Free Rad. Res., Vol. 29, pp. 441–449
Reprints available directly from the publisher
Photocopying permitted by license only

© 1998 OPA (Overseas Publishers Association) N.V.
Published by license under
the Harwood Academic Publishers imprint,
part of The Gordon and Breach Publishing Group.
Printed in Malaysia.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/19/11
For personal use only.

Electron Spin Resonance Detection of Extracellular Superoxide Anion Released by Cultured Endothelial Cells

JEAN-PIERRE SOUCHARD[a], MARIE-ALINE BARBACANNE[a,b], EMMANUEL MARGEAT[a], ARLETTE MARET[b], FRANÇOISE NEPVEU[a] and JEAN-FRANÇOIS ARNAL[b,*]

[a]*Laboratoire de Synthèse, Physico-Chimie et Radiobiologie, Université Paul Sabatier, 31062 Toulouse Cedex, France;* [b]*INSERM U397, Institut Louis Bugnard, Laboratoire de Physiologie, CHU Rangueil, 31054 Toulouse Cedex, France*

Accepted by Prof. H. Sies

(Received 27 March 1998; In revised form 24 July 1998)

Objective and Methods Endothelium produces oxygen-derived free radicals which play a major role in vessel wall physiology and pathology. Whereas $NO^•$ production from endothelium has been extensively characterized, little is known about endothelium-derived $O_2^{-•}$. In the present study, we determined the $O_2^{-•}$ production of bovine aortic endothelial cells (BAEC) using the spin trap 5,5-dimethyl-1 pyrroline-N-oxide (DMPO) and electron spin resonance (ESR) spectroscopy.

Results An ESR adduct DMPO-OH detected in the supernatant of BAEC after stimulation with the calcium ionophore A23187 originated from the trapping of extracellular $O_2^{-•}$, because coincubation with superoxide dismutase (30 U/ml) completely suppressed the ESR signal, whereas catalase (2000 U/ml) had no effect. A23187 stimulated extracellular $O_2^{-•}$ production in a time- and dose-dependent manner. The coenzymes NADH and NADPH both increased the ESR signal, whereas a flavin antagonist, diphenylene iodonium, abolished the ESR signal. Phorbol myristate acetate potentiated, whereas bisindolylmaleimide I inhibited the A23187-stimulated $O_2^{-•}$ production, suggesting the involvement of protein kinase C. These signals were not altered L-NAME, a NO-synthase inhibitor, suggesting that the endogenous production of $NO^•$ did not alter $O_2^{-•}$ production. Finally, the amount of $O_2^{-•}$ generated by A23187-stimulated post-confluent BAEC was one order of magnitude higher than that evoked by rat aortic smooth muscle cells stimulated under the same conditions.

Keywords: Endothelium, superoxide anion, electron spin resonance, nitric oxide

Abbreviations: nitric oxide ($NO^•$), superoxide anion ($O_2^{-•}$), electron spin resonance (ESR), bovine aortic endothelial cells (BAEC), 5,5-dimethyl-1 pyrroline-N-oxide (DMPO), diethylenetriaminepentaacetic acid (DTPA), nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), N^w-nitro-L-arginine methyl ester (L-NAME)

INTRODUCTION

McCord and Fridovich[1] showed that superoxide free radical anion ($O_2^{-•}$) is produced enzymatically and demonstrated that superoxide dismutase catalyzed the dismutation of $O_2^{-•}$. It was demonstrated by several groups that

* Corresponding author. Tel.: 33 5 61 32 21 47. Fax: 33 5 61 32 21 41. E-mail: arnal@rangueil.inserm.fr.

RIGHTSLINK

endothelium releases a free radical, nitric oxide ($NO^{\bullet}$), which relaxes vascular smooth muscle.[2–4] Whereas endothelium-derived $NO^{\bullet}$ has been extensively characterized,[5,6] little is known about the source and the functions of endothelium-derived $O_2^{-\bullet}$. However, endothelial $O_2^{-\bullet}$ generation could be of major clinical importance.[7]

Two major sources of $O_2^{-\bullet}$ have been distinguished in mammalian cells. In most cell types, the mitochondrial respiratory chain is traditionally viewed as the major source of intracellular $O_2^{-\bullet}$ which is readily converted to hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutase (SOD). However, the phagocytes (neutrophils, eosinophils, monocytes and macrophages) possess an NADPH oxidase complex, which is able to generate very large amounts of $O_2^{-\bullet}$ extracellularly. But other cell types, such as vascular smooth muscle and fibroblasts, also have an NADH/NADPH oxidase system which seems to be able to generate extracellular $O_2^{-\bullet}$, but in lesser amounts than the phagocytes. Little is known about the molecular structure of the non-phagocytic NADPH oxidase system, except that one subunit of phagocyte NADPH oxidase (p22phox) appears to be involved in its activity in the vascular smooth muscle cells.[8]

Although lucigenin luminescence has been widely used to assess the low endothelial production of $O_2^{-\bullet}$,[9–12] the reliability of this technique has been recently questioned.[13–15] The other commonly used method for the detection of $O_2^{-\bullet}$ is the reduction of ferricytochrome c.[16–19] In contrast, few studies have reported the generation of $O_2^{-\bullet}$ by vascular endothelial cells using the spin trap 5,5-dimethyl-1 pyrroline-N-oxide (DMPO).[20,21] However, it has been demonstrated that spin trapping is at least 20-fold more sensitive than ferricytochrome c for the measurement of $O_2^{-\bullet}$.[22]

Thus, in the present study, we applied electron spin resonance (ESR) spectroscopy to assess the production of $O_2^{-\bullet}$ in cultured bovine aortic endothelial cells (BAEC) stimulated with various doses of the calcium ionophore A23187. The effect on endothelial $O_2^{-\bullet}$ production of NADH, NADPH, diphenylene iodonium (DPI) and protein kinase C modulators was studied, as these factors have been shown to modulate the activity of membrane NADH/NADPH oxidase systems. We also examined the potential influence of endothelium-derived $NO^{\bullet}$ on $O_2^{-\bullet}$ production in post-confluent BAEC. Finally, we compared the production of $O_2^{-\bullet}$ in BAEC with that of cultured rat aortic smooth muscle cells.

METHODS

Cell Culture and Materials

BAEC were obtained as described previously[23] and cultured in DME medium supplemented with 10% heat-inactivated calf serum (CS) at 37°C and 1 ng/ml basic fibroblast growth factor (bFGF) under a 10% CO_2 humidified atmosphere. The cells used in this study were between the 5th and the 15th passage. All passages were made using a splitting ratio of 1 : 6. Confluency was determined by visual inspection of the cells, and deemed present when > 95% of the cells were in contact with adjacent cells. Under these culture conditions, the cells invariably reached confluency 3 days after passage. Each measure was performed in a 25 cm^2 flask containing 2.5×10^6 post-confluent BAEC (i.e. 4–6 days after confluency to allow maturation of the cells).

Rat aortic smooth muscle cells were isolated from rat aorta as previously described.[24] Briefly, the thoracic aortae were excised and rinsed and the fat and collateral vessels removed. The adventitia were separated and the remaining media plus intima sliced into fine rings and incubated in DMEM containing collagenase (1248 IU/ml) for 40 min at 37°C. The rings were then flushed and filtered to remove the endothelial cells, and placed in a second enzymatic bath (37°C, water bath agitation) containing collagenase (87.5 IU/ml) plus elastase (17.5 IU/ml) in medium 199 (Boehringer). After 60 min of this

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/19/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/19/11
For personal use only.

second digestion, the cell suspension was briefly centrifuged (300 g, 5 min). The cell suspension was plated for 24 h in a plastic flask coated with 0.1% collagen to produce a pure cell preparation (> 95% of smooth muscle cells), as confirmed by immunostaining with a monoclonal antibody for smooth muscle α-actin. Confluent rat aortic smooth muscle cells were detached using trypsin/EDTA, and propagated in DME medium supplemented with 15% fetal cs, 50 IU/ml penicillin, and 50 µg/ml streptomycin. Cells from passages 2–4 were used in the present study. Each measure was performed in 25 cm^2 flask containing 2.5×10^6 just-confluent smooth muscle cells.

Except when specified, all reagents were purchased from Sigma (St. Louis, MO): superoxide dismutase (ref. S-2515), catalase (ref. C-40), 5,5-dimethyl-1 pyrroline-N-oxide (DMPO), diethylenetriaminepentaacetic acid (DTPA, ref. D-751) and activated charcoal. DMPO was purified before use by treatment with activated charcoal for 15 min as reported in the literature,[25] passed through a membrane filter (0.2 µm, Sartorius), aliquoted, protected from light and stored frozen at −20°C.

ESR Measurements

BAEC cultured in a 25 cm^2 flask were washed with PBS, and then incubated with a mix containing 150 mM DMPO, 5 mM glucose, 2 mM $CaCl_2$, 15 µM DTPA, 2 mM NaCl, 5 mM KCl in sodium phosphate buffer (19 mM NaH_2PO_4/53 mM Na_2HPO_4, pH 7.4), A23187 for 15 min (except for the dose–response runs). The supernatant was then transferred to a flat quartz cell which was inserted in a TM 110 Bruker cavity. ESR spectra were recorded at room temperature with an ER 200 D Bruker spectrometer by starting a 3 min scan 5 min after the end of the incubation with the cells. The ESR spectrometer operated at 9.66 GHz with high frequency at 100 kHz, modulation amplitude 1 Gauss, time constant 0.5 s, microwave power 10 mW, field: mid-range at 3500 Gauss, scan range 20 Gauss/min. The intensity of the ESR signal was calculated by adding the height of the four peaks, and expressed in arbitrary units.

Statistical Analysis

The data are expressed as mean ± standard error. Comparisons of data between 2 groups were made using Student's *t*-test and between more than two groups by ANOVA and a Scheffe's post-hoc test when appropriate. p values < 0.05 were considered significant.

RESULTS

Characterization of the ESR Signals Detected using DMPO as Spin Trap

The ESR signal given by unstimulated (basal) cells was less than 2-fold the baseline (Figure 1B). After 15 min incubation of DMPO with BAEC stimulated by the calcium ionophore A23187 (10 µM), a typical ESR signal was obtained resulting from the DMPO-OH adduct (Figure 1A). This adduct could result from hydroxyl radical trapping by DMPO in the extracellular medium. However, it is well known that the DMPO-OOH adduct, resulting from the trapping of $O_2^{-\bullet}$, decomposes rapidly into a more stable adduct: DMPO-OH.[26] The effects of superoxide dismutase and catalase were tested as previously reported[27,28] to identify the reactive oxygen intermediate released by the BAEC and initially trapped by DMPO. When superoxide dismutase 30 U/ml was coincubated, the ESR signal was completely suppressed ($n = 3$, Figure 1C). Denatured superoxide dismutase (15 min boiling) did not affect the ESR signal ($n = 3$, not shown). In contrast, coincubation of DMPO, catalase (2000 U/ml) and A23187 (10 µM) did not alter the morphology or the amplitude of the ESR signal given by BAEC ($n = 3$, Figure 1D). The catalase we used has been reported not to be contaminated by superoxide dismutase-like impurities.[29] We verified the selectivity of catalase, which did not

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/19/11
For personal use only.

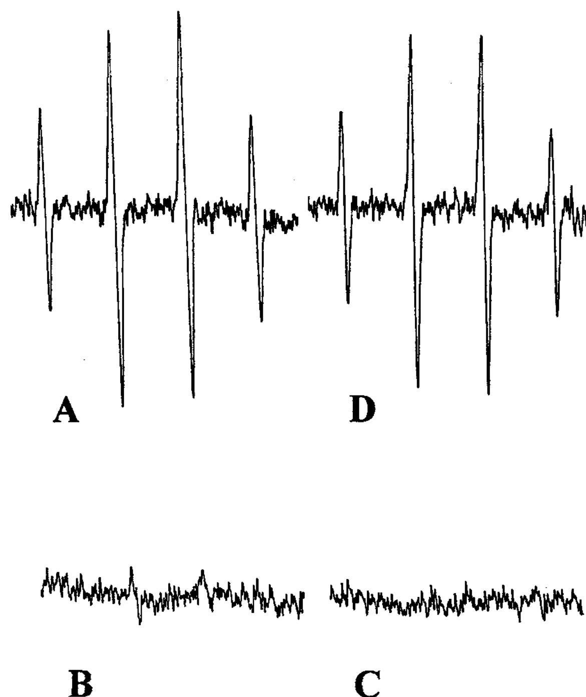

FIGURE 1 Effect of A23187 on the ESR spectra obtained from BAEC. The cells (2.5×10^6) were incubated in the presence of the spin trap DMPO and stimulated with (A) or without (B) 10 µM A23187 for 15 min. In parallel experiments, cells were coincubated with (C) A23187 and 30 U/ml superoxide dismutase or with (D) A23187 and catalase (2000 U/ml) as described in Materials and Methods.

affect the DMPO-OOH signal generated by the xanthine/xanthine oxidase system, as previously reported.[30] We have performed a control for a possible $OH^{\bullet}$ radical involvement using dimethylsulfoxide (DMSO). However, coincubation of BAEC with DMPO, DMSO 5% and A23187 (10 µM) did not decrease the amplitude of the DMPO-OH signal neither did alter the morphology of the signal (the spin-adduct DMPO-CH3 was not detected). Altogether, these results demonstrate that the ESR adduct DMPO-OH detected in the supernatant of BAEC originated from the trapping of extracellular $O_2^{-\bullet}$.

Regulation of $O_2^{-\bullet}$ Generation

The time-course of the ESR signal intensity was studied with 3 and 10 µM A23187 (Figure 2). The

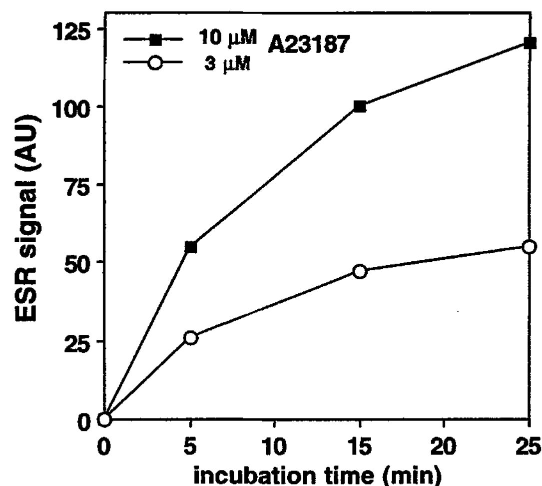

FIGURE 2 Time-course of A23187 (3 and 10 µM) for the intensity of the ESR signal (AU, arbitrary units) from BAEC. Data are the average of triplicate incubations, and representative of 2 different experiments.

signal was already detectable after 5 min after which the ESR signal intensity continued to increase, but more slowly. A 15 min incubation time was used in subsequent studies.

The intensity of the ESR signal increased dose-dependently in response to A23187 (Figure 2). The effect of superoxide dismutase and catalase on the ESR signal produced from BAEC stimulated by 3 µM A23187 was similar to that described above with 10 µM A23187 (not shown).

The effect of the coenzymes, NADH and NADPH, was also tested. NADH (100 µM) doubled the unstimulated (basal) $O_2^{-\bullet}$ production, as well as the A23187-stimulated $O_2^{-\bullet}$ production (Figure 3). NADPH (100 µM) increased both unstimulated (basal) and A23187-stimulated $O_2^{-\bullet}$ production by about 50% (Figure 3).

Diphenylene iodonium (DPI, 30 µM), a flavin antagonist, completely suppressed the unstimulated (basal) and the A23187-stimulated ESR signal (not shown). Once the DMPO-OOH adduct or the DMPO-OH adduct has been generated by the xanthine/xanthine oxidase system,[25] no alteration was induced by subsequent addition of 100 µM DPI (3-fold the concentration used in our study). When cells were preincubated with

RIGHTSLINK

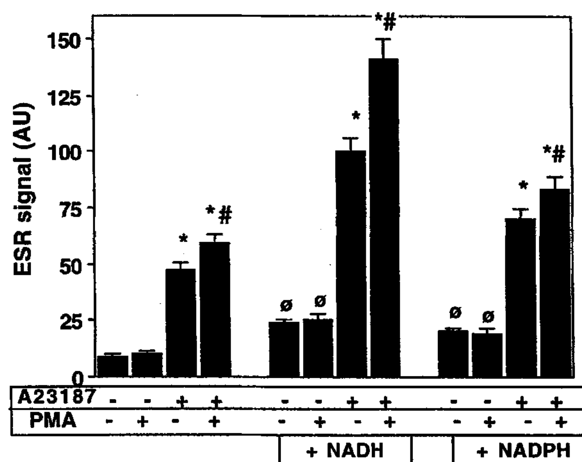

FIGURE 3 Effect of the calcium ionophore A23187 (3 μM), the phorbol ester PMA (1 μM), NADH (100 μM) and NADPH (100 μM) on the intensity of the ESR signal (AU, arbitrary units) from BAEC (2.5×10^6). Data are the mean ± SE of sixplicate post-confluent BAEC incubations. *$p < 0.05$ vs respective basal (unstimulated) BAEC, #$p < 0.05$ vs respective A23187-stimulated cells, ø $p < 0.05$ vs basal (first column) BAEC.

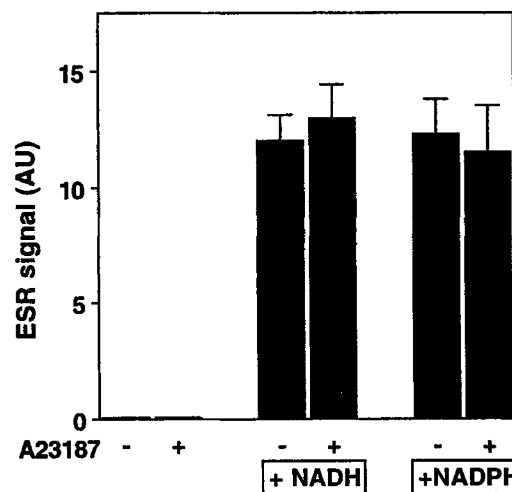

FIGURE 4 Effect of the calcium ionophore A23187 (3 μM), NADH (100 μM) and NADPH (100 μM) on the intensity of the ESR signal (AU, arbitrary units) from smooth mucle cells (2.5×10^6). Data are the mean ± SE of sixplicate confluent smooth muscle cells incubations. Note that the scale is 10-fold smaller.

30 μM DPI for 15 min, and when the cells were then incubated in the buffer containing A23187 and DMPO but not DPI, the production of $O_2^{-\bullet}$ was still inhibited. The DMPO-OH signal elicited by A23187 and NADH in BAEC was also completely inhibited by DPI (not shown).

The effect of an activator of protein kinase C, phorbol 12-myristate 13-acetate (PMA, 1 μM) and of an inhibitor of protein kinase C, bisindolylmaleimide I (5 μM, 15 min preincubation) was evaluated. Whereas PMA alone did not alter $O_2^{-\bullet}$ generation as detected by ESR, it slightly but significantly potentiated the stimulatory effect of A23187, both in the presence and absence of NADH or NADPH (Figure 3). Conversely, preincubation of BAEC with bisindolylmaleimide I for 15 min decreased the ESR signal by 55%. These data suggest that protein kinase C activation is involved in the $O_2^{-\bullet}$ generation. Finally, preincubation with the xanthine oxidase inhibitor allopurinol (120 μM) for 20 h did not alter the DMPO-OH signal elicited by A23187, either in the presence or absence of NADH (not shown).

Effect of Arginine-depletion and of a NO-synthase Inhibitor on $O_2^{-\bullet}$ Production in Just- and Post-confluent BAEC

The NO-synthase inhibitor L-NAME (N^w-nitro-L-arginine methyl ester, 100 μM) did not alter $O_2^{-\bullet}$ generation induced by A23187 in BAEC (L-NAME: 52 ± 5 AU vs control: 50 ± 4 AU, NS). BAEC were preincubated for 3 h in a medium with or without 1 mM L-arginine. We have previously shown that 3 h preincubation in arginine-free medium reduces the intra-cellular arginine to about 80–100 μM.[31] Arginine depletion did not affect the A23187-induced ESR signal (54 ± 7 AU vs control: 50 ± 4 AU, NS). These data do not suggest that NO-synthase activity influences $O_2^{-\bullet}$ generation.

Comparison with Rat Aortic Smooth Muscle Cells

Under the same conditions, rat aortic smooth muscle cells stimulated with 10 μM A23187 for 15 min did not induce any detectable ESR signal, whatever the confluency (Figure 4). However,

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/19/11
For personal use only.

RIGHTSLINK

NADH (100 μM) and NADPH (100 μM) induced a detectable ESR signal in unstimulated (basal) $O_2^{-\bullet}$ production, as well as in A23187-stimulated rat aortic smooth muscle cells (Figure 4). However, the amplitude of the signal was 5-fold (when the $O_2^{-\bullet}$ production is indexed to cell number) to 10-fold (when the $O_2^{-\bullet}$ production is indexed to cell surface) less than that of the 10 μM A23187-stimulated BAEC. DPI (30 μM) completely suppressed NADH and NADPH-elicited ESR signal (not shown).

DISCUSSION

The major findings of the present study are: (1) Extracellular $O_2^{-\bullet}$ production of calcium ionophore-stimulated BAEC can be detected using ESR; (2) This calcium ionophore A231987 is a potent stimulus of this $O_2^{-\bullet}$ production, which is also dependent on protein kinase C activation; (3) Both basal and A23187-stimulated production are potentiated by adding NADH or NADPH, and are completely inhibited by the flavin antagonist DPI, suggesting that an NADH/NADPH oxidase system accounts for this extracellular $O_2^{-\bullet}$ production; (4) The inhibition of NO-synthase activity does not alter $O_2^{-\bullet}$ production; and (5) Finally, the amount of $O_2^{-\bullet}$ generated by stimulated BAEC was one order of magnitude higher than that evoked by rat aortic smooth muscle cells stimulated under the same conditions.

Few studies have reported the generation of $O_2^{-\bullet}$ by vascular endothelial cells using the spin trap DMPO. Rosen and Freeman[20] used the EPR technique to detect $O_2^{-\bullet}$ in endothelial cells exposed to menadione, an uncoupling agent of the cellular reductases, and Zweier *et al.*[21] detected $O_2^{-\bullet}$ in endothelial cells on reoxygenation after anoxia. These studies did not however explore the capacity of BAEC to generate $O_2^{-\bullet}$ under physiological conditions (normoxia). Because $NO^\bullet$ and $O_2^{-\bullet}$ both contain an unpaired electron, they react rapidly together to form peroxynitrite. $O_2^{-\bullet}$ is thus recognized to be a powerful inactivator of the messenger $NO^\bullet$, and the extracellular release of $O_2^{-\bullet}$ could further decrease the half-life of $NO^\bullet$ *in vivo*.[32] $O_2^{-\bullet}$ is known to react rapidly with $NO^\bullet$ in solutions, the rate constant, k, being $6.7 \times 10^9\,M^{-1}s^{-1}$, which is about 3-times higher than that of SOD-catalyzed dismutation ($2 \times 10^9\,M^{-1}\,s^{-1}$).[32] Thus, we can speculate that the $O_2^{-\bullet}$ production generated by endothelium helped to shorten the half-life of $NO^\bullet$, thereby enabling this radical to fulfill its role of local messenger.

Conversely, $O_2^{-\bullet}$ was acknowledged as early as 1986 to inactivate EDRF.[33,34] We therefore tried to determine whether or not endogenous $NO^\bullet$ influenced $O_2^{-\bullet}$ production by studying the effect of intracellular arginine depletion on $O_2^{-\bullet}$ generation. This treatment could alter $O_2^{-\bullet}$ generation in at least two ways: (1) it might decrease $NO^\bullet$ production by decreasing the availability of NO-synthase substrate. We and others have already shown that arginine depletion slightly decreases $NO^\bullet$ production in response to a calcium ionophore;[31] (2) arginine depletion might also induce the generation of $O_2^{-\bullet}$ from NO synthase.[35–37] Although this mechanism has never been reported for endothelial NO synthase, it has been demonstrated both *in vitro* and in cells for the neuronal isoform.[35–37] We could not however detect any effect of arginine depletion on $O_2^{-\bullet}$ generation from BAEC using ESR, possibly due to the capacity of endothelial cells to recycle citrulline into arginine, thus avoiding the extensive depletion of NO-synthase substrate.[38] In addition, we did not detect any effect of L-NAME, an inhibitor of NO-synthase, on $O_2^{-\bullet}$ generation from BAEC. It has previously been reported that neither the basal nor acetylcholine-stimulated production of endogenous $NO^\bullet$ is sufficient to reduce $O_2^{-\bullet}$ levels.[9,39,40] These studies and the present data both support the idea that the amount of endothelium-derived $NO^\bullet$,[41] although quite detectable in these cultured BAEC, does not significantly alter the released $O_2^{-\bullet}$.

Phagocytic cells, such as polymorphonuclear cells or monocytes and macrophages, generate large amounts of $O_2^{-\bullet}$ through activation of the multimeric plasma membrane enzyme NADPH

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/19/11
For personal use only.

RIGHTS LINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/19/11
For personal use only.

oxidase. More recently, it has been reported that non-phagocytic cells, such as fibroblasts or vascular smooth muscle cells, also generate $O_2^{-\bullet}$,[42,43] at least in part to an NADH/NADPH oxidase system. However, the molecular identity of the NADH/NADPH oxidase system present in vascular smooth muscle is only partially understood. Ushio-Fukai *et al.*[8] provided the initial evidence that p22phox was a critical component of the $O_2^{-\bullet}$-generating NADH/NADPH oxidase system in vascular smooth muscle cells. The nonphagocytic and phagocytic oxidases nevertheless appear to be structurally and functionally different.[43]

Mohazzab-H *et al.*[11] have previously shown that an NADH-oxidase system accounts for most of the $O_2^{-\bullet}$ generated in cultured bovine coronary endothelial cells. Jones *et al.*[44] demonstrated that cultured human endothelial cells express both mRNA and protein for cytosolic components of the phagocyte NADPH oxidase, but, as they could not detect the cytochrome b558 heme, they concluded that contribution of the phagocyte NADPH oxidase to $O_2^{-\bullet}$ generation would be unlikely. In the present study, the endothelial NADH/NADPH oxidase system appears to preferentially utilize NADH rather than NADPH, and to be completely inhibited by DPI, thus suggesting the absolute requirement for a flavin. These characteristics are in agreement with those reported previously using lucigenin-enhanced in chemiluminescence in bovine coronary endothelial cells[11] and in human endothelial cell homogenates.[44] Furthermore, in contrast to the phagocytic NADPH oxidase, calcium ionophore is a powerful stimulus of the endothelial NADH/NADPH oxidase system, compared to phorbol myristate acetate which seems to be weak. This profile of production is very similar to that reported in cultured human fibroblasts.[43] In the present study, the xanthine oxidase inhibitor allopurinol did not alter the extracellular $O_2^{-\bullet}$ generation. However, xanthine oxidoreductase, and in particular the dehydrogenase form of the enzyme, can generate $O_2^{-\bullet}$ from NADH.[45,46] Furthermore, this generation appears to be inhibited by DPI and not by allopurinol. Thus, although xanthine oxidoreductase is an intracellular enzyme, we cannot exclude that part of the BAEC extracellular $O_2^{-\bullet}$ may be generated by this enzyme.

What is the major source of extracellular $O_2^{-\bullet}$ in the vessel wall? The present work suggest that, at least in culture conditions, the endothelium appears to generate far more extracellular $O_2^{-\bullet}$ than the vascular smooth muscle. This *in vitro* study is in agreement with an *in vivo* study in rat aorta, which demonstrated that $O_2^{-\bullet}$ generation was decreased by endothelium removal and DPI, unaltered by NO synthase inhibition, and increased more with NADH than with NADPH.[39] These results are somewhat different from those obtained in rabbit aorta, in which $O_2^{-\bullet}$ generation was decreased by DPI and unaltered by NO-synthase inhibition, but where $O_2^{-\bullet}$ generation was not reduced, or was even increased, by endothelium removal, and was mainly dependent on NADPH.[9,47,48] Thus, considerable differences may exist between species, and bovine endothelium (present study) appears to resemble human endothelium more closely[44] in this respect than rabbit endothelium.[9,47,48]

Li *et al.* concluded that lucigenin-derived chemiluminescence still appeared to be a valid probe for detecting $O_2^{-\bullet}$ production by enzymatic and cellular sources.[49] It should be noted that the previously reported regulations of $O_2^{-\bullet}$ production by vascular endothelial cells using lucigenin-enhanced chemiluminescence[9–11] appear to parallel those of the present study using using DMPO and ESR spectroscopy. In any case, because of the difficulty in reliably assessing $O_2^{-\bullet}$ production in biological systems, we believe that the technique described herein will provide a useful tool for subsequent studies.

References

[1] J.M. McCord and I. Fridovich (1969) Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry*, **244**, 6049–6055.

[2] R.F. Furchgott and P.M. Vanhoutte (1989) Endothelium-derived relaxing and contracting factors. *FASEB Journal*, **3**, 2007–2018.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/19/11
For personal use only.

[3] L. Ignarro, G. Buga, K. Wood, R. Byrns and G. Chanduri (1987) Endothelial derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences of the U.S.A.*, **84**, 9265–9269.

[4] R.M.J. Palmer, A.G. Ferrige and S. Moncada (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature (Lond.)*, **327**, 524–526.

[5] O.W. Griffith and D.J. Stuehr (1995) Nitric oxide synthases: properties and catalytic mechanism. *Annual Review of Physiology*, **57**, 707–736.

[6] C. Nathan and Q.W. Xie (1994) Regulation of biosynthesis of nitric oxide. *Journal of Biological Chemistry*, **269**, 13725–13728.

[7] K. Griendling and R.W. Alexander (1996) Endothelium control of the cardiovascular system: recent advances. *FASEB Journal*, **10**, 283–292.

[8] M. Ushio-Fukai, A.M. Zafari, T. Fukui, N. Ishizaka and K.K. Griendling (1996) p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulate angiotensin II-induced activity hypertrophy in vascular smooth muscle cells. *Journal of Biological Chemistry*, **271**, 23317–23321.

[9] P.J. Pagano, K. Tornheim and R.A. Cohen (1993) Superoxide anion production by rabbit thoracic aorta: effect of endothelium-derived nitric oxide. *American Journal of Physiology*, **265**, H707–H712.

[10] N.W. Kooy and J.A. Royall (1994) Agonist-induced peroxynitrite production from endothelial cells. *Archives of Biochemistry and Biophysics*, **310**, 352–359.

[11] K. Mohazzab-H, P. Kaminski and M. Wolin (1994) NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *American Journal of Physiology*, **266**, H2568-H2572.

[12] T. Münzel, H. Sayegh, B.A. Freeman, M.M. Tarpey and D.G. Harrison (1995) Evidence for enhanced vascular superoxide anion production in nitrate tolerance: A novel mechanism underlying tolerance and cross-tolerance. *Journal of Clinical Investigation*, **95**, 187–194.

[13] S.I. Liochev and I. Fridovich (1997) Lucigenin luminescence as a measure of intracellular superoxide dismutase activity in Escherichia coli. *Proceedings of the National Academy of Sciences of the U.S.A.*, **94**, 2891–2896.

[14] S.I. Liochev and I. Fridovich (1997) Lucigenin (Bis-N-methylacridinium) as a mediator of superoxide anion production. *Archives of Biochemistry and Biophysics*, **337**, 115–120.

[15] J. Vasquez-Vivar, N. Hogg, K.A. Pritchard Jr, P. Martasek and B. Kalyanaraman (1997) Superoxide anion formation from lucigenin: an electron spin resonance spin-trapping study. *FEBS Letters*, **403**, 127–130.

[16] T. Matsubara and M. Ziff (1986) Superoxide anion release by human endothelial cells: synergism between a phorbol ester and a calcium ionophore. *Journal of Cellular Physiology*, **127**, 207–210.

[17] U.P. Steinbrecher (1988) Role of superoxide in endothelial-cell modification of low-density lipoproteins. *Biochimica and Biophysica Acta*, **959**, 20–30.

[18] J.A. Holland, K.A. Pritchard, M.A. Pappolla, M.S. Wolin, N.J. Rogers and M.B. Stemerman (1990) Bradykinin induces superoxide anion release from human endothelial cells. *Journal of Cellular Physiology*, **143**, 21–25.

[19] H. Murphy, J. Shayman, G. Till, M. Mahroughi, C. Owens, U. Ryan and P. Ward (1992) Superoxide responses of endothelial cells to C5a and TNF-a: divergent signal transduction pathways. *American Journal of Physiology*, **263**, L51–59.

[20] G.M. Rosen and B.A. Freeman (1984) Detection of superoxide generated by endothelial cells. *Proceedings of the National Academy of Sciences of the U.S.A.*, **81**, 7269–7273.

[21] J.L. Zweier, P. Kuppusamy and G.A. Lutty (1988) Measurement of endothelial cell free radical generation: evidence for a central mechanism of free radical injury in post ischemic tissues. *Proceedings of the National Academy of Sciences of the U.S.A.*, **85**, 4046–4050.

[22] S. Sanders, S. Harrison, P. Kuppusamy, J. Sylvester and J. Zweier (1994) A comparative study of ESR spin trapping and cytochrome c reduction techniques for the measurement of superoxide anions. *Free Radical Biology and Medicine*, **16**, 753–761.

[23] D. Gospodarovicz, J. Moran, D. Braun and C.R. Birdwell (1976) Clonal growth of bovine endothelial cells in tissue culture : Fibroblast growth factor as a survival agent. *Proceedings of the National Academy of Sciences of the U.S.A.*, **73**, 4120–4124.

[24] T. Battle, J.F. Arnal, M. Challah and J.B. Michel (1994) Selective isolation of rat aortic wall layers and their cell types in culture – application to converting enzyme activity measurement. *Tissue and Cell*, **26**, 943–955.

[25] G.R. Buettner and L.W. Oberley (1978) Consideration in the spin trapping of superoxide and hydroxyl radicals in aqueous systems using 5,5-dimethyl-1-pyrroline-1-oxide. *Biochemical and Biophysical Research Communication*, **83**, 69–74.

[26] B. Halliwell and J.M.C. Gutteridge (1989) Detection of hydroxyl radicals in biological systems. In *Free Radicals in Biology and Medicine* 2nd Ed., Clarendon Press, Oxford, pp. 47–53.

[27] R.P. Mason and K.M. Morehouse (1994) Spin trapping – the ideal method for measuring oxygen radical formation? In *Free Radicals: Methodology and Concepts.* (C. Rice-Evans and B. Halliwell eds) Richelieu, London, pp. 157–168.

[28] E. Finkelstein, G.M. Rosen, E.J. Rauckman and J. Paxton (1979) Spin trapping of superoxide. *Molecular Pharmacology*, **16**, 676–685.

[29] R.V. Lloyd and R.P. Mason (1990) Evidence against transition metal-independent hydroxyl radical generation by xanthine oxidase. *Journal of Biological Chemistry*, **265**, 16733–16736.

[30] B.E. Britigan, S. Pou, G.M. Rosen, D.M. Lilleg and G. Buettner (1990) Hydroxyl radical is not a product of the reaction of xanthine oxidase and xanthine. *Journal of Biological Chemistry*, **265**, 17533–17538.

[31] J.F. Arnal, T. Münzel, R.C. Venema, N.L. James, C.L. Bay, W. Mitch and D.G. Harrison (1995) Interactions between L-arginine and L-glutamine modulate endothelial NO production: an effect independent of NO synthase substrate availability. *Journal of Clinical Investigation*, **95**, 2565–2572.

[32] J.S. Beckman and W.H. Koppenol (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *American Journal of Physiology*, **271**, C1424-C1437.

[33] R.J. Gryglewski, R.M.J. Palmer and S. Moncada (1986) Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature*, **320**, 454–456.

[34] G.M. Rubanyi and P.M. Vanhoutte (1986) Superoxide anion and hyperoxia inactivate endothelium-derived

RIGHTSLINK

relaxing factor. *American Journal of Physiology*, **250**, H822–H827.

[35] M. Culcasi, M. Lafon-Cazal, S. Pietri and J. Bockaert (1994) Glutamate receptors induce a burst of superoxide via activation of nitric oxide synthase in arginine-depleted neurons. *Journal of Biological Chemistry*, **269**, 12589–12593.

[36] S. Pou, W.S. Pou, D.S. Bredt, S.H. Snyder and G.M. Rosen (1992) Generation of superoxide by purified brain nitric oxide synthase. *Journal of Biological Chemistry*, **267**, 24173–24176.

[37] Y. Xia, V.L. Dawson, T.M. Dawson, S.H. Snyder and J.L. Zweier (1996) Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proceedings of the National Academy of Sciences of the U.S.A.*, **93**, 6770–6774.

[38] M. Hecker, W.C. Sessa, H.J. Harris, E.E. Anggard and J.R. Vane (1990) The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proceedings of the National Academy of Sciences of the U.S.A.*, **87**, 8612–8616.

[39] S. Rajagopalan, S. Kurz, T. Münzel, M.M. Tarpey, B.A. Freeman, K.K. Griendling and D.G. Harrison (1996) Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. *Journal of Clinical Investigation*, **97**, 1916–1923.

[40] R.P. Brandes, M. Barton, K.M.H. Philippens, G. Schweitzer and A. Mügge (1997) Endothelium-derived superoxide anions in pig coronary arteries: evidence from lucigenin chemiluminescence and histochemical techniques. *Journal of Physiology*, **500**, 331–342.

[41] J.F. Arnal, S. Clamens, C. Pechet, A. Nègre-Salvayre, C. Allera, J.P. Girolami, R. Salvayre and F. Bayard (1996) Ethinylestradiol does not enhance the expression of nitric oxide synthase in bovine aortic endothelial cells but increases the release of bioactive nitric oxide by inhibiting superoxide anion production. *Proceedings of the National Academy of Sciences of the U.S.A.*, **93**, 4108–4113.

[42] K.K. Griendling, C.A. Mineri, J.D. Ollerenshaw and R.W. Alexander (1994) Angiotensin II stimulates NADH and NADPH activity in cultured vascular smooth muscle cells. *Circulation Research*, **74**, 1141–1146.

[43] B. Meier, A.J. Jesaitis, A. Emmendorffer, L. Roesler and M.T. Quinn (1993) The cytochrome b-558 molecules involved in the fibroblast and polymorphonuclear leucocyte superoxide-generating NADPH oxidase systems are structurally and genetically distinct. *Biochemical Journal*, **289**, 481–486.

[44] S.A. Jones, V.B. O'Donnell, J.D. Wood, J.P. Broughton, E.J. Hughes and O.T.G. Jones (1996) Expression of phagocyte NADPH oxidase components in human endothelial cells. *American Journal of Physiology*, **271**, H1626-H1634.

[45] Z. Zhang, D. Blake, C. Stevens, J. Kanczler, P. Winyard, M. Symons, M. Benboubetra and R. Harisson (1998) A reappraisal of xanthine dehydrogenase and oxidase in hypoxic reperfusion injury: the role of NADH as an electron donor. *Free Radical Research*, **28**, 151–164.

[46] S. Sanders, R. Eisenthal and R. Harisson (1997) NADH oxidase activity of human xanthine oxidoreductase. Generation of superoxide anion. *European Journal of Biochemistry*, **245**, 541–548.

[47] Y. Ohara, T.E. Peterson and D.G. Harrison (1993) Hypercholesterolemia increases endothelial superoxide anion production. *Journal of Clinical Investigation*, **91**, 2546–2551.

[48] P.J. Pagano, I. Yasushi, K. Tornheim, P.M. Gallop, A.I. Tauber and R.A. Cohen (1995) An NADPH oxidase superoxide-generating system in the rabbit aorta. *American Journal of Physiology*, **268**, H2274–H2280.

[49] Y. Li, H. Zhu, P. Kuppusamy, V. Roubaud, J. Zweier and M. Trush (1998) Validation of lucigenin (Bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *Journal of Biological Chemistry*, **273**, 2015–2023.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/19/11
For personal use only.

RIGHTSLINK