicate that intracellular conversion of ethidium to oxyethidium may occur, likely via reduction of ethidium to dihydroethidium and a reaction of the latter with O$_2^-$ (Scheme 1). Although 60-min incubation of ethidium (3.3 µM) with 0.1 mM ascorbate, 1 mM glutathione, or BAEC homogenate with NADPH did not significantly affect the ethidium concentration (3.2, 3.1, and 3.1 µM, correspondingly), we cannot completely exclude formation of oxyethidium by the reaction of O$_2^-$ with one-electron reduced ethidium (Scheme 1). The concentration of ethidium, however, is much lower under normal experimental conditions and does not exceed 1 µM, minimizing the significance of these chemical reactions. Indeed, when endothelial cells were loaded with 1 µM ethidium, carefully washed, and incubated for 5–60 min, HPLC analysis indicated that intracellular ethidium was very stable (5 min, 1.1 µM; 30 min, 0.97 µM; 60 min, 0.97 µM) and did not reveal the formation of oxyethidium from ethidium.

Our data support stability and the absence of interconversion between ethidium and oxyethidium (Table 1 and Scheme 1). O$_2^-$ did not react with ethidium or oxyethidium, because the treatment of ethidium with either xanthine/xanthine oxidase or potassium superoxide did not produce oxyethidium. In addition, the reaction of dihydroethidium with O$_2^-$ linearly increased the concentration of oxyethidium but did not generate ethidium (Table 1). Our experiments with dihydroethidium-free oxyethidium did not show reduction by intact BAEC, BAEC homogenates (4 mg/ml), ascorbate, or glutathione (Table 1).

Increased conversion of dihydroethidium to oxyethidium in endothelial cells with stimulated O$_2^-$ production. The above data support the concept that the oxidation of dihydroethidium to oxyethidium can be used to quantify production of O$_2^-$ in studies of isolated enzymes and that oxyethidium remains stable once formed in endothelial cells. To determine whether the formation of oxyethidium can reflect elevated levels of intracellular O$_2^-$, endothelial cells in culture were incubated with 25 µM dihydroethidium for 20 min and the dihydroethidium was then removed and replaced with either control medium or medium containing the redox cycling agent (20 µM) menadione (14, 23) for 1 h. Subsequent HPLC analysis indicated that menadione increased oxyethidium formation ninefold while not altering ethidium levels (Fig. 5). The formation of oxyethidium in response to menadione was significantly inhibited by PEG-SOD (Fig. 5).

To determine whether dihydroethidium might be used to detect O$_2^-$ produced in response to a pathological stimulus, endothelial cells were studied using an identical protocol, except that the cells were exposed to 200 nM angiotensin II rather than menadione. Angiotensin II caused a twofold increase in oxyethidium formation (Fig. 6), and this increase was completely inhibited in cells pretreated for 1 h with PEG-SOD. This increase in oxyethidium formation is compatible with the

![Fig. 4. Intracellular metabolism of ethidium (E') in cultured BAEC. BAEC were incubated with 5 µM authentic ethidium in 20 mM Krebs-HEPES buffer (pH 7.4). Detection of DHE, ethidium, and Oxy-E was performed after harvesting of cultured BAEC as described in methods. E', ethidium; nd, not detectable. Data are means ± SE (n = 4 experiments).](http://ajpcell.physiology.org/content/287/10/A573/F1)
Previously, it was reported that the activation of ANG II also stimulated an increase in ethidium, which was observed previously using electron spin resonance (16). An- formation ($P$ pretreatment of endothelial cells with 100 U/ml PEG-SOD-blunted Oxy-E stimulated oxyethidium formation by 30% (Fig. 7, A and B)). Thus, unlike the situation with DCF-DA, in which augmentation of glutathione levels abolished the fluorescence signal, glutathione manipulation had only a modest effect on oxyethidium formation. The cause for a reduction in oxyethidium formation by NAC treatment may be due in part to reactions of glutathione with a dihydroethidium radical intermediate or may be due to an increase in direct thiol scavenging of $O_2^\cdot$ (5). The fact that depletion of glutathione caused only a minor change in basal oxyethidium formation suggests that reactions of intracellular glutathione with dihydroethidium radical intermediate (Fig. 7A) are not as significant as they are in the case of the DCF radical intermediate. It should be noted that the concentration of NAC used in these experiments has been shown to double intracellular glutathione (10), while buthionine sulfoximine in the concentration used depletes glutathione by 50% (10, 12).

Detection of intracellular $O_2^\cdot$ production in intact vascular segments. The above experiments demonstrate that the formation of oxyethidium can be used to detect $O_2^\cdot$ in cultured cells. An estimation of intracellular $O_2^\cdot$ production in intact tissues such as vascular segments would be highly desirable. To determine whether conversion of dihydroethidium to oxyethidium could serve this purpose, segments of murine aorta were exposed to dihydroethidium for 20 min. The dihydroethidium-containing buffer was then replaced with dihydroethidium-free Krebs-HEPES buffer and 20 µM menadione for 60 min. The vascular segments were then homogenized and analyzed using HPLC for the detection of dihydroethidium and its oxidized metabolites. Menadione increased levels of oxyethidium three- to fourfold in these intact vascular segments (Fig. 8). This increase in oxyethidium was inhibited by...
preincubation of vascular segments with 100 U/ml PEG-SOD for 1 h.

To determine whether the formation of oxyethidium might be used to detect O$_2^-$ in response to a known pathophysiological stimulus, we studied two models of hypertension associated with increased vascular O$_2^-$ production (16, 17). Vascular segments were removed from mice treated with chronic angiotensin II infusion (0.7 mg·kg$^{-1}$·day$^{-1}$ for 14 days) and studied as described above. Levels of oxyethidium were increased twofold in these vessels (Fig. 9A), corresponding to the increase in O$_2^-$ detected by cytochrome c reduction (Fig. 9C).

Angiotensin II is known to activate NADPH oxidase within vascular smooth muscle, adventitial, and endothelial cells and therefore produces an increase in O$_2^-$ production in all layers of the vascular wall (2, 7, 8). In some pathophysiological processes, the increase in O$_2^-$ is more localized. For example, in DOCA-salt hypertension in mice, there is a predominant increase in endothelial cell O$_2^-$ production due in large part to uncoupling of the endothelial nitric oxide synthase (17). We were interested in whether dihydroethidium might be used to detect an increase in O$_2^-$ production that was more localized to a specific cell type within the vessel wall. Mice were made to have DOCA-salt hypertension as previously described (16). After 14 days, their aortas were removed and studied as described above. HPLC analysis revealed a 2.5-fold increase in oxyethidium formation compared with controls from vessels after control (Fig. 9B). This increase was largely prevented if vessels were incubated with N$^\eta$-nitro-l-arginine methyl ester, in keeping with a role of uncoupled nitric oxide synthase as the predominant source of O$_2^-$ (Fig. 9B). In vessels from DOCA-salt-hypertensive mice, the percent increase in oxyethidium formation compared with controls was similar to the percent increase in O$_2^-$ production measured by cytochrome c reduction (Fig. 9C).

**DISCUSSION**

Despite the availability of a variety of different assays, the measurement of O$_2^-$ in intact cells and tissues remains very challenging (6). The work of Zhao et al. (25) demonstrated the formation of specific product in the reaction of dihydroethidium with O$_2^-$ (rate constant of 2.6 × 10$^5$ M$^{-1}$s$^{-1}$), previously assumed to be ethidium (13). This product has a distinct fluorescence emission spectrum and retention time during HPLC (25). It differs from ethidium by the presence of an additional oxygen atom in its molecular structure and therefore has been termed oxyethidium. In the present experiments, we have demonstrated that this product can readily be detected by HPLC and have defined conditions that permit its separation from ethidium. We have further found that the production of oxyethidium from dihydroethidium is increased in situations in which O$_2^-$ is increased in both cultured endothelial cells and intact vessels. Taken together, our results strongly suggest that this HPLC-based assay might provide a fairly straightforward approach to estimate rates of O$_2^-$ production in intact tissues.

An important requirement for a marker of radical production in vivo is that it remain stable throughout the duration of the assay. Our studies with authentic oxyethidium indicate that upon bolus administration to endothelial cells, this marker achieves a stable intracellular concentration within several minutes and remains at this level for 60 min (Table 1).
Furthermore, our data show no interconversion between ethidium and oxyethidium (Table 1 and Scheme 1). In additional experiments, we have found that oxyethidium remains stable in a methanol extract of cells and in vessels kept at −20°C for ≤1 mo (data not shown). This is advantageous because it permits analysis of samples by HPLC days or weeks after the experiments are performed.

In endothelial cells and vascular segments, a small amount of ethidium was also detected after incubation with dihydroethidium. This was never increased by manipulation to enhance O$_2^-$ production, nor was the signal reduced by PEG-SOD. Because ethidium is an oxidation product of dihydroethidium (Scheme 1), formation of ethidium might reflect the redox status of the cell rather than O$_2^-$ production per se. When cells were treated with 5 μM authentic ethidium, a small amount of oxyethidium was observed. We think that this was due to a reduction of ethidium to dihydroethidium and subsequent formation of oxyethidium by O$_2^-$ reaction with dihydroethidium, because we did not observe direct formation of oxyethidium upon exposure of ethidium to O$_2^-$ (Table 1), while formation of oxyethidium was inhibited by PEG-SOD. The concentration of ethidium, however, is much lower under normal experimental conditions and does not exceed 1 μM, minimizing the significance of these reactions. Indeed, experiments did not reveal the formation of oxyethidium in BAEC when the intracellular levels of ethidium were ≤0.1 μM.

As is evident in Fig. 9C, measurements of vascular O$_2^-$ production using oxyethidium formation and cytochrome c reduction paralleled one another in terms of the percent increase cause by either angiotensin II-induced hypertension or DOCA-salt hypertension. In cell-free experiments with known quantities of O$_2^-$, the formation of oxyethidium was 28% of the level of O$_2^-$ generation (Fig. 2). Given this information, the absolute values of O$_2^-$ estimated by oxyethidium formation exceed 3.6-fold those estimated by cytochrome c reduction. This discrepancy might reflect the fact that the sources of O$_2^-$ in these pathological conditions, the NAPDH oxidase and the endothelial nitric oxide synthase, largely release O$_2^-$ intracellularly and that the cytochrome c assay detects only extracellular O$_2^-$.

It is important to note that assignment of precise values to O$_2^-$ production is inherently inaccurate in using any assay because, as a result of competition with antioxidants such as superoxide dismutases, it is unlikely that all O$_2^-$ in a biological system reacts with the detecting probe. Nevertheless, the fact that the oxyethidium measurements provide data that are directionally similar to the well-validated cytochrome c assay supports the validity of the oxyethidium measurements. HPLC-based detection of oxyethidium has advantages over the cytochrome c assay in that it can be used to detect intracellular O$_2^-$, and the HPLC assays need not be performed immediately when the cells or vessels are being studied.

In summary, the current data demonstrate that HPLC-based analysis of cells and tissue homogenates provides a simple and accurate method of monitoring the conversion of dihydroethidium to oxyethidium, a reaction that reflects the rate of intracellular O$_2^-$ production. Oxyethidium is stable, and its formation from dihydroethidium is proportionate to the rate of superoxide production. Given that oxyethidium is not formed by other common oxidants, this assay could provide a “gold standard” for quantifying O$_2^-$ in intact tissues.

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Present address of B. Fink: Noxygen Science Transfer & Diagnostics, Ferdinand-Porsche-Str. 5/1, 79211 Denzlingen, Germany (E-mail: Bruno.Fink@noxygen.de).

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