Analysis of DHE-derived oxidation products by HPLC in the assessment of superoxide production and NADPH oxidase activity in vascular systems

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Fernandes DC, Wosniak J Jr, Pescatore LA, Bertoline MA, Liberman M, Laurindo FR, Santos CX. Analysis of DHE-derived oxidation products by HPLC in the assessment of superoxide production and NADPH oxidase activity in vascular systems. Am J Physiol Cell Physiol 292: C413–C422, 2007. First published September 13, 2006; doi:10.1152/ajpcell.00188.2006.—Dihydroethidium (DHE) is a widely used sensitive superoxide (O2•−) probe. However, DHE oxidation yields at least two fluorescent products, 2-hydroxyethidium (EOH), known to be more specific for O2•−, and the less-specific product ethidium. We validated HPLC methods to allow quantification of DHE products in usual vascular experimental situations. Studies in vitro showed that xanthine/xanthine oxidase, and to a lesser degree peroxynitrite/carbon dioxide system led to EOH and ethidium formation. Peroxidase/H2O2 but not H2O2 alone yielded ethidium as the main product. In vascular smooth muscle cells incubated with ANG II (100 nM, 4 h), we showed a 60% increase in EOH/DHE ratio, prevented by PEG-SOD or SOD1 overexpression. We further validated a novel DHE-based NADPH oxidase assay in vascular smooth muscle cell membrane fractions, showing that EOH was uniquely increased after ANG II. This assay was also adapted to a fluorescence microplate reader, providing results in line with HPLC results. In injured artery slices, shown to exhibit increased DHE-derived fluorescence at microscopy, there was a 1.5- to 2-fold increase in EOH/DHE and ethidium/DHE ratios after injury, and PEG-SOD inhibited only EOH formation. We found that the amount of ethidium product and EOH/ethidium ratios are influenced by factors such as cell density and ambient light. In addition, we indirectly disclosed potential roles of heme groups and peroxidase activity in ethidium generation. Thus HPLC analysis of DHE-derived oxidation products can improve assessment of O2•− production or NADPH oxidase activity in many vascular experimental studies.

hydroethidine; dihydroethidium; peroxidase; vascular smooth muscle cells; angioplasty

REACTIVE OXYGEN SPECIES (ROS) production in biological systems was initially approached mostly as an accidental pathological process occurring at high uncontrolled levels or as an extracellular burst at microbicidal micromolar levels in phagocytes (2). More recently, ROS generation has been characterized in many cell types as a nanomolar level, mainly intracellular, agonist-triggered, and enzyme-dependent physiological process (11, 26, 37). This distinct situation upgraded the challenge of accurately identifying and quantifying such transient intermediates. In vascular cells, superoxide radical (O2•−) generation, mainly due to NADPH oxidase isoforms (11, 15), is of great pathophysiological relevance and its quantification particularly problematic (16, 40). Contrary to spin traps, which react with O2•− through nucleophilic addition or combination, most other probes explore redox reactions of superoxide. In this context, the high reaction rates of O2•− with nitric oxide or superoxide dismutase (SOD) must be overcome by the use of high probe concentrations, which exacerbate many artifacts. Indeed, probes exploring the reductive chemistry of O2•−, such as cytochrome c, nitroblue tetrazolium, and particularly, lucigenin, are prone to redox cycling due to reaction of partially reduced probe with oxygen, leading to artificial superoxide generation (19). Other probes, such as luminol, coelenterazine, and 2-methyl-6-phenyl-3,7-dihydroimidazol(1,2-α)pyrazin-3-one explore the oxidative chemistry of O2•−, but are prone to poor specificity due to oxidation by other species. While the use of adequate controls can minimize such problems enough to allow comparative estimates of O2•−, its practical accurate quantification in vascular and other cells is essentially yet unavailable. Recently, dihydroethidium (hydroethidine, DHE) fluorescence has been used as a O2•− probe (5, 6, 14, 28, 43), with the rationale that 2-electron oxidation of membrane-permeable DHE by O2•− yields the fluorescent, DNA-binding membrane-impermeable compound ethidium (5, 6, 28). DHE oxidation in vitro is not subjected to artifactual superoxide recycling (31); is unaffected by reductants such as glutathione (14) and provides reasonable accurate O2•− detection in vivo (4). Indeed, DHE-derived fluorescence became a widely used test in vascular biology, both in cells and tissue slices (24, 28, 40). However, DHE may be oxidized by other oxidants such as H2O2 in the presence of heme proteins (30) and potentially by other reactive species, although not by peroxynitrite or H2O2 alone (14, 42). Moreover, studies (42, 43) using HPLC clearly demonstrated that DHE is oxidized by O2•− primarily to a compound different from ethidium, recently characterized as 2-hydroxyethidium (EOH). While the EOH product is more specific for O2•−, the ethidium product is not linearly related to O2•− concentration, at least because it is inducible by other oxidants and/or secondary to more complex pathways of DHE oxidation. Similarly to ethidium, EOH binds to DNA, forming a strong fluorescent complex displaying red fluorescence under rhodamine filter at microscopy (42, 43). Thus, total DHE-derived fluorescence represents the overlapping of oxidation products due to specific (e.g., O2•−) and nonspecific sources, e.g., heme proteins (30, 43). Consequently, the detection of EOH using HPLC fluorescence approach was proposed to
more accurately demonstrate O$_2^-$ production in vascular systems and initial validation studies have been undertaken (14, 43). The purpose of the present study was to report a broader and more systematic validation analysis of this technique under assay conditions typical of those used for studies of vascular redox signaling. Our studies include not only isolated vascular smooth muscle cells (VSMC) challenged with agonists of redox pathways, but also the novel validation of an NADPH oxidase activity assay in membrane fractions and analysis of vessel slices. Information on the usefulness and confounding/additional information provided by such techniques may have considerable implication regarding the practical accurate assessment of vascular superoxide.

**METHODS**

**Chemicals.** DHE was purchased from Invitrogen (Carlsbad, CA; catalog number D-116), xanthine and xanthine oxidase from Calbiochem (San Diego, CA), and H$_2$O$_2$ from Merck (Darmstadt, Germany). All other reagents, including DNA from calf thymus (catalog number D-8515), were from Sigma (St. Louis, MO). Peroxynitrite was synthesized from 0.6 M sodium nitrite and 0.65 M H$_2$O$_2$ in a quenched-flow reactor and its concentration was determined spectrophotometrically ($\epsilon_{302\text{nm}} = 1.670 \text{ M}^{-1}\text{cm}^{-1}$) (3). Dihydroethidium stock solutions (10 mM) were prepared in DMSO and stored under nitrogen in the dark at −20°C. All solutions were prepared with distilled water further purified in a Millipore Milli-Q system and treated with Chelex-100 before use.

**HPLC conditions of analysis.** Separation of DHE, EOH, and ethidium was performed as described previously (42) with the specified modifications. To assess a picture of possible interferents affecting overall DHE-derived fluorescence, we considered important to assess both EOH and ethidium products of DHE oxidation. Thus, while the optimal range of EOH detection is 570–580 nm (emission), 595 nm has been established as the optimal emission wavelength for both hydroxethidium and ethidium detection (42, 43). Chromatographic separation was carried out with the use of a NovaPak C$_{18}$ column (3.9 × 150 mm, 5 μm particle size) in a HPLC system (Waters) equipped with a rhodamine injector and photodiode array (W2996) and fluorescence (W2475) detectors. Solutions A (pure acetonitrile) and B (water/10% acetonitrile/0.1% trifluoroacetic acid) were used as a mobile phase at a flow rate of 0.4 ml/min. Runs were started with 0% solution A, increased linearly to 40% solution A during the initial 10 min, kept at this proportion for another 10 min, changed to 100% solution A for additional 5 min, and to 0% solution A for the final 10 min. DHE was monitored by ultraviolet absorption at 245 nm. EOH and ethidium were monitored by fluorescence detection with excitation 510 nm and emission 595 nm. Quantification was performed by comparison of integrated peak areas between the obtained and standard solutions under identical chromatographic conditions. EOH standard was prepared as previously described (42). Typically, 100 μM DHE was incubated with xanthine/xanthine oxidase (0.5 mM/0.05 U/ml) in PBS buffer composed of (in mM) 7.78 Na$_2$HPO$_4$, 2.20 KH$_2$PO$_4$, 140 NaCl, and 2.73 KCl, pH 7.4, containing 100 μM diethylenetriamine pentaacetic acid (PBS/DTPA) at 37°C for 30 min. EOH was separated by HPLC as described above, collected, and lyophilized to dryness. The purple-pink solid was further resuspended in DMSO and used as standard. EOH purity was confirmed by HPLC and stock concentration assessed spectrophotometrically ($\epsilon_{475\text{nm}} = 9.400 \text{ M}^{-1}\text{cm}^{-1}$) (44).

**DHE oxidation in vitro.** DHE oxidation was assessed after DHE (100 μM) incubation in phosphate buffer (100 mM, pH 7.4, DTPA 100 μM) with the following solutions: 1) H$_2$O$_2$ (1–5 mM) in the presence or not of Fe${^{2+}$/EDTA (0.5 mM/0.5 mM); 2) peroxynitrite (1 mM), added to DHE solution in absence or presence of sodium bicarbonate (25 mM); and 3) horseradish peroxidase/H$_2$O$_2$ (0.2 U/ml/5 mM). After 30 min (1) and (3) or 5 min (2) of incubation at room temperature and dim light, samples were kept on ice in the dark until analysis. Visible spectroscopic studies were recorded in a Beckman DU-640 spectrophotometer and HPLC analyses were performed as described above. Concentration of CO$_2$ was calculated from the added bicarbonate concentrations by using pK$_a$ 6.4 (7).

**Cell extract analysis by HPLC.** Rabbit VSMC were obtained from a previously established selection-immortalized line (10). Cells grown in 6-well dishes (well area of 9.6 cm$^2$) in F-12 medium supplemented with fetal bovine serum (10%), streptomycin (100 μM), and penicillin (100 U/ml) were stimulated or not with ANG II (100 nM) for 4 h. In some experiments, 3 h after ANG II stimulus, we added for the remaining 1 h, SOD conjugated to polyethylene glycol (PEG-SOD, 25 U/ml) or PEG alone, at a concentration corresponding to that of PEG-SOD (2.4 nmol/ml); or catalase conjugated to PEG (PEG-CAT, 200 U/ml) or PEG alone, at a concentration corresponding to that of PEG-CAT (0.8 nmol/ml). Cells were washed twice with PBS and incubated in PBS/DTPA (0.5 ml) at final DHE concentration of 50 μM (2.5 μl of DHE 10 mM stock solution) for additional 30 min. Of those VSMC were serum deprived only during the 30-min DHE incubation. Cells were washed twice with cold PBS, harvested in acetonitrile (0.5 ml/well), and centrifuged (12,000 g for 10 min at 4°C). It is important to point out that VSMC incubation for 30–45 min in PBS/DTPA did not promote cell detachment in our experimental conditions, although we observed partial cell detachment if we employed this methodology with primary culture of rabbit VSMC. In parallel experiments, we verified the effects of substitution of PBS/DTPA by Hanks’ buffer composed of (in mM) 1.3 CaCl$_2$, 0.8 MgSO$_4$, 5.4 KCl, 0.4 KH$_2$PO$_4$, 4.3 NaHCO$_3$, 137 NaCl, 0.3 Na$_2$HPO$_4$, and 5.6 glucose, pH 7.4, containing DTPA (100 μM). While Hanks’ buffer was able to prevent primary rabbit VSMC detachment, the EOH/DHE and ethidium/DHE ratios after DHE extraction in control and ANG II-stimulated cells were similar to those observed with PBS/DTPA (data not shown). All extractions were performed with acetonitrile, which was able to extract EOH from intact cells or isolated DNA more efficiently than methanol, whereas ethidium extraction was unchanged for the two solvents (data not shown). To maximize reproducibility, we extracted no more than two samples at a time. Simultaneous extraction of more samples appeared to add more variability in the results, and increased the risk of effects of light exposure (see below). Supernatants were dried under vacuum (Speed Vac Plus model SC-110A, Thermo Savant) and pellets maintained at −20°C in the dark until analysis. Samples were resuspended in 120 μl PBS/DTPA and injected (100 μl) into HPLC system. Simultaneous detection of DHE and its derived oxidation products (EOH and ethidium) using, respectively, ultraviolet and fluorescence detection, allowed the ideal situation of using DHE as an internal control during organic extraction of each sample. Thus, DHE-derived products were expressed as ratios of EOH and ethidium generated per DHE consumed (initial DHE concentration minus remaining DHE; EOH/DHE and ethidium/DHE). The overall baseline values obtained under optimal conditions with 50 μM DHE at 80% VSMC confluence were 180 ± 40 nmol EOH/μmol DHE and 200 ± 50 nmol ethidium/μmol DHE. In all protocols, the data were also normalized for protein levels or the number of cells. Results were analogous, although variability tended to be higher. Because of some confounding effects of light and sonication recently observed during DHE oxidation in vitro (21), we compared EOH and ethidium generation in VSMC acetonitrile extracts submitted or not to sonication or visible light exposure. In our system, sonication had no detectable effect in EOH and ethidium formation. However, after 10 min of exposure to ambient light (2 fluorescence 40-W tubular lamps, 1 to 1.5 m distance), extracts exhibited an almost 4-fold increase in EOH generation compared with extracts maintained in the dark or dim light. In our experiments, we avoided the interference of light by uniformly keeping all samples protected or under dim light throughout all steps of manipulation.
SODI overexpression in VSMC. The mammalian cell expression vector encoding SOD1 (pCMV with a c-myc tag) was kindly provided by Dr. Mariano Janiszewski, from the University of São Paulo School of Medicine. VSMC (3 × 10^6 cells) grown for 24 h in 6-well dishes were transiently transfected with 5 μg of SOD cDNA using Lipofectamine 2000 (10 μl; Invitrogen) according to the manufacturer’s instruction. Twenty-four hours after transfection, ANG II was added. Transfection efficiency was confirmed by Western blot analysis and increased SOD activity (see Fig. 3).

**NADPH oxidase assay in VSMC membrane fraction.** VSMC membrane homogenates were obtained by sequential centrifugation as described previously (22). Briefly, cells grown in 100-mm dishes were washed with cold PBS, harvested, homogenized in lysis buffer composed of 50 mM Tris, pH 7.4, containing 0.1 M EDTA, 0.1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonfyl fluoride, sonicated (10 s of 3 cycles at 8 W), and centrifuged (18,000 × g for 15 min) to separate mitochondria and nuclei. Supernatants were further centrifuged at 100,000 × g for 1 h to obtain a membrane-enriched fraction. Membrane fraction was used in three distinct assays: 1) NADPH oxidase activity by HPLC; 2) in a microplate reader (described below), and in 3) NADPH-stimulated oxygen uptake. For HPLC analysis, membrane fraction (20 μg of protein) was incubated with DHE (50 μM) at 37°C (final volume of 120 μl) in the dark for different time periods after the addition of NADPH (300 μM) in PBS/DTPA, in absence or presence of SOD (25 U/ml). The reaction was stopped on ice until HPLC injection (volume of 100 μl). Oxygen uptake studies with membrane fractions were performed using Clark-type oxygen electrode (Hansatech, Norfolk, UK) in a water-jacketed chamber containing 0.75 ml of PBS at 37°C, pH 7.4, without air above the buffer solution. The saturation oxygen concentration at this temperature was 210 μM (32). Briefly, after basal stabilization, membrane fraction (100 μg protein) was added to the buffer and changes in O2 concentration recorded continuously. Ten minutes after a flat baseline, NADH or NADPH (0.3–1 mM) was added to the chamber, followed after further 10 min by lucigenin or DHE, in several concentrations (0.05–1 mM). Oxygen consumption was followed for at least 60 min. No significant increase in oxygen uptake was detected after addition of VSMC membrane fraction, whether or not stimulated with ANG II (100 nM, 4 h). A negligible increase, barely distinguishable from basal noise, was detectable after further addition of NADH or NADPH. While up to 1 mM DHE addition essentially had no change in oxygen consumption, at the same experimental conditions 250 μM lucigenin addition showed oxygen consumption rates of 40 μM O2·min⁻¹·mg protein⁻¹, a value consistent with previously reported rates of lucigenin redox cycling in vascular tissue samples (19).

**DHE-derived fluorescence assay of NADPH oxidase in the microplate reader.** Membrane fraction (10 μg of protein) was incubated with DHE (10 μM) and DNA (1.25 μg/ml) in PBS/DTPA with the addition of NADPH (50 μM), at a final volume of 120 μl. Incubations were performed for 30 min at 37°C in the dark. Fluorescence was followed in a microplate reader using two different filters: 1) excitation 490 nm and emission 590 nm (same wavelength used in microscopy studies for rhodamine red fluorescence) and 2) excitation 490 nm and emission 570 nm (corresponding to that of acridine filter) in a spectrophluorometer (Wallac Victor2 1420-Multilabel Counter, PerkinElmer).

**VSMC heme quantification.** Fresh VSMC homogenates (~200 μg protein) were incubated with Drabkin solution [K2Fe(CN)6 600 μM/ KCN 770 μM/NaHCO3 12 mM] for 10 min at room temperature. Total heme [ferroheminochrome formation, Hm(CN)3] was estimated spectrophotometrically (εmaxnm = 1.13 × 10^4 M⁻¹·cm⁻¹) (12).

**Rabbit iliac artery injury model.** Iliac artery overdistension injury was performed in pentobarbital-anesthetized normolipemic male New Zealand White rabbits, as described previously (22, 23) using a coronary angioplasty-type balloon with diameter of 2.75 mm, inflated at 8.0 atm. After 14 days, the rabbits were euthanized with pentobarbital sodium and the lower abdominal aorta was gently perfused with PBS to remove remaining blood. Both the injured right and uninjured left iliac arteries were removed and cut into segments, which were used immediately for HPLC analysis, peroxidase activity, and total DHE-derived fluorescence.

**Tissue extract analysis by HPLC.** Iliac artery segments (~3 mm in length) were incubated in 0.5 ml of PBS/DTPA in the presence or absence of PEG-SOD or PEG-CAT for 15 min in a 1.5-ml Eppendorf vial. A volume of 2.5 μl of DHE 10 mM stock solution was added to the buffer to achieve final concentration of 50 μM, and a final DMSO concentration of 0.5% vol/vol. Incubation was carried out for 30 min at 37°C in the dark. Artery segments were washed in PBS, transferred to liquid nitrogen, and homogenized with mortar and pestle. The homogenate was resuspended in acetonitrile (0.5 ml), sonicated (3 cycles at 8 W for 10 s), and centrifuged (12,000 × g for 10 min at 4°C). Supernatants were processed similarly to those of cell extracts described above. Since repeated tissue extract injections led to increase in column pressure much more than cell extracts, probably due to hydrophobic compounds, exhaustive and repeated cleaning of HPLC column was required to prevent altered retention times. For fluorescence analysis, tissue sections (30 μm), incubated or not with PEG-SOD (25 U/ml) or azide (0.5%) for 15 min, were incubated with DHE (2 μM) at 37°C for further 20 min. Images were obtained with the use of a fluorescence microscope (Zeiss Axiovert 200M) with Axiosvision 3.0 software, equipped with rhodamine filter (excitation 546 nm, emission 590 nm). Parallel image acquisition of control and injured sections were performed with fixed parameters.

**Peroxidase activity.** VSMC (3–30 × 10⁶) grown in 150-mm dishes or 2-mm segments of iliac arteries were incubated with phosphate buffer (50 mM, pH 6.2), containing o-dianisidine (0.8 mM) and H2O2 (1.5 mM). In VSMC incubations, saponin (0.1%) was added to the buffer and dishes submitted to slight motion. The rate of substrate oxidation was monitored spectrophotometrically at 460 nm in 1.0-cm light path cuvettes for 30 min. One unit of peroxidase activity was defined as the amount of enzyme necessary to produce 1 μmol/min oxidized o-dianisidine (ε460nm = 1.13 × 10⁴ M⁻¹·cm⁻¹) at 25°C (9).

**Statistical analysis.** Values are expressed as means ± SE. Statistical comparisons were performed with Student’s t-test for unpaired data or one-way ANOVA, followed by Student-Newman-Keuls multiple-range test, at a 0.05 significant level using the Primer of Biostatistics program (14a).

**RESULTS AND DISCUSSION**

**In vitro studies of dihydroethidium oxidation.** The precise mechanisms underlying the formation of EOH, ethidium, and potentially other intermediates from DHE oxidation are still incompletely understood (14, 43). In addition, DHE oxidation by oxidants other than O2⁰⁻ may be relevant in vascular pathophysiological conditions has been only partially characterized (5–6, 14, 30, 31, 42, 43). Thus we first assessed whether DHE-derived fluorescent products can potentially be formed by such oxidizing systems. For this purpose, in vitro studies were performed using visible spectroscopy and HPLC fluorescence detection (Fig. 1). We confirmed that DHE was oxidized at significant rates by O2⁰⁻ generated by xanthine/xanthine oxidase system, producing EOH at high (~35%) yields (Table 1), in agreement with previously reported yields (14). Although we confirmed that DHE is not oxidized to EOH or ethidium either by H2O2 or peroxynitrite, our results showed that DHE was oxidized by peroxynitrite in the presence of CO2, and also by Fenton reagent (Fe2⁺-EDTA/H2O2) and peroxidase system (HRP/H2O2) (Fig. 1, Table 1). Both EOH and ethidium were formed by peroxynitrite/CO2 and Fenton
Dihydroethidium (DHE) oxidation in vitro. A: visible spectra obtained after DHE incubation with different oxidizing systems. DHE (100 μM) was incubated in 100 mM phosphate buffer [pH 7.4; 100 μM diethylenetriamine pentaacetic acid (DTPA)] with 1 mM H2O2, peroxynitrite (ONOO−, 1 mM) in the presence or absence of 2.5 mM CO2; 0.1 mM H2O2 in the presence of Fe2+/EDTA (Fe2+/EDTA/H2O2, 0.5 mM/0.5 mM/5 mM) and horseradish peroxidase in the presence of H2O2 (HRP/H2O2, 0.2 U/ml/5 mM). Incubation time was 5 min for peroxynitrite reactions and 30 min for all the others. B: HPLC chromatogram showing 2-hydroxyethidium (EOH) and ethidium (E) separation obtained by fluorescence detection (excitation 510 nm, emission 595 nm) for the same reactions. Chromatographic separation was as described in METHODS.

Table 1. Quantification of 2-hydroxyethidium and ethidium from dihydroethidium incubation with different oxidizing systems in vitro

<table>
<thead>
<tr>
<th>System</th>
<th>EOH, μM</th>
<th>Ethidium, μM</th>
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<tbody>
<tr>
<td>X/XO</td>
<td>34.1±1.2</td>
<td>ND*</td>
</tr>
<tr>
<td>H2O2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ONOO−</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ONOO−/CO2</td>
<td>5.3±1.6</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>Fe2+/EDTA/H2O2</td>
<td>12.3±2.2</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>HRP/H2O2</td>
<td>ND</td>
<td>2.0±0.1</td>
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</tbody>
</table>

Values are means ± SE; n = 4 experiments. ND, not detected; X/XO, xanthine/xanthine oxidase; HRP, horseradish peroxidase. Dihydroethidium (100 μM) was incubated in phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μM) with X/XO (0.5 mM/0.05 U/ml) H2O2 (1 mM), H2O2 in the presence of Fe2+/EDTA (Fe2+/EDTA/H2O2; 0.5 mM/0.5 mM), peroxynitrite (ONOO−; 1 mM) in the presence or absence of CO2 (2.5 mM); and HRP/H2O2 (0.2 U/ml/5 mM). Incubation time was 5 min for peroxynitrite reactions and 30 min for all others. Concentration of carbon dioxide was calculated from the added bicarbonate concentrations by using pK, 6.4 (8). *EOH < 1.5 μM and ethidium < 50 nM under HPLC conditions.
which there is more limited information about DHE/HPLC analysis (14). ANG II is a well-known NAD(P)H oxidase agonist relevant to the pathophysiology of most vascular diseases (17). We confirmed that ANG II incubation (100 nM, 4 h) results in strong DHE-derived fluorescence in cells at microscopy (rhodamine filter), which is completely inhibited by preincubation with PEG-SOD (data not shown and ref. 20). Initially, in control experiments using cells without any stimulus, we estimated that almost all DHE added (98%) was incorporated into intact VSMC after 30 min of incubation, with negligible amounts of DHE, EOH, and ethidium remaining in extracellular PBS incubation buffer (data not shown). To analyze DHE-derived oxidation products, we performed organic extraction using acetonitrile, as described in METHODS, and injected the extract into the HPLC system (Fig. 2, inset). After ANG II, the rates of DHE oxidation to EOH were significantly increased ~2-fold vs. control, with ethidium signal increasing to the same degree. To analyze specific formation of superoxide radical anion during this stimulus, VSMC were incubated for 15 min with PEG-SOD (25 U/ml) before DHE addition. Unexpectedly, PEG-SOD incubation in control cells increased ~40% vs. basal EOH/DHE ratios in unstimulated VSMC (data not shown). Thus, we performed VSMC incubation with PEG alone, in a concentration corresponding to that from PEG-SOD (2.4 nmol/ml), and we mainly found that incubations for 15 min significantly interfered with DHE oxidation in control VSMC and decreased EOH/DHE and ethidium/DHE ratios in ANG II-stimulated VSMC at levels similar to PEG-SOD (data not shown). In fact, this time period of 15 min seems to be critical for PEG or PEG-SOD penetration into cells, being accompanied by disturbances in membrane fluidity, as has been well described by Robinson et al. (4). However, incubations with PEG-conjugated compounds for 1 h resulted, as expected, in a significant decrease in EOH/DHE and E/DHE in control cells and in ANG II stimulated cells (Fig. 2). At these conditions, PEG alone had no effect at baseline, while it interfered with effects of ANG II stimulus (inhibition of ~40% of EOH/DHE) and its conjugated PEG-SOD and PEG-CAT had an additional inhibition of ~30% over PEG alone (Fig. 2). In summary, even though PEG alone may not be a good control of conjugated PEG because the later should have less exposure of glycol groups, our results clarified the distinctive roles of SOD activity, distinguishing them from PEG effects.

Specific formation of EOH by O$_2^-$ was confirmed through SOD1 overexpression in VSMC, which promoted decrease in EOH/DHE ratio to basal levels after ANG II (Fig. 3), compared with empty vector transfection. Of note, EOH and ethidium formation increases up to twofold even after empty vector transfection, implying that the transfection procedure by itself may affect VSMC redox properties enough to be detected by our HPLC analysis. In Fig. 2, incubation with PEG-CAT decreased both EOH and ethidium formation. Therefore, while the increased EOH/DHE ratio is attributable to DHE oxidation by O$_2^-$, the inhibitory effect of CAT remained puzzling, considering that our in vitro and previous results clearly showed that H$_2$O$_2$ does not oxidize DHE even at high concentrations (Table 1) and that peroxidase-mediated DHE oxidation yields mainly ethidium (Fig. 1B). Thus, we hypothesized that H$_2$O$_2$ might trigger or sustain ANG II-mediated increase in O$_2^{-}$ production, in line with the known byphasic nature of NADPH oxidase activation due to ANG II and other agonists (13, 15, 27). Such byphasic pattern involves an initial H$_2$O$_2$ peak production, which is important to sustain later enzymatic activity. To address this issue, we preincubated VSMC with PEG-CAT for 1 h, followed by further incubation with ANG II. PEG-CAT completely prevented NADPH oxidase activity increase due to ANG II, with values similar to baseline (data not shown), indicating a role for H$_2$O$_2$ in maintaining VSMC NADPH oxidase activity. This result was also confirmed through CAT overexpression in VSMC, which inhibited ANG II-mediated NADPH oxidase activity to basal levels (data not shown).
to oxidize DHE by two electrons, such as peroxidase activity (this study and Ref. 30) and heme levels (31), were affected by cell confluence in directions potentially consistent with their triggering of ethidium product (Table 2). Overall peroxidase activity and activity of the major peroxidase in cells glutathione peroxidase were decreased at high vs. low cell density, thus suggesting that peroxidases were unlikely to account for the larger ethidium product at high confluence in our preparation. On the other hand, total heme level increased at higher confluences and remains a potential source of ethidium in this circumstance (Table 2), although this proposal cannot be validated further.

Although our results validated basic aspect of the application of this method to vascular cells and tissues, it should be considered that optimal conditions for analysis of EOH and potentially other DHE oxidation products are still open to further investigation. For example, the optimal emission wavelength for EOH is 570–580 nm, which could be used if one seeks a more exclusive quantitation of this product (14). Also, specific advantages of the 395-nm excitation wavelength have recently been proposed (33). In both cases, quantitation of ethidium is less than ideal. The chromatographic conditions in our study were generally similar to previously published reports (42, 43) and led to a comparable efficiency of separation.

**DHE oxidation as a probe of vascular NADPH oxidase activity.** As a major source of reactive oxygen species in VSMC, assessment of vascular NADPH oxidase function is of prime interest. Despite improved molecular tools to assess the contribution of specific NOX isoforms to O$_2^-$ generation, measurement of overall NADPH-triggered enzyme activity in membrane or other subfraction homogenates remains a simple and quite informative test. The practical option currently available for this purpose utilizes lucigenin, which can undergo redox-cycling in the presence of electron transfer enzymes, particularly flavoenzymes, especially when NADH is used as a substrate (19). Electron paramagnetic resonance spin trapping is the alternative option, but is either less practical or poorly sensitive. Probes such as coelenterazine or 2-methyl-6-phenyl-3,7-dihydroimidazo(1,2-$c$)pyrazin-3-one have proven unsuitable for NADPH oxidase assays. We therefore sought to

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**Table 2. Effect of cell density in SOD, GPx, and total peroxidase activities and total heme in vascular smooth muscle cells**

<table>
<thead>
<tr>
<th>Cell Density</th>
<th>Low (&lt;5 × 10$^4$ cell/cm$^2$)</th>
<th>High (&gt;1 × 10$^4$ cell/cm$^2$)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD activity, U/mg protein</td>
<td>20.9 ± 4.6</td>
<td>32.7 ± 1.0</td>
<td>0.047</td>
</tr>
<tr>
<td>GPx activity, µM</td>
<td>137 ± 5.5</td>
<td>70 ± 3</td>
<td>0.06</td>
</tr>
<tr>
<td>Peroxidase activity</td>
<td>1.0 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.003</td>
</tr>
<tr>
<td>Total heme</td>
<td>33 ± 3</td>
<td>49 ± 3</td>
<td>0.005</td>
</tr>
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Values are means ± SE; n = 4 experiments. SOD, superoxide dismutase; GPx, glutathione peroxidase. During initial cell density, experiments were performed after 48 h of cell plating. SOD activity was determined by inhibition of cytochrome c$^{3+}$ reduction (see Ref. 16); GPx activity was determined by NADPH consumed × min$^{-1}$ × mg protein (see Ref. 36); peroxidase activity is the percentage vs. low density (determined by o-dianisidine oxidation; see METHODS); and total heme is measured in µM heme/mg protein (determined by ferrohemochromogen formation; see METHODS and Ref. 12).
validate a DHE-based NADPH oxidase assay using HPLC analysis.

Our results showed that isolated membrane fraction from ANG II-stimulated VSMC (100 nM, 4 h) when incubated with NADPH (300 μM) resulted in an increased rate of DHE oxidation, reaching an EOH/DHE maximum after 30 min (Fig. 4A, inset). Thus further studies were performed with 30-min incubations. In this condition, ANG II increased EOH/DHE ratios ~40–50% vs. control, which is within the range previously obtained by other methods such as lucigenin (20, 36).

Importantly, the addition of SOD to the incubation mixture led to marked decrease in EOH/DHE ratios (Fig. 4A), as well as in ethidium/DHE ratios. Similar results were obtained with the membrane fraction of VSMC stimulated with lipopolysaccharide, another agonist of vascular NADPH oxidase (data not shown) (29).

Since the HPLC method is costly, time consuming, and not widely available, we sought to validate a DHE-based assay in a fluorometer using a microplate reader assay. In the particular case of such enzymatic assay, this proposal is backed up by the fact the increase in DHE-derived fluorescence was due almost exclusively to EOH, thus obviating the contribution of ethidium product to total fluorescence. First, we confirmed that both EOH and ethidium compounds displayed basal fluorescence, which was highly increased in the presence of DNA (data not shown) (42, 43). We next performed experiments to assess NADPH-triggered DHE oxidation-derived fluorescence in the membrane fraction of VSMC previously stimulated by ANG II (Fig. 4B). Addition of NADPH (50 μM) to VSMC membrane fraction in the presence of DNA (1.25 μg/ml) led to significant increase in total fluorescence, measured after 30 min at 37°C utilizing either the rhodamine or acridine filters (the later reportedly more specific for EOH) (42, 43).

Similarly to HPLC method, addition of SOD completely inhibited this fluorescence, thus showing a suitable correlation between HPLC and microplate assay methods (compare Fig. 4, A and B). In addition, the flavoprotein inhibitor diphenyleneiodonium chloride (20 μM) was able to inhibit NADPH oxidase activity to the same extent as SOD (Fig. 4B), while the O$_2^•$ scavenger tiron (20 μM) completely inhibited such signals (data not shown). Of note, contrarily to lucigenin, DHE does not undergo redox cycling (31), as revealed by lack of increase in O$_2$ consumption in experiments in which DHE (0.05–1 mM) was incubated with membrane fraction (100 μg) of VSMC stimulated or not with ANG II in the presence of NADPH (300–1,000 μM) (data not shown). These results validate both HPLC/DHE and fluorescence microplate assays for measuring vascular NADPH oxidase activity in isolated membrane fraction under the experimental conditions of the present study. It is important, however, to point out that the microplate assay may possibly be less accurate in conditions (specific cellular types or subfractions or specific oxidase agonists) in which ethidium is formed in addition to EOH.

**DHE-derived fluorescence analysis in vessels.** In situ detection of O$_2^•$” in tissue sections was one of the first described applications of DHE-derived fluorescence (6, 28). It is particularly useful to disclose specific cells or regions potentially producing ROS within a vessel section (24, 28). We (24) and others (38) have reported that total DHE oxidation-derived fluorescence observed at microscopy is increased 14 days after angioplasty vs. control vessels, particularly at the neointima (Fig. 5A). Our goal was to assess whether HPLC analysis and quantification of DHE-derived fluorescent products could improve specificity and help understand redox events during vascular remodeling. HPLC analysis revealed increased levels of EOH/DHE and ethidium/DHE ratios (100% and 40%, respectively) in injured vessels compared with basal arteries (Fig. 5B). The addition of PEG-SOD to artery slices strongly inhibited EOH/DHE, whereas the addition of PEG-CAT decreased both EOH/DHE and ethidium/DHE ratios (Fig. 5B) in a way analogous to intact cells, although quantitatively dissimilar. It was notable that in tissue slices, the ethidium product was particularly dominant over EOH. This suggested to us that tissue sources of ethidium such as peroxidases (see Fig. 1) might be contributing to fluorescence. In fact, peroxidase activity was increased 5-fold in injured vessels compared with control arteries (control vessels = 4.7 ± 1.1 vs. injured vessels = 23.2 ± 3.6 μU/mg protein; values are means ± SE;
Peroxidase activity was strongly inhibited by 0.5% azide addition, which was also able to decrease fluorescence at microscopy (Fig. 5A). Several peroxidase sources might account for increased ethidium generation. In particular, analysis of myeloperoxidase expression by Western blot analysis and immunostaining with RAM-11 for macrophages (data not shown) were negative at this time of injury. Peroxidase activity of SOD (8, 23) remains one possible source. Whatever the peroxidase source, however, our data stress the fact that peroxidases are particularly active and thus may have an important role in the redox signaling of vascular repair (22).

Summary. In conclusion, our results showed validation of HPLC analysis of DHE oxidation-derived products for assessment of $O_2^{-}$ production in cultured VSMC and vascular tissue slices. In addition, we described the novel validation of a DHE-based assay for NADPH oxidase in VSMC membrane fraction using either HPLC analysis or spectrofluorometry. By and large, our data add to the recent literature (14, 43) and extend to the vascular system data, indicating that this method is a valuable tool to improve the accuracy of $O_2^{-}$ analysis. Our results also identified endogenous pathways that can either work as potentially confounding factors or indicate potentially new developments that can amplify the scope of analysis. Such pathways are summarized in Fig. 6, which shows that EOH formation in cells and tissues is largely contributed by $O_2^{-}$. Our data raise the possibility that, in specific situations, contributions of peroxynitrite/$CO_2$ may potentially occur. The origin of the ethidium product remains unsettled, but our results indicate possible complex pathways contributing to its formation, including heme levels and peroxidase activity. Such proposals, however, while backed up by other studies from the literature (30, 31), cannot be fully proven in complex in vivo systems and remain hypothetical.

Our data raise the possibility that, in specific situations, contributions of peroxynitrite/$CO_2$ may potentially occur. The origin of the ethidium product remains unsettled, but our results indicate possible complex pathways contributing to its formation, including heme levels and peroxidase activity. Such proposals, however, while backed up by other studies from the literature (30, 31), cannot be fully proven in complex in vivo systems and remain hypothetical. The multiple pathways accounting for ethidium generation stress the importance and the increase in specificity provided by HPLC analysis, considering that total fluorescence assesses the contribution of all DHE-derived products. Since our data and previous results (6, 42) suggested that $H_2O_2$ is likely to be a major precursor underlying ethidium formation, observed decreases in total DHE-derived fluorescence by antioxidants at microscopy or flow citometry must be taken with care regarding discrimination of $O_2^{-}$ contribution, even in the case of SOD, which is known mainly to decrease steady-state $H_2O_2$ levels in cells (41). Our results also identified that some intracellular interactions could be mistaken by direct chemical reactions of DHE, particularly the $H_2O_2$-stimulated NADPH oxidase activation (13, 15) apparently rendering the EOH formation sensitive to CAT. Furthermore, we identified extrinsic factors that significantly affect the levels of DHE-derived products in vivo. Changes in

Fig. 5. DHE-derived fluorescence in iliac arteries of rabbits after balloon injury. Fourteen days after injury, the rabbits were euthanized, and the left (control) and right (injured) iliac arteries were immediately analyzed. A: fluorescence microscopy of iliac artery slices after incubation with DHE (2 μM) for 20 min, observed under rhodamine filter for control (C) or injured (I) arteries, and for injured arteries after addition of inhibitors PEG-SOD (25 U/ml) (IS) or azide (0.5%) (IA). Inhibitors were incubated 15 min before DHE addition. B: ratios of EOH/DHE and E/DHE obtained by HPLC analysis from acetonitrile extracts of iliac artery segments. Segments were incubated with DHE (50 μM) in PBS/DTPA in the presence or absence of inhibitors (PEG-SOD 25 U/ml or PEG-CAT 200 U/ml) for 15 min, extracted with acetonitrile, dried, resuspended in PBS, and analyzed by HPLC. Analysis of azide-treated arteries was not performed, since we noted that azide interferes with HPLC analysis. Inset, HPLC chromatogram of an injured iliac tissue extract. Values are means ± SE ($n$ = 4 experiments). *$P < 0.05$ vs. control; **$P < 0.05$ vs. injured; #$P < 0.1$ vs. injured.

Fig. 6. Schematic representation of possible endogenous pathways and extrinsic modifiers of DHE oxidation. The intensity of arrows roughly parallels the importance of the pathway, as judged from the strength of our data as well as previous works (see Summary).
cell density are particularly relevant, although the mechanism is unclear and could be multiple. There is surprising little information regarding the role of cell density in ROS levels, although a prior study (25) detected confluence-dependent changes in neuron cell redox properties. In addition, artifacts resulting from exposure to light and probably from the method of extraction can be quite significant and should be carefully considered. Interestingly, our finding of light-induced increase in 2-hydroxyethidium in cells contrasts with the reported increase in ethidium signal due to light in the in vitro situation (21). This is probably due to yet unclear in vivo routes of decay and interplay of DHE-derived intermediates. Accordingly, other yet unknown intermediates of DHE oxidation reaction are likely to exist and may also disclose relevant information. Therefore, HPLC analysis of DHE-derived oxidation products can be used in many vascular experimental settings and provides an advance in the assessment of \( \text{O}_2^- \) production or NADPH oxidase activity. Yet, given the many potential sources of ambiguity common in the redox area, cross-checking of results against another method of ROS assessment remains a necessity. (18, 35, 39)

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