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Evidence for a NADH/NADPH Oxidase in Human Umbilical Vein Endothelial Cells Using Electron Spin Resonance

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ABSTRACT

A growing body of evidence has suggested that a membrane-bound NADH/NADPH oxidase is the predominant source of reactive oxygen species (ROS) in vascular cells. Prior studies have used indirect assessments of superoxide including lucigenin-enhanced chemiluminescence, cytochrome *c*, and fluorescent dye techniques. The present study was performed to determine if NADH/NADPH oxidase function could be detected human endothelial cells using electron spin resonance. Human umbilical vein endothelial cells (HUVEC) were homogenized and fractionated into cytosolic and membrane components. Cell fractions were incubated in buffer containing either NADH or NADPH (100 μM for each) and the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO). EPR signals were obtained in a Bruker EMX spectrometer. Cytoplasmic fractions were devoid of activity. In contrast, incubation of membrane fractions with NADH produced a signal with a total peak intensity of 1,038 ± 64, which was significantly greater than that observed with NADPH (540 ± 101). The signal was completely inhibited by either manganese superoxide dismutase (MnSOD, 100 U/ml) or the flavoprotein inhibitor diphenylene iodinium (DPI, 100 μM). Rotenone (100 μM) did not significantly alter the signal intensity, (833 ± 88). These data demonstrate direct evidence for a functional NADH/NADPH oxidase in human endothelial cells and show that electron spin resonance is a useful tool for study of this enzyme system. Antiox. Redox Signal. 2, 779–787.

INTRODUCTION

DURING THE PAST DECADE, it has become evident that vascular cells, including endothelial cells (Mohazzab *et al.*, 1994; Jones *et al.*, 1996), vascular smooth muscle cells (Griendling *et al.*, 1994; Ushio-Fukai *et al.*, 1996), and cells of the adventitia (Wang *et al.*, 1998) are capable of producing reactive oxygen species (ROS). Of note, vascular production of superoxide ($^{\cdot}O_2^-$), H_2O_2, $OH^{\cdot}$, and other ROS have been shown to be increased in several pathophysiological states. These include hypercholesterolemia (Warnholtz *et al.*, 1999), hypertension (Rajagopalan *et al.*, 1996a), ischemia followed by reperfusion (Zweier *et al.*, 1987, 1989; Zweier, 1988; Bolli *et al.*, 1989), diabetes (Tesfamariam, 1994), and heart failure (Mallat *et al.*, 1998). This phenomenon is thought to contribute to altered vasomotion (Somers and Harrison, 1999), vascular inflammation (Khan *et al.*, 1994), remodeling (Rajagopalan *et al.*, 1996b), and vascular smooth muscle growth and hypertrophy (Ushio-Fukai *et al.*, 1996).

The source of ROS in vascular cells has been the subject of substantial interest. Prior research has implicated xanthine oxidase, mitochondrial electron transport, cyclooxygenase, and lipoxygenase as contributing to production

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of ROS in vascular cells. Recently, it has been shown that nitric oxide synthases (NOS), when deprived of their substrate L-arginine or the cofactor tetrahydrobiopterin, can produce superoxide (Xia *et al.*, 1996, 1998; Xia and Zweier, 1997; Vasquez-Vivar *et al.*, 1998) and indirect evidence suggests that the endothelial isoform of NOS may produce superoxide under pathophysiological conditions *in vivo* (Cosentino and Katusic, 1995).

Several studies have suggested that a major source of ROS in both endothelial cells and vascular smooth muscle cells is a membrane-bound NADH/NADPH oxidase (Mohazzab *et al.*, 1994; Pagano *et al.*, 1995). This enzyme system is thought to have similarities to the neutrophil NADPH oxidase. Indeed, mRNAs for all of the NADPH oxidase subunits have been detected in human umbilical vein endothelial cells (HUVEC) using polymerase chain reactions (Jones *et al.*, 1996). In vascular smooth muscle cells, the NADPH oxidase subunit p22phox has been shown to play a critical role in production of $^{\cdot}O_2^-$ and H_2O_2 in response to angiotensin II (Ushio-Fukai *et al.*, 1996; Zafari *et al.*, 1998). Inhibition of expression of p22phox using antisense approaches inhibits vascular smooth muscle cell hypertrophy in response to angiotensin II.

A potential criticism of prior studies examining vascular $^{\cdot}O_2^-$ enzyme sources is that many have employed lucigenin-enhanced chemiluminescence. This method is exquisitely sensitive and may be used to detect $^{\cdot}O_2^-$ in intact tissues and cells. Unfortunately, lucigenin has been shown to redox cycle in the presence of flavoproteins, leading to artifactual production of $^{\cdot}O_2^-$ (Vasquez-Vivar *et al.*, 1997). Other approaches, including the reduction of cytochrome *c* or use of fluorescent indicators, may lack sensitivity or specificity.

On the basis of these considerations, the present study was performed using electron spin resonance (ESR) spectroscopy to examine specifically sources of ROS in human umbilical vein endothelial cells. We employed the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO) (Roubaud *et al.*, 1997), and specifically examined substrates that would drive $^{\cdot}O_2^-$ production in cell homogenates and subcellular fractions.

MATERIALS AND METHODS

Materials

Penicillin, streptomycin,, and L-glutamine were purchased from Life Technologies (Gaithersburg, MD). Medium 199 was purchased from Mediatech, Inc. (Herndon, VA). Endothelial cell growth supplement was used from Collaborative Biomedical Products (Bedford, MA). DEPMPO was purchased from Oxis (Portland, OR). Heparin was from Elkins-Sinn, Inc. (Cherry Hill, NJ). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

HUVECs were grown in Medium 199 containing 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg of streptomycin, 2 m*M* L-glutamine, 5 mg/ml endothelial cell growth supplement, and 20,000 U heparin. Cells were used between passage 2 and passage 6 at 100% confluence.

Cell fractionation

HUVECs were washed twice and scraped with 10 ml of 1 × phosphate-buffered saline (PBS). The cells were centrifuged at 1,000 × *g* for 10 min at 4°C in a swinging bucket rotor, and the supernatant was removed. The pelleted cells were resuspended in 100 μl of buffer (50 m*M* Tris, 0.1 m*M* EDTA, 0.1 m*M* EGTA, pH 7.4) containing 1 μ*M* pepstatin, 2 μ*M* leupeptin, 1 m*M* phenylmethylsulfonyl flouride (PMSF), and 2 μ*M* Bestatin. The cells were sonicated on ice at 500-msec bursts for 10 sec using a Kontes Micro Ultrasonic Cell Disruptor at 20% power. Lysed HUVECs were centrifuged at 250 × *g* for 10 min to remove unbroken cells. The supernatant containing lysed cells was centrifuged at 20,000 × *g* to pellet the nonmembrane particulate fraction, and the supernatant was centrifuged at 100,000 × *g* to pellet the membrane fraction. Membranes were then resuspended for study in isotonic PBS, pH 7.4, containing Ca-EDTA (1 m*M*) for study. Protein concentration was determined using the method described by Bradford (Bradford, 1976).

Cellular homogenate subfractions were incubated with DEPMPO (10 m*M*), diethyl-

dithiocarbamic acid (DETC, 100 μM), and NADH (100 μM) unless otherwise specified. In some reactions, NADH was substituted with NADPH (100 μM), xanthine (100 nm), succinate (5 mM) + antimycin A (30 μM) or arachidonic acid (100 μM). In another set of reactions, NADH was used as a substrate after the membrane fraction was incubated with an oxidase inhibitor for 5 min. Inhibitors used were rotenone (100 μM), oxypurinol (100 μM), indomethacin (10 μM), *N*-nitro-L-arginine (L-NNA) (10 μM), or the flavoprotein inhibitor diphenylene iodinium (DPI) (100 μM). Reactions were allowed to proceed at 37°C for 30 min. The reactions were then stopped by rapid freezing in liquid nitrogen and samples were thawed within 4 hr for ESR spectroscopy.

Electron spin resonance

Spectra were recorded in a quartz flat cell at room temperature using a Bruker EMX spectrometer at a microwave frequency of 9.78 GHz, a microwave power of 20 mW, and a modulation amplitude of 1.0 G. Scan time was 84 sec, and 10 sequential scans were signal averaged. Data from each spectrum were quantified as the sum of the total eight-peak intensity and expressed as mean ± SEM.

RESULTS

Free radical production from human endothelial subcellular fractions

In preliminary studies of whole-cell homogenates, minimal ESR signal was obtained in the presence of NADH or NADPH. Likewise, no signal was obtained from cytosolic subfractions (Fig. 1, left upper panel). In contrast, membranes obtained by 100,000 × g for 60 min of centrifugation produced a distinct eight-peak signal when incubated with NADH (100 μM) (Fig. 1, left lower panel). Mean data from five experiments are shown in Fig. 1 (right panel).

Although the homogenization method used likely ruptured most mitochondria, this crude membrane preparation may contain some intact mitochondria, which could serve as a source of ROS derived from the electron transport chain. Further experiments were performed to determine if mitochondrial sources contributed to our ESR signal. The cell homogenates were subjected to an initial ultracentrifugation at 20,000 × g for 20 min, followed by a subsequent centrifugation at 100,000 × g for 60 min. The 20,000 × g fraction is known to contain both mitochondria and peroxysomes, as well as other membrane vesicles. This 20,000 × g subfraction yielded an ESR signal of roughly equal intensity to that of the subsequent 100,000 × g fraction (data not shown). Interestingly, in neither of these subfractions was the signal affected by rotenone, an inhibitor of mitochondrial electron transport (data not shown). These results indicate that an NADH-driven oxidase is present in the particulate fraction, but not the cytosolic fraction of HUVEC, and this enzyme activity is unrelated to mitochondrial electron transport. Subsequent experiments were performed on membrane fractions obtained from the 100,000 × g centrifugation of the supernatant from the 20,000 × g centrifugation.

Characterization of free radicals produced from HUVECs

The eight-peak spectrum obtained was characteristic of the DEPMPO-OH adduct. This adduct is the result of either $\cdot O_2^-$ reacting with DEPMPO to form DEPMPO-OOH, which then degenerates into DEPMPO-OH, or from direct reaction of hydroxl radical with DEPMPO to form DEPMPO-OH. To determine which radical species initially reacted with DEPMPO, oxidase activity was measured in the presence of catalase, to scavenge hydrogen peroxide (H_2O_2) and thus prevent hydroxyl radical formation via Fenton or Haber-Weiss reactions, or in the presence of superoxide dismutase (SOD), to scavenge superoxide. Since DETC was used in the assay to inhibit native Cu/Zn SOD, Mn-SOD was used for this purpose. As shown in Fig. 2, DEPMPO-OH adduct formation was unchanged in the presence of catalase (1,105 ± 93) compared to control (1,027 ± 73). However, scavenging of superoxide with MnSOD resulted in a complete quenching of the ESR signal. This provides evidence that the ROS formed by the HUVEC homogenate is $\cdot O_2^-$.

In additional experiments using a xanthine/

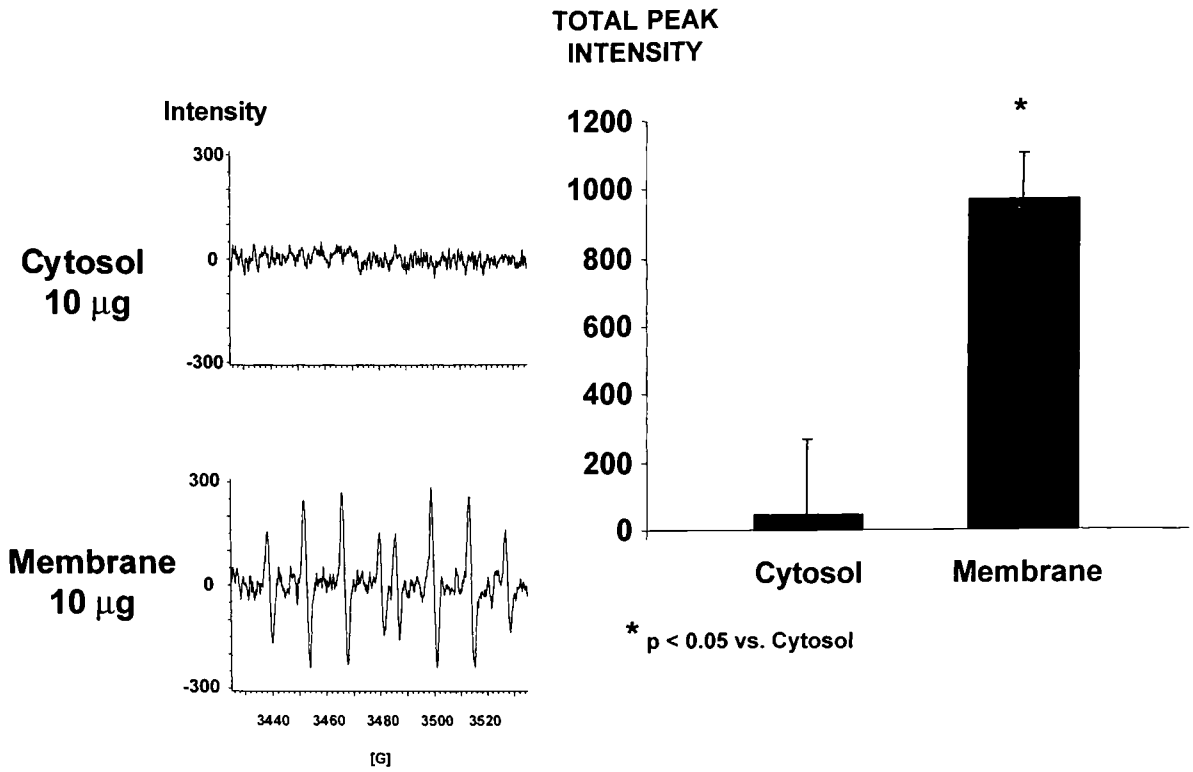

FIG. 1. ESR detection of a DEPMPO-spin adduct produced by human umbilical vein endothelial subcellular fractions. The membrane component was separated from the cytosolic component by ultracentrifugation. A total of 10 μg of protein was incubated with 100 μM DETC, 10 mM DEPMPO, and 100 μM NADH in 1 mM Ca/EDTA PBS at 37°C for 30 min. (**Left**) Authentic ESR spectra obtained as described in Materials and Methods. (**Right**) Mean ± 5E total peak intensity for five experiments.

xanthine oxidase $^{\cdot}O_2^-$-generating system, the degradation of the DEPMPO-OOH adduct to the DEPMPO-OH adduct was found to be more rapid at the physiological temperature of 37°C than at room temperature. Furthermore, while DETC enhanced the $^{\cdot}O_2^-$ detected in this assay, additional studies indicated that it also enhanced the conversion of the DEPMPO-OOH adduct to DEPMPO-OH. To determine if a $^{\cdot}O_2^-$ adduct could be obtained from HUVEC membranes, additional experiments were performed using 50 μg of protein incubated with NADH (100 μM) in the absence of DETC at room temperature. Under these experimental conditions, an obvious DEPMPO-OOH adduct was observed superimposed upon a smaller DEPMPO-OH adduct (Fig. 3).

HUVEC oxidase dose–response

Superoxide production was measured in the presence of 5, 10, and 20 μg of membrane protein. As shown in Fig. 4, superoxide production increased with increasing concentrations of homogenate protein (504 ± 79, 1,026 ± 151, 1,745 ± 306, respectively).

Substrate specificity

Superoxide production was measured using substrates for known biologic sources of superoxide. The most vigorous superoxide production was seen with NADH (1,038 ± 68). NADPH drove a measurable but significantly lower amount of superoxide production compared to NADH (540 ± 101). Xanthine, succinate/antimycin-A, and arachidonic acid produced minimal ESR signal, suggesting minimal involvement of xanthine oxidase, mitochondrial electron transport, or cyclooxygenase/lipoxygenase as the source of ROS from the HUVEC homogenate (Fig. 5).

Oxidase inhibitors

To delineate further the identity of the superoxide-producing enzyme, inhibitors of other oxidase enzymes were used (Fig. 6). No

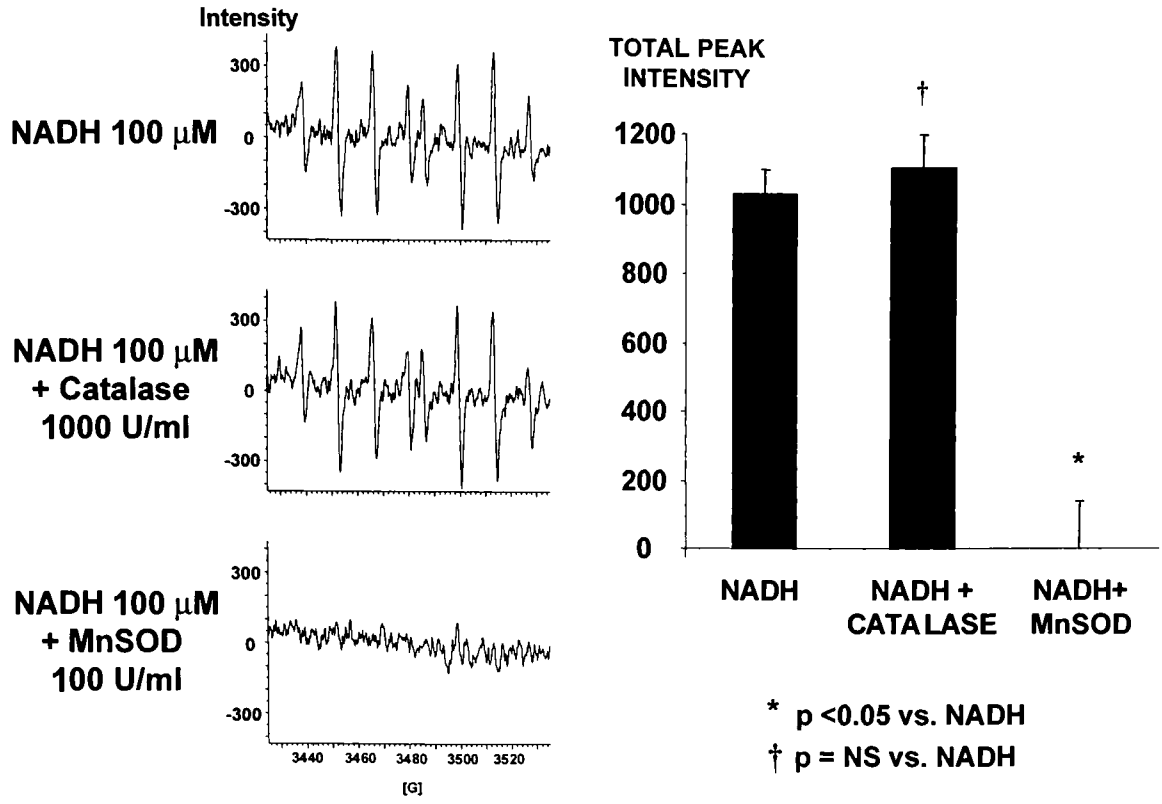

FIG. 2. Identity of the radical species formed by HUVEC oxidase. Incubations of membrane homogenates with NADH, DETC, and DEPMPO in Ca/EDTA PBS were performed in the presence or absence of catalase (1000 U/ml) or Mn-SOD (100 U/ml). (**Left**) Actual ESR spectra. (**Right**) Data from five separate experiments.

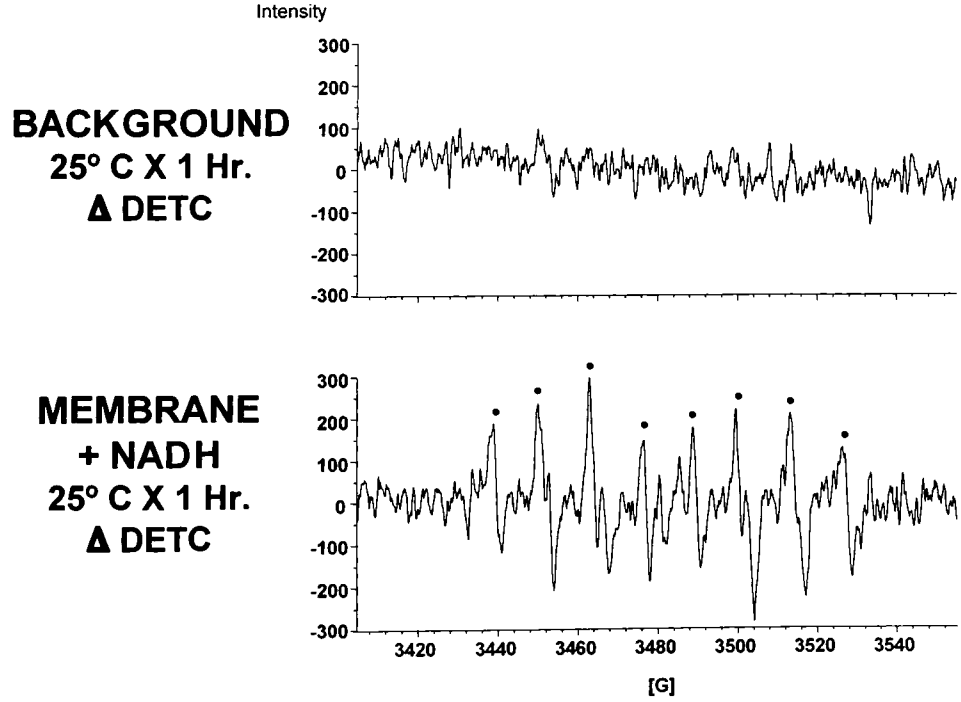

FIG. 3. ESR spectra showing evidence for $^{\cdot}O_2^-$ production by HUVEC membranes. HUVEC membranes (50 μg) obtained by differential centrifugation (see text) were incubated with NADH (100 μM) and DEPMPO (10 mM) at room temperature in the absence of DETC at room temperature for 1 hr. ESR spectra were obtained as in Fig. 1. (**Upper panel**) Absence of an ESR spectrum in the absence of protein. (**Lower panel**) Predominant DEPMPO-OOH spectrum (·) superimposed upon a DEPMPO-OH spectrum.

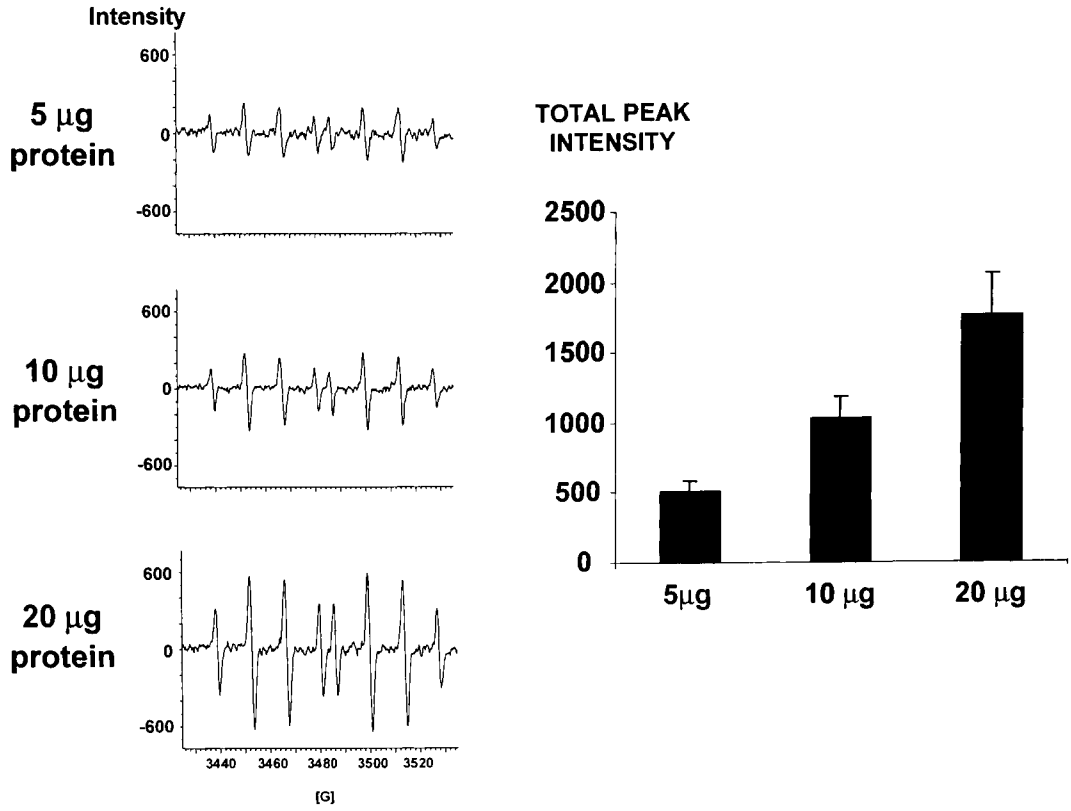

FIG. 4. Concentration dependence of membrane fraction protein and oxidase activity. A total of 5, 10, and 20 μg of protein were incubated in 1 ml containing NADH, DETC, and DEPMPO in Ca/EDTA PBS as described in the text. (**Left**) Actual ESR spectra from one experiment. (**Right**) Average of five separate experiments.

significant decrease in the ESR signal was seen with rotenone, indomethacin, oxypurinol, or L-NNA, suggesting that the source of superoxide is unlikely to be mitochondrial electron transport, cyclooxygenase/lipoxygenase, xanthine oxidase, or NOS. A significant decrease in the signal was seen with the flavoprotein inhibitor DPI (268 ± 81 compared to 1,052 ± 68 for NADH control).

DISCUSSION

In the present study, ESR spectroscopy was used to establish clearly the presence of an NADH/NADPH-driven oxidase in human endothelial cells. When NADH, or to a lesser extent NADPH, was added to membrane fractions of these cells, a DEPMPO spin adduct was easily detectable using ESR. The formation of this adduct was inhibited by addition of SOD and was detected only in membrane subcellular fractions. Importantly, $^{\cdot}O_2^-$ production in response to NADH or NADPH was not prevented by specific inhibitors of other well-described oxidases.

Our current findings are in keeping with previous studies using lucigenin-enhanced chemiluminescence to demonstrate the presence of a membrane-bound NADH-dependent oxidase in vascular cells (Mohazzab *et al.*, 1994; Ushio-Fukai *et al.*, 1996). Recently, lucigenin-enhanced chemiluminescence has been critized, because lucigenin may react with intracellular flavoproteins, indirectly leading to the production of superoxide (Vasquez-Vivar *et al.*, 1997). Our current approach avoids this potential pitfall. Previous studies have found varying relative potencies of NADH and NADPH in driving $^{\cdot}O_2^-$ as detected by lucigenin and other approaches. This inconsistency may be due to different sources of endothelial cells, differences in cell preparation, or due to the differences in detection methods. Despite these minor differences, the findings in the current study are qualitatively similar to prior studies using other

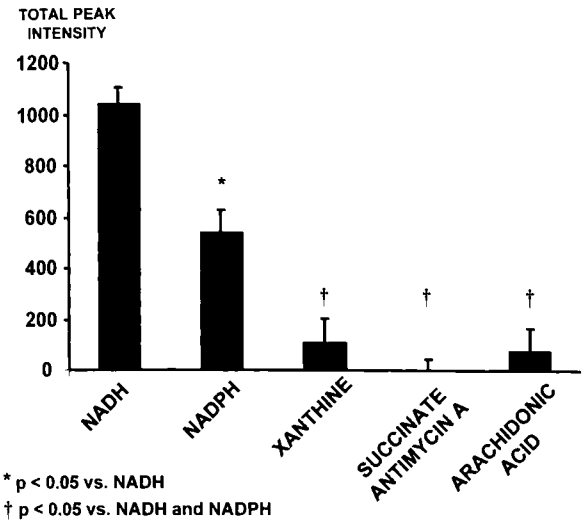

FIG. 5. Substrate preference of the HUVEC oxidase. The HUVEC membrane fraction was incubated with DETC and DEPMPO in Ca/EDTA PBS in the presence of known oxidase substrates 100 μM NADH, 100 μM NADPH, 100 nM xanthine, 5 mM succinate with 30 μM antimycin A or 100 μM arachidonic acid. Only NADH and to a lesser extent NADPH were able to drive significant superoxide production.

measurement approaches (Mohazzab *et al.*, 1994; Pagano *et al.*, 1995; Ushio-Fukai *et al.*, 1996).

A significant finding in this study is the identity of the ROS produced by HUVEC. Enzymatic reduction of oxygen can potentially lead to the production of superoxide, H_2O_2, or hydroxyl anion, each of which can have specific effects on vascular processes. By showing complete quenching of the ESR signal with SOD and by demonstrating the characteristic DEPMPO-OOH superoxide adduct, we have confirmed the identity of the product of the HUVEC oxidase to be superoxide. Superoxide has been shown to play a major role in vascular disease, including direct scavenging of NO to form peroxynitrite (White *et al.*, 1994; Pryor and Squadrito, 1995), vascular growth (Ushio-Fukai *et al.*, 1996), and initiation of inflammatory pathways via the NF-κB transcription factor (Hishikawa and Luscher, 1997). In addition, dismutation of superoxide by SOD leads to formation of H_2O_2 (Fridovich, 1997), formation of hydroxyl, and peroxidation of lipids (Darley-Usmar *et al.*, 1992).

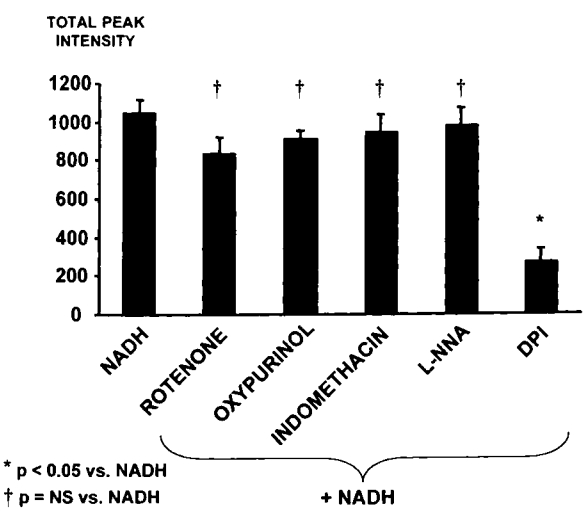

FIG. 6. Inhibition of HUVEC superoxide production. A total of 10 μg of protein was incubated in 1 ml containing NADH, DETC, and DEPMPO in Ca/EDTA PBS and either 100 μM rotenone, 100 μM oxypurinol, 10 μM indomethacin, 10 μM L-NNA, or the 10 μM flavoprotein inhibitor DPI. Only DPI was able to reduce to superoxide signal significantly

To examine for other potential sources of $^{\cdot}O_2^-$ in endothelial cell homogenates, we used two approaches. First, we examined $^{\cdot}O_2^-$ production in response to a variety of substrates for other superoxide-producing oxidases. Substrates for xanthine oxidase, cyclooxygenase, and the mitochondrial electron transport chain were unable to produce a detectable ESR signal. NOS is capable of producing $^{\cdot}O_2^-$ (Vasquez-Vivar *et al.*, 1998; Xia *et al.*, 1998); however this enzyme solely uses NADPH as an electron donor, in contrast to our present findings. Second, we used inhibitors of several enzymes or enzyme systems previously shown to produce ROS. Neither indomethacin, rotenone, oxypurinol, nor L-nitroarginine affected the signals produced by addition of NADH. Importantly, the nonspecific flavoprotein inhibitor diphenylene iodinium markedly inhibited NADH-driven $^{\cdot}O_2^-$ production, compatible with the responsible enzyme containing flavins. Of note, treatment of cells with apocynin, an inhibitor of the neutrophil oxidase (Stolk *et al.*, 1994), failed to inhibit $^{\cdot}O_2^-$ production in homogenates of endothelial cells in several preliminary studies (data not shown). This strongly suggests that the endothelial cell oxidase may not be identical to the neutrophil enzyme system.

Numerous previous studies have shown that mitochondria can produce ROS. This seems to be accentuated in pathological conditions such as heart failure (Ide *et al.*, 1999), ischemia reperfusion (Ueta *et al.*, 1990), and following various

toxins (Ogura *et al.*, 1991; Pryor *et al.*, 1992). Our failure to observe a spin adduct in the presence of succinate and antimycin does not exclude the potential role of mitochondria as source of ROS in intact endothelial cells. The homogenization approach employed in this study was designed to optimize examination of nonmitochondrial membranes and cytoplasmic fractions, rather to study mitochondrial sources specifically. It is possible that in various disease states, mitochondrial production of ROS may be more prominent than observed in the present studies.

In conclusion, the present study clearly establishes the presence of an NADH/NADPH oxidase in human endothelial cells using a specific ESR-based approach. The activity of this enzyme appears far greater than that of other traditionally described sources of $^{\cdot}O_2^-$, and it is likely that it contributes to oxidative events within the endothelium in both normal and pathophysiological conditions.

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ABBREVIATIONS

DETC, Diethyldithiocarbamic acid; DEPMPO, diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; DPI, diphenylene iodinium; ESR, electron spin resonance; GHz, gigahertz; HUVEC, human umbilical vein endothelial cells; L-NNA, *N*-nitro-L-arginine; NADH, nicotine adeninine diphosphate (reduced form); NADPH, nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate; NOS, nitric oxide synthase; $O2^-$, superoxide anion; PMSF, phenylmethylsulfonyl flouride; SOD, superoxide dismutase.

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