High rates of extracellular superoxide generation by cultured human fibroblasts: involvement of a lipid-metabolizing enzyme

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Expression of NADPH oxidase and low superoxide generation (approx. 0.06 nmol/min per 10^6 cells) by cytokine- or ionophore-stimulated human fibroblasts is known. However, we here show that these cells also contain an ectoplasmic enzyme, distinct from NADPH oxidase, which can generate superoxide (2.19 ± 0.14 nmol/min per 10^6 cells) at levels similar to phorbol ester-stimulated monocytes on exogenous NADH addition. Superoxide generation was temperature-dependent, insensitive to chelation (desferal), and had a K_m(app)(NADH) of 11.5 μM. Inhibitor studies showed that there was no involvement of NADPH oxidase (diphenylene iodonium, diphenyl iodonium), prostaglandin H synthase (indomethacin), xanthine oxidase (allopurinol), cytochrome *P*-450 (metyrapone) or mitochondrial respiration (rotenone, antimycin A). NAD^+ was a competitive inhibitor, whereas NADPH supported 40 % of the rate seen with NADH. No luminescence was observed after the addition of lactate, malate, pyruvate, GSH or L-cysteine. NADH-stimulated superoxide generation was enhanced by the addition of (3–30 μM) arachidonic acid, linoleic acid or (5*S*)-hydroxyeicosatetraenoic acid [(5*S*)-HETE] but not palmitic acid, (15*S*)-hydroperoxyeicosatetraenoic acid [(15*S*)-HPETE], (15*S*)-HETE or (12*S*)-HETE. Several features suggest involvement of an enzyme related to 15-lipoxygenase, and, in support of this, we show superoxide generation and NADH oxidation by recombinant rabbit reticulocyte 15-lipoxygenase. The large amounts of superoxide measured suggest that the fibroblast extracellular enzyme could be a major source of reactive oxygen species after tissue damage.

INTRODUCTION

Extracellular superoxide generation is primarily associated with phagocyte defence against disease. In both neutrophils and monocytes, this is accomplished by a trans-plasma-membrane enzyme, NADPH oxidase, which oxidizes intracellular NADPH *via* FAD and cytochrome b_{-245} and reduces oxygen on the outer surface to form superoxide. Typical rates of superoxide generation for NADPH oxidase of isolated neutrophils and monocytes are 9–10 nmol/min per 10^6 cells and 1.8 nmol/min per 10^6 cells respectively (see ref. [1] for a review). Generation of low levels of superoxide by several non-phagocytic cell types, including endothelial cells [2], fibroblasts [3,4] and mesangial cells [5,6], has been described. In common with phagocytes, this requires activating stimuli such as cytokines, phorbol esters and calcium ionophores, but it only reaches 1 % of the oxidant generation of activated neutrophils. The involvement of an NADPH oxidase-like enzyme has been suggested since components of this system (cytochrome b_{-245}, and mRNA transcripts of protein) have been detected in many cases [4,7–9]. The physiological reason for NADPH oxidase expression in non-phagocytic cells is not known.

Oxidation of external NAD(P)H by oxygen has been shown for several diverse cellular systems, including corn root protoplasts [10], blood platelets [11], osteoclasts [12], erythrocytes and malaria parasites [13]. Recently, several studies have also demonstrated the presence of NAD(P)H-dependent superoxide- or hydrogen peroxide-generating enzymes in cellular homogenates (smooth muscle, endothelial) [14–16]. So far, neither the source nor function of this superoxide or hydrogen peroxide is known.

Low levels of reactive oxygen species (ROS) have non-toxic effects on physiological processes which can lead to alterations in intracellular signalling [17], gene expression [18] and cell proliferation [3]. Numerous signalling pathways are known to be affected by ROS, for example arachidonate metabolism enzymes [19], protein kinases [19,20] and transcription factors [21]. Therefore continuous exposure to low levels of ROS could have major consequences on normal cell growth and differentiation. The involvement of oxidant stress has been suggested for several disease states, for example neurological (Alzheimer's disease, Parkinson's disease [22,23]), viral (HIV and AIDS expression [24]) and degenerative (atherosclerosis, cancer, cataract). The identification of potential sources of ROS in cellular systems is therefore of major importance.

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise stated. (15*S*)-Hydroperoxyeicosatetraenoic acid [(15*S*)-HPETE], (15*S*)-hydroxyeicosatetraenoic acid [(15*S*)-HETE], (12*S*)-HETE, (5*S*)-HETE were from Cascade Ltd. (Reading, Berks., U.K.). Xanthine oxidase (buttermilk) was from Boehringer-Mannheim (Schweiz, AG). Trolox was from Roche (Basel, Switzerland). Diphenyl iodonium was from Fluka Chemie AG (Buchs, Switzerland). Diphenylene iodonium was a gift from Professor O. T. G. Jones (University of Bristol, Bristol, U.K.). Desferal (desferrioxamine) was a gift from Ciba-Geigy (Basel, Switzerland). Recombinant rabbit reticulocyte 15-lipoxygenase was a gift from Dr. H. Kühn (Humboldt-Universität, Berlin, Germany).

Cell culture

Human skin fibroblasts (CCD-39sk) obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) were routinely

Abbreviations used: HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; ROS, reactive oxygen species; SOD, superoxide dismutase; PMA, phorbol 12-myristate 13-acetate; NDGA, nordihydroguaiaretic acid.

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cultured in modified Eagle's medium containing 10 % foetal calf serum and penicillin/streptomycin (200 units/ml, 100 units/ml). Cells were never cultured beyond passage 18. After trypsin treatment, cell viability was assessed by Trypan Blue exclusion.

Chemiluminescence

Lucigenin-enhanced chemiluminescence was measured using the system described [25]. Light was detected by single photon counting (accumulated over 0.9 s intervals) using a photomultiplier (model 9635; EMI) operated on high voltage (810 V) and coupled via a 0.01 nF capacitor to an amplifier/discriminator and photon counter (Princeton Applied Research, Princeton, NJ, U.S.A.; models 1120 and 1109). All reactions (except where stated) were carried out with stirring at 37 °C. Additions of substrates and inhibitors as indicated in Figure legends were made via a light-tight access port using a 10 μl Hamilton syringe during the assay period. Where original chemiluminescence data are shown, results are representative of a typical experiment carried out several times on the same cell isolate and repeated on different occasions on at least two additional isolates.

Lucigenin-enhanced chemiluminescence of xanthine oxidase, human fibroblasts, phorbol-stimulated monocytes and rabbit 15-lipoxygenase

Xanthine oxidase

For calibration of cell-mediated superoxide-generation rates, xanthine oxidase was added to 1 ml of Krebs-Ringer buffer (20 mM Hepes, 5 mM D-glucose, 127 mM NaCl, 5.5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, pH 7.4) containing lucigenin (200 μM) and xanthine (500 μM), and the chemiluminescence response recorded as described above, before and after the addition of superoxide dismutase (SOD; 40 μg). For calculation of lipoxygenase-mediated superoxide generation rates, separate calibrations were performed in phosphate (0.1 M, pH 7.4) containing sodium cholate (0.2 %) at 20 °C. Protein content of xanthine oxidase was measured using the method of Bradford [26].

Fibroblasts

Adherent fibroblasts were treated with trypsin, gently washed, and resuspended in Krebs–Ringer buffer. At this point, viability was always greater than 98.5 % as judged by Trypan Blue exclusion. Cells were kept on ice until assayed and used within 2 h of harvesting. For chemiluminescence, 2×10^5–10×10^5 cells were added to 1 ml of prewarmed (37 °C) Krebs–Ringer buffer containing lucigenin (200 μM). For chemiluminescence of adherent fibroblasts, cells were seeded on to collagen-precoated plastic strips (custom designed for these cuvettes) and allowed to adhere overnight before assay. Luminescence was measured before and after the addition of SOD (20–40 μg).

Monocytes

Monocytes (2.5×10^5 per assay) isolated as previously described [27] were added to 1 ml of Krebs-Ringer buffer containing lucigenin (200 μM). Rates of luminescence were monitored before and after the addition of phorbol 12-myristate 13-acetate (PMA) (160 nM) and SOD (40 μg/ml).

Recombinant rabbit reticulocyte lipoxygenase

Enzyme (2.8 μg) was added to 1 ml of phosphate buffer (0.1 M, pH 7.4) containing sodium cholate (0.2 %) and linoleate (575 μM) with or without NADH (100–300 μM) at 20 °C with stirring. As a negative control, SOD (40 μg/ml) was added to samples before the enzyme. Before use, phosphate buffer was pretreated with chelating resin by the batch method to remove adventitious metal ions [28].

Superoxide generation measured using cytochrome *c* reduction

Xanthine oxidase

Activity was measured by the SOD-sensitive rate of reduction of cytochrome *c* using an Aminco DW/2a spectrophotometer. Briefly, small amounts of enzyme were added to 2 ml of Krebs–Ringer buffer containing cytochrome *c* (50 μM) and xanthine (500 μM) and absorbance was monitored at 550–540 nm using $\epsilon = 19.1\ mM^{-1} \cdot cm^{-1}$ [29]. SOD (40 μg/ml) was added at the end and the residual rate subtracted.

Fibroblasts

Trypsin-treated cells (5×10^5) were added to 2 ml of Krebs–Ringer buffer containing cytochrome *c* (50 μM) at 37 °C with stirring. Reduction was monitored (as above) before and after the addition of NADH (100 μM) and SOD (40 μg/ml).

Monocytes

Cells (2.5×10^5) were added to 2 ml of Krebs buffer containing cytochrome *c* (50 μM) at 37 °C with stirring. Reduction was monitored (as above) before and after the addition of PMA (160 nM) and SOD (40 μg/ml).

Calculation of kinetic parameters

Chemiluminescence data were analysed using Enzfitter (R. J. Leatherbarrow, Elsevier-Biosoft, 1987).

Measurement of hydroperoxide content of arachidonic acid preparations

Total hydroperoxides of fatty acid stock solutions were determined using the modified iodometric method of Darrow and Organisciak [30].

NADH oxidation by intact fibroblasts and recombinant rabbit 15-lipoxygenase

Oxidation of NADH was measured at 340 nm using $\epsilon = 6.22\ mM^{-1} \cdot cm^{-1}$ [31].

Recombinant rabbit reticulocyte lipoxygenase (3.5 μg) was added to 1 ml of phosphate buffer (0.1 M, pH 7.4) containing sodium cholate (0.2 %), linoleate (575 μM) and NADH (200 μM) at 20 °C. Before use, phosphate buffer was pretreated with chelating resin by the batch method to remove adventitious metal ions [28].

Fibroblast monolayers (3.3×10^5 per well) in six-well plates were washed with Krebs–Ringer buffer then incubated at 37 °C with 1 ml of Krebs buffer containing NADH (200 μM). At the indicated times, buffer samples were removed and NADH concentrations measured.

RESULTS

Calibration of chemiluminescence

Lucigenin-enhanced chemiluminescence was calibrated using xanthine oxidase the activity of which has been determined $[2.19(\pm 0.14) \times 10^{-7}$ mol of superoxide produced/min per mg of

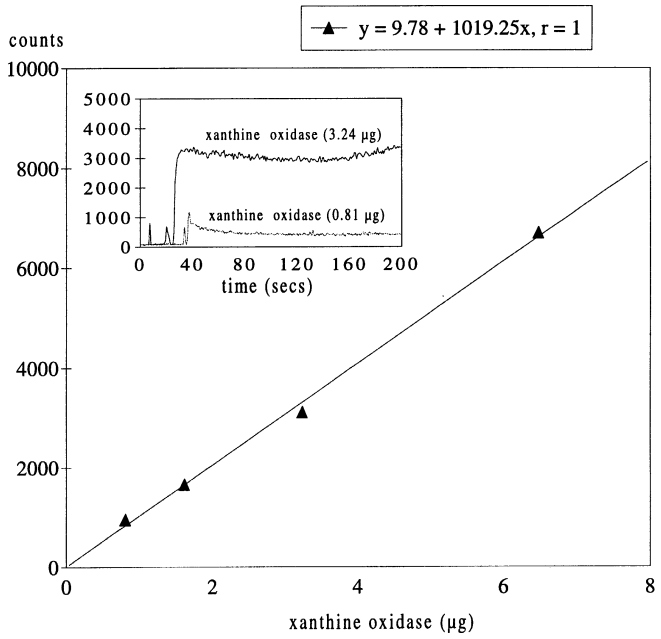


Figure 1 Calibration of chemiluminescent response using xanthine oxidase

Superoxide generation was assayed using lucigenin-enhanced chemiluminescence as in the Materials and methods section. Dilutions of enzyme were added to 1 ml of Krebs buffer (37 °C, stirring) with lucigenin (200 μM) and xanthine (500 μM), and the initial rate of photon emission was recorded ($n = 2$ for each dilution). Inset: example of xanthine oxidase assay using two different enzyme concentrations.

Table 1 Comparison of superoxide-generation rates of PMA-stimulated monocytes and fibroblasts + NADH

Superoxide generation was measured using SOD-inhibitable rates of cytochrome *c* reduction or lucigenin-enhanced chemiluminescence as described in the Materials and methods section. Rates for lucigenin chemiluminescence for monocytes and fibroblasts were estimated using the xanthine oxidase calibration curve (Figure 1). For fibroblasts and monocytes, rates were calculated on the same cell isolate within 1–2 h. Results are means ± S.D. ($n = 3$).

	Superoxide generation (nmol/min per 10^6 cells)	
	Lucigenin	Cytochrome *c*
Monocytes + PMA	3.1 ± 0.5	2.7 ± 0.33
Fibroblasts + NADH	0.64 ± 0.1	0.49 ± 0.05

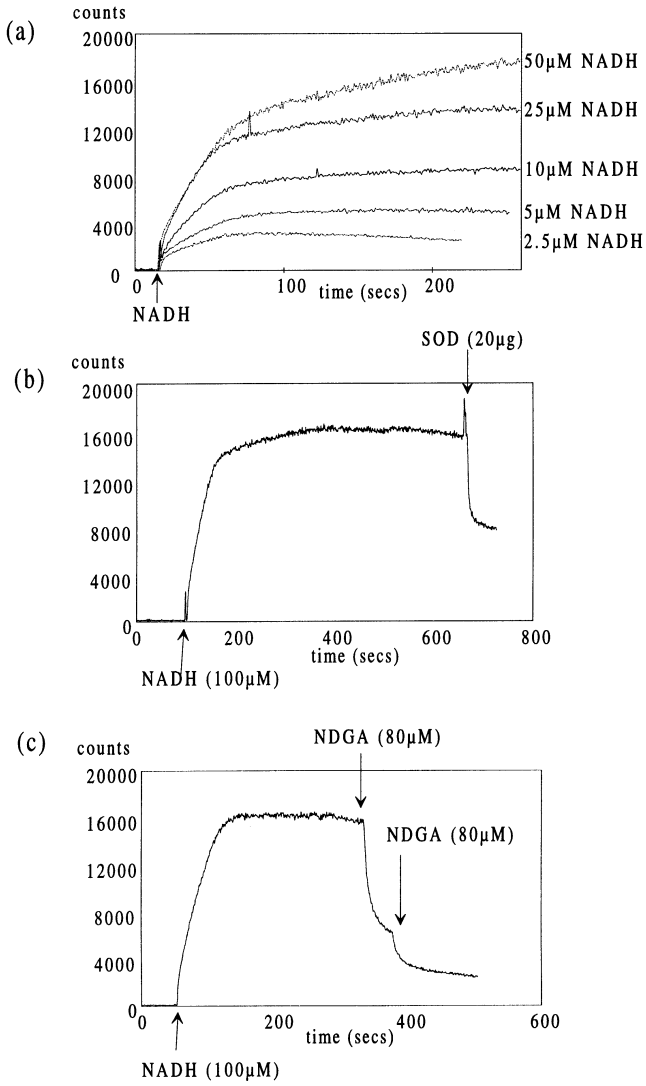


Figure 2 Fibroblast superoxide generation in response to exogenous NADH

Trypsin-treated cells (10^6) were added to prewarmed Krebs buffer containing lucigenin (200 μM) and placed in the chemiluminometer sample holder (stirring, 37 °C). Chemiluminescence was recorded before and after the addition of NADH. All additions were made via a light-tight port using a 10 μl Hamilton syringe. (**a**) Dose-dependence of chemiluminescence in response to NADH. (**b**) Effect of SOD on chemiluminescence. (**c**) Effect of the lipoxygenase inhibitor and radical scavenger nordihydroguairaretic acid (NDGA) on chemiluminescence.

protein; $n = 3$] by the SOD-sensitive rate of cytochrome *c* reduction. The addition of small amounts of enzyme to 1 ml of Krebs–Ringer buffer containing xanthine (500 μM) and lucigenin (200 μM) resulted in a rate of chemiluminescence which was linear over the range of enzyme concentrations tested (Figure 1 and inset), and was totally abolished by the addition of SOD (40 μg). To check our calibrations, rates of superoxide generation by PMA-stimulated monocytes were compared using both cytochrome *c* reduction and xanthine oxidase-calibrated lucigenin-enhanced chemiluminescence (Table 1). Recalibrations carried out at intervals demonstrated that the sensitivity of the luminometer did not change significantly over the course of the study (not shown).

Superoxide generation by human fibroblasts in response to NADH

After the addition of NADH to suspensions of intact human fibroblasts (2×10^5–10×10^5), light emission was observed which was immediate, dose-dependent (Figure 2a) and continued at a constant rate for at least 20 min (not shown). There was no response if cells or lucigenin were omitted from the cuvette. Chemiluminescence was 70–75 % inhibited by the addition of SOD (20–40 μg) (Figure 2b), but unaffected by catalase. Using the calibration curve (Figure 1), the rate of superoxide generation (SOD-sensitive chemiluminescence) was estimated to be 2.4 ± 0.75 nmol/min per 10^6 cells (mean ± S.D., pooling data from nine different isolates). In the absence of added NADH, there was no chemiluminescence above background levels. In-

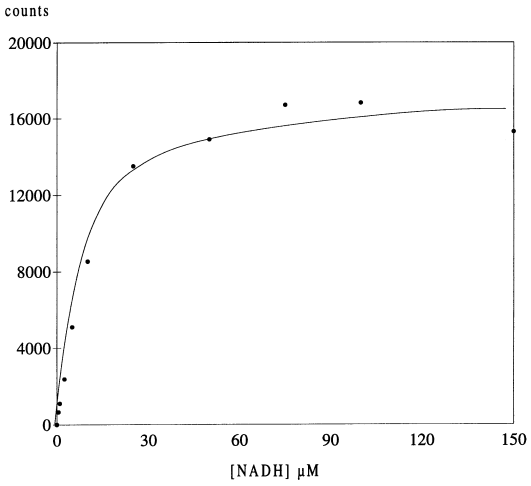


Figure 3 Calculation of Michaelis–Menten parameters

NADH-induced chemiluminescence of trypsin-treated cells (10^6 per sample) was measured as described for Figure 2. The maximum rate of photon emission (at 200–300 s) was recorded using several concentrations of NADH and K_m(app) calculated at 11.6 μM using Enzfitter (Biosoft), according to $v = V_{max}[S]/(K_m + [S])$, where v is velocity, S is substrate.

hibition of the residual SOD-insensitive chemiluminescence could be accomplished by the addition of the radical scavenger and lipoxygenase inhibitor, NDGA. In addition, NDGA could inhibit luminescence in the absence of SOD (Figure 2c). Partial inhibition was seen on use of the radical scavenger Trolox (results not shown). Inclusion of the metal chelator desferrioxamine (0.5 mM) had no effect on luminescence, indicating that redox cycling of free metal ions was unlikely to be involved. Cooling of the sample (approx. 15 °C) resulted in only 7% of the activity seen at 37 °C (not shown). These data suggest a direct involvement of cellular enzyme processes. By varying the concentration of NADH, we calculated K_m(app) for NADH at 11.6 μM (Figure 3).

Specificity of chemiluminescence for NAD(P)H and inhibition by NAD^+

To investigate for substrate specificity, superoxide generation was assessed after the addition of a variety of potential substrates to intact fibroblasts. NADPH (100 μM) caused light emission that was 40% of the NADH-stimulated rate (results not shown), whereas no response was seen with lactate (1 mM), pyruvate (1 mM), malate (1 mM) or the thiol ligands glutathione (100 μM) and L-cysteine (100 μM) (results not shown). The addition of NAD^+ did not lead to chemiluminescence; however, it was an efficient competitive inhibitor of the NADH-stimulated response, increasing K_m(app) to 30.8 μM at 240 μM NAD^+ with no effect on V_{max} (results not shown).

Effect of inhibitors of oxidant-generating enzymes

To assess the involvement of known cellular oxidant-generating systems, we tested a variety of inhibitors for effects on chemiluminescence. No inhibition was observed using rotenone (1 μM), allopurinol (100 μM), indomethacin (2 μM) or metyrapone (200 μM). Also, neither diphenylene iodonium (20 μM) nor diphenyl iodonium (200 μM) caused inhibition, whether added directly to turning-over enzyme (i.e. in the presence of NADH) or preincubated with cells for 20 min before assay. These data rule out any involvement of the mitochondrial respiratory chain,

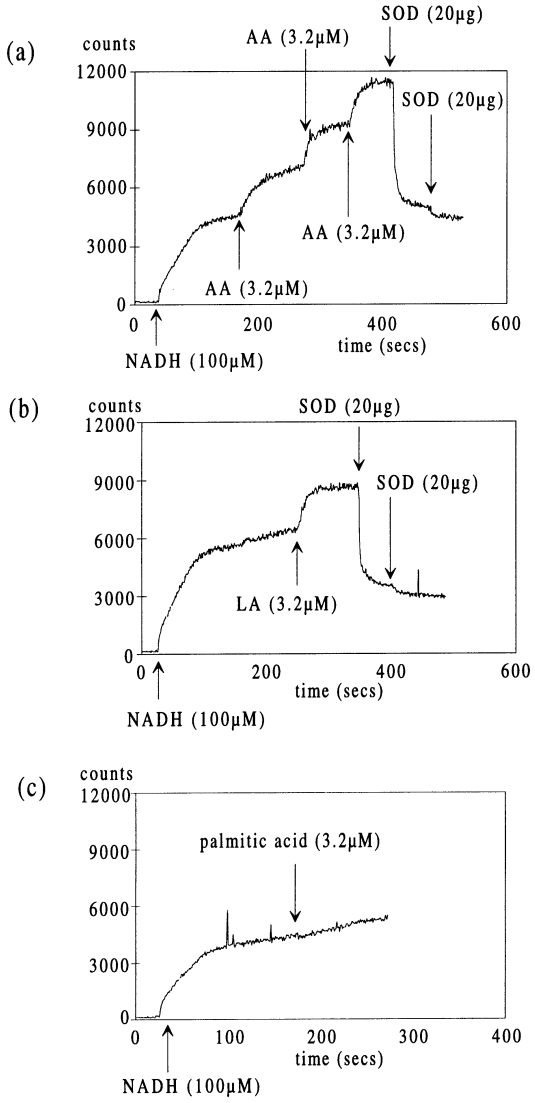


Figure 4 Effect of lipoxygenase substrates on NADH-stimulated luminescence

Chemiluminescence (5×10^5 cells per assay) was measured as described for Figure 2. (**a**) Arachidonic acid (AA; 3.2 μM) was added after NADH, as shown. (**b**) Linoleic acid (LA; 3.2 μM) was added after NADH, as shown. (**c**) The non-lipoxygenase substrate palmitic acid (3.2 μM) was added after NADH, as shown.

prostaglandin H synthase, xanthine oxidase, NADPH oxidase and cytochrome *P*-450 in this system. The addition of the thiol-blocking reagent *p*-chloromercuribenzylsulphonic acid (pCMBS) (100 μM) led to immediate and complete inhibition of the luminescent response (not shown). In contrast, another thiol-blocking reagent, *N*-ethylmaleimide (60 μM), had no effect.

Effect of lipoxygenase fatty acid substrates and products on superoxide generation

Superoxide generation by lipoxygenases in the presence of NADH and a fatty acid substrate is reported elsewhere [32,33]. Therefore we examined the effects of arachidonic acid, linoleic acid and the non-lipoxygenase substrate palmitic acid on the chemiluminescence response. If given to cells alone, no chemiluminescence was observed (results not shown). However, in the

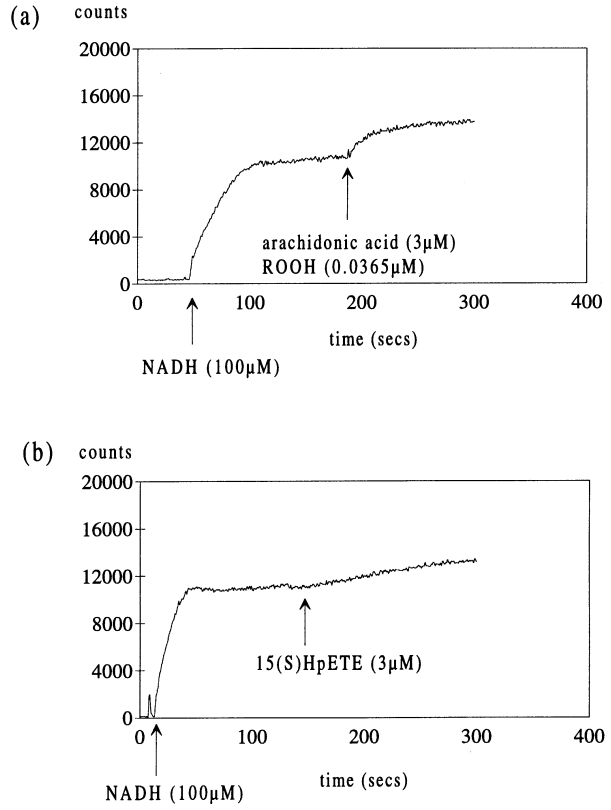


Figure 5 Effect of an arachidonate hydroperoxide on NADH-stimulated chemiluminescence

Luminescence (10^6 cells per sample) was measured as described for Figure 2. (**a**) Arachidonic acid (3 μM) with 0.036 μM contaminating hydroperoxide was added after NADH as shown. (**b**) (15*S*)HPETE was added after NADH as shown.

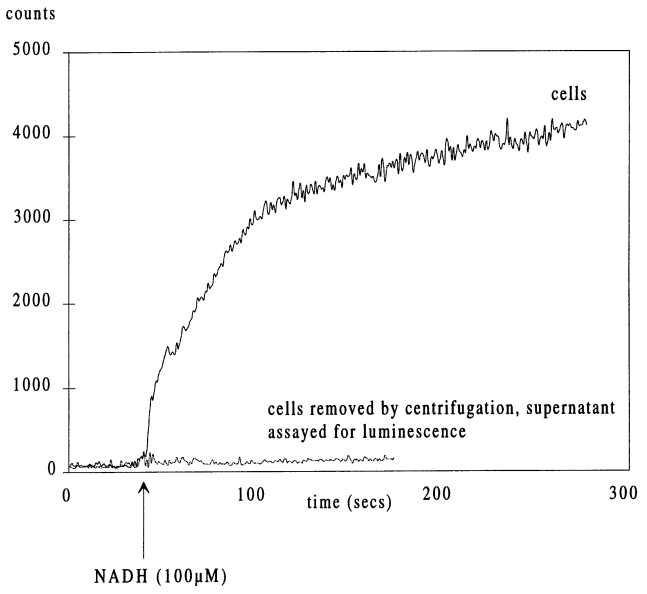


Figure 6 Effect of removal of intact cells on chemiluminescence response

Trypsin-treated cells were diluted to 5×10^5 cells per ml of Krebs buffer. After a low-speed centrifugation (1000 rev./min, 10 min) to remove intact cells, the supernatant was assayed for NADH-stimulated luminescence as described for Figure 2. A sample which had not been centrifuged is shown as a control.

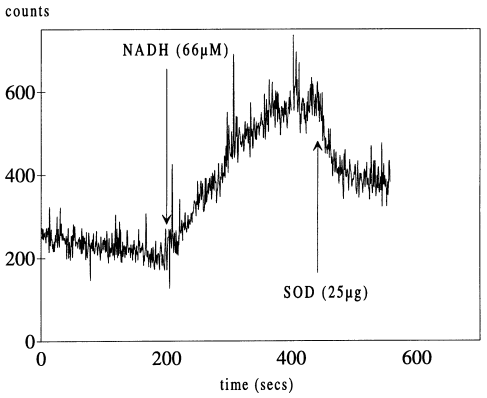


Figure 7 NADH-stimulated chemiluminescence of adherent fibroblasts

Cells were seeded on to collagen-precoated plastic inserts and cultured overnight. After this, duplicate inserts were treated with trypsin and counted (5.5×10^4 cells per insert). Inserts containing cells were then rinsed gently ($\times 2$) in prewarmed (37 °C) Krebs buffer, placed in sample cuvettes containing 1.5 ml of Krebs buffer and 200 μM lucigenin, and assayed for luminescence as described for Figure 2.

presence of NADH, additions of either linoleic or arachidonic acid, but not palmitic acid or ethanol (solvent control), led to large increases in both total chemiluminescence and superoxide generation (Figures 4a–4c). These increases were dose-dependent to at least 30 μM added fatty acid (not shown). Since fatty acid preparations may contain significant amounts of lipid hydroperoxides, we investigated the effects of the lipoxygenase product (15*S*)-HPETE on superoxide generation. When added at a 100-fold excess of contaminating hydroperoxide in the arachidonate preparations (determined using an iodometric assay), no increase in NADH-stimulated chemiluminescence was observed (Figure 5). Finally, addition of (5*S*)-HETE during assay caused small increases in chemiluminescence (considerably less than for arachidonate or linoleate addition), while there was no effect of either (12*S*)-HETE or (15*S*)HETE (results not shown). If xanthine plus xanthine oxidase was used instead of fibroblasts and NADH as a source of superoxide, no effect of either linoleic or arachidonic acid on luminescence was observed. This indicates that these responses did not result from reactions of superoxide with unsaturated fatty acids and were specific for the fibroblast enzyme (results not shown).

Superoxide generation by fibroblasts in response to NADH using cytochrome *c* as electron acceptor

To confirm superoxide-generation rates, cytochrome *c* was used instead of lucigenin as electron acceptor. In this assay, significant reduction of cytochrome *c*, which was only 25% SOD-inhibitable, was seen immediately on NADH addition. Calculation of the SOD-inhibitable portion confirmed that superoxide-generation rates were similar to those calculated using xanthine oxidase-calibrated luminescence, for the same cell isolate (Table 1).

Cellular localization of superoxide generation

Neither NADH, cytochrome *c* nor SOD effectively cross intact membranes, and, because of intracellular absorbance of photons,

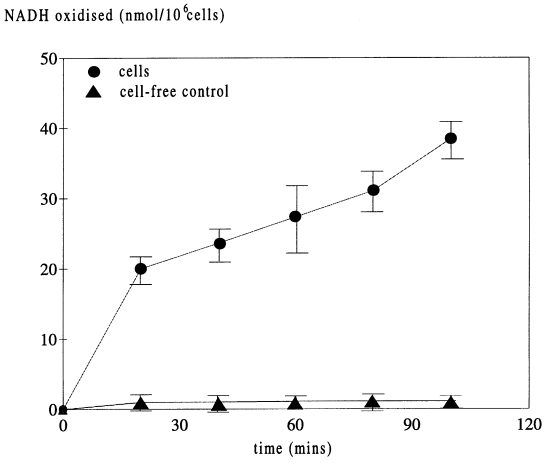


Figure 8 NADH oxidation by adherent fibroblasts

Fibroblast monolayers (3.3×10^5 cells per sample) were incubated with Krebs buffer containing NADH for the indicated times at 37 °C. Buffer was removed and NADH oxidation measured as described in the Materials and methods section. Results are means ± S.D. ($n = 3$).

Table 2 Superoxide generation by recombinant rabbit reticulocyte lipoxygenase

Superoxide generation was measured using lucigenin-enhanced chemiluminescence as described in the Materials and methods section. Results are means ± S.D. ($n = 3$).

	Activity (nmol/min per mg)		
	Enzyme + linoleate	Enzyme + linoleate + NADH (100 μM)	Enzyme + linoleate + NADH (300 μM)
Superoxide generation	44.9 ± 9.6	135.7 ± 5.7	354 ± 53
NADH oxidation	0	480 ± 37	691 ± 32

lucigenin is probably not capable of detecting radicals in intact cells [34]. Since these cells were always more than 98.5 % intact (excluding Trypan Blue), two sources of superoxide are possible: (i) extracellular via enzymes exposed on the outside of the plasma membrane; (ii) intracellular or cytosolic via enzymes released from the small number of non-viable cells (1.5 % maximum). To examine this, cells were diluted in Krebs buffer, then centrifuged [800 ***g*** (1000 rev./min), 10 min]. The resulting supernatant was assayed for NADH-stimulated superoxide generation. As seen (Figure 6), all activity was cell associated. Experiments using adherent cells (100 % excluding Trypan Blue) also confirmed that superoxide generation is extracellular (Figure 7), although average rates were slightly lower (0.94 ± 0.13 nmol/min per 10^6 cells, $n = 4$) than those observed after trypsin treatment.

NADH oxidation by intact fibroblasts

Adherent fibroblasts oxidized external NADH at rates similar to superoxide generation (0.4–1 nmol/min per 10^6 cells) (Figure 8).

NADH oxidation and superoxide generation by recombinant rabbit reticulocyte 15-lipoxygenase

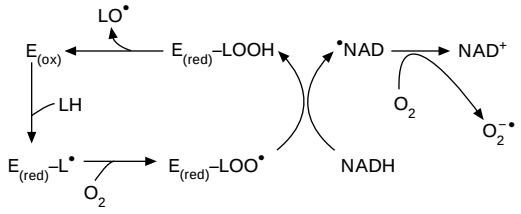
In the presence of linoleate, recombinant rabbit reticulocyte 15-lipoxygenase oxidized NADH and generated superoxide (Table 2). Interestingly, with linoleate alone, a low rate of superoxide generation (SOD-inhibitable chemiluminescence) was also observed. Inclusion of the NADPH oxidase inhibitors, diphenylene iodonium (20 μM) or diphenyl iodonium (200 μM), did not lead to inhibition of this activity. A kinetic analysis of this will be published in full elsewhere.

DISCUSSION

This study shows that human primary fibroblasts contain an outer-membrane-exposed enzyme which can generate large and sustained (continuous up to 20 min) amounts of superoxide in response to exogenous reduced nicotinamide nucleotides [0.94 ± 0.13 nmol/min per 10^6 cells and 2.4 ± 0.75 nmol/min per 10^6 cells for adherent ($n = 4$) and trypsin-treated cells ($n = 9$) respectively]. These rates represent up to 25 % of that of PMA-stimulated NADPH oxidase in neutrophils, and are therefore considerable. The magnitude is also highlighted by the observation that neutrophil NADPH oxidase activation by physiological stimuli (e.g. fMet-Leu-Phe, interleukin 8, C5a) only results in 10 % of PMA-stimulated rates. Also, in contrast with the fibroblast enzyme, neutrophil superoxide generation is short lived (3–5 min or 20–30 s using either PMA or interleukin 8/fMet-Leu-Phe respectively). Not all the chemiluminescence was SOD-inhibitable, indicating that other species generated by this system may also react with lucigenin. Inhibition of the SOD-insensitive portion by the scavenger NDGA suggests that these may also be radicals. Possible candidates could include lipid alkoxyl radicals, generated during hydroperoxidase activity of lipoxygenase after NADH oxidation (see later). Some examples of non-superoxide lucigenin-enhanced chemiluminescence are discussed by Barber et al. [34].

Using specific inhibitors, no role was found for mitochondrial respiratory enzymes, NADPH oxidase, prostaglandin H synthase, cytochrome *P*-450 or xanthine oxidase in this process. However, several features suggest involvement of a lipoxygenase or related enzyme. Previous work has shown NADH oxidation and superoxide generation by soyabean lipoxygenase-5 and prostaglandin H synthase in the presence of fatty acid substrates and NAD(P)H [32,33], and here we demonstrate superoxide generation and NADH oxidation by recombinant rabbit reticulocyte 15-lipoxygenase under similar conditions. Enhancement of luminescence by certain non-esterified fatty acids (which were unable to stimulate luminescence alone) suggests that these may be cosubstrates for activity. Stimulation by arachidonate or linoleate, but not by derivatives modified at C-12 or C-15, suggests involvement of a 15-lipoxygenase, since primary oxygenation by these enzymes occurs at these sites [35]. Superoxide generation by purified 15-lipoxygenases and prostaglandin H synthase in response to NAD(P)H also requires fatty acid substrate [32,33]. Since 15-lipoxygenases (unlike 12- or 5-lipoxygenases) are capable of direct oxygenation of phospholipid-bound fatty acids [35], cellular NADH-stimulated superoxide generation in the absence of added arachidonate or linoleate may involve direct plasma-membrane oxidation. Although HETE generation was not measured here, human fibroblasts have been shown to generate 15-HETE (inhibited by 5,8,11,14-eicosatetranoic acid, but not indomethacin) [36], indicating the presence of 15-lipoxygenase activity in these cells. Finally, the thiol-blocking reagent pCMBS is a known inhibitor of lipoxygenases [37].

Two mechanisms have been suggested for superoxide generation by lipoxygenases in response to NAD(P)H [32,33]. These enzymes can mediate reductant-dependent pseudoperoxidase reactions in the presence of a suitable reducing substrate, RH



Scheme 1 Mechanism for superoxide generation by lipoxygenases in response to NAD(P)H

E, lipoxygenase; LH, unsaturated lipid; LOOH, lipid hydroperoxide; LOO•, lipid peroxyl radical; LO•, lipid alkoxyl radical.

[38]. Therefore superoxide generation could occur after one-electron oxidation of NAD(P)H to •NAD(P) by oxidized enzyme ($E_{(ox)} \rightarrow E_{(red)}$) and subsequent reduction of molecular oxygen by •NAD(P) [39,40]. To regenerate active enzyme, this mechanism also requires hydroperoxide ($E_{(red)} + LOOH \rightarrow E_{(ox)} + LO^{\bullet}$). However, since an arachidonate hydroperoxide was without effect here, our data are more in agreement with the model of Roy et al. [32], which postulates that the enzyme–substrate intermediate $E_{(red)}$–$LOO^{\bullet}$ is the oxidant and not $E_{(ox)}$. In this model, unsaturated fatty acid is required for reaction. The suggested mechanism is shown in Scheme 1.

Previously, Meier et al., [4,5] demonstrated low-level superoxide generation (approximately 0.06 nmol/min per 10^6 cells) by human fibroblasts in response to certain cytokines (tumour necrosis factor α, interleukin 1). Since expression of several components of the neutrophil NADPH oxidase was found, an involvement of this enzyme was suggested [5,6]. Interestingly, Meier et al. [4] also observed an enhancement of fibroblast oxidant generation on addition of external NAD(P)H to superoxide-generating cells. This led them to postulate an external active site for NADPH oxidase. However, we suggest that, although NADPH oxidase may be a source of low-level superoxide after cytokine stimulation, high rates of radical formation in response to external nicotinamide nucleotides most likely originate from an unrelated enzyme for several reasons. First, external NADH-stimulated superoxide generation was not inhibited by iodonium compounds, which are potent NADPH oxidase inhibitors [41]. Secondly, the enzyme demonstrated here displays a preference for NADH over NADPH, in contrast with NADPH oxidase [K_m (NADH) 495 μM, K_m (NADPH) 57 μM] [42] and may utilize added non-esterified fatty acids as cosubstrates. Thirdly, external oxidation of nicotinamide nucleotides by the phagocyte NADPH oxidase is unknown. Finally, the phagocyte NADPH oxidase is not constitutively active and requires intracellular assembly of at least three cytosolic and two membrane proteins for activation (by cytokines, phorbol esters, calcium ionophores etc.).

Cytosolic (rat liver cells) concentrations of NADH and NADPH have been determined to be 270 and 367 μM respectively, with mitochondrial concentrations at 638 and 4233 μM respectively [43]. These would obviously support the system described here [K_m(app)(NADH) = 11.6 μM], although, since NAD(P)H cannot easily cross biological membranes, access would not be expected under normal conditions. However, if tissue damage occurs, this situation could alter dramatically. During periods of prolonged ischaemia (> 60 min), major membrane ultrastructural changes leading to mitochondrial swelling, vesicle formation and ultimately loss of plasma-membrane integrity occur [44,45]. Also, cellular levels of reduced nicotinamide nucleotides increase, since mitochondrial respiration is blocked [46,47]. Once oxygen supply is restored, exposure of the enzyme to NAD(P)H released from damaged cells may then allow uncontrolled ROS generation, as seen on reperfusion after ischaemia. Consistent with this, membrane lipid peroxidation and NAD(P)H depletion have all been observed during reoxygenation of ischaemic heart [48,49]. A role for xanthine oxidase, generated by proteolytic cleavage of xanthine dehydrogenase, in some cases of ischaemia/reperfusion damage (e.g. small bowel) is known. However, in many tissues (e.g. brain, heart), catalytic tissue levels of this enzyme cannot be demonstrated and the source of damaging ROS is still unknown (reviewed in ref. [50]).

Recently, several studies have demonstrated NAD(P)H oxidases in homogenates and particulate fractions of several cell types (endothelial, smooth muscle) capable of superoxide or hydrogen peroxide generation in response to the addition of reduced nicotinamide nucleotides [14–16,51]. In addition, induction of NADH-stimulated hydrogen peroxide generation by transforming growth factor β in human lung fibroblasts has been found [52]. Unlike the fibroblast enzyme described here and the purified lipoxygenases, inhibition by iodonium compounds has been observed for all these systems. This suggests the involvement of flavoenzymes, possibly related to the phagocyte NADPH oxidase, and indicates that they are distinct from the activity observed here.

Until now, rates of extracellular superoxide generation equivalent to those observed here have been demonstrated only in phagocytic cells (*via* NADPH oxidase). However, the existence of extracellular heparan sulphate-binding SOD suggests that other cell types may also contain outer-membrane enzymes capable of generation of this radical. Since reactions of superoxide with nitric oxide could effectively prevent endothelium-dependent vasorelaxation, it will be important to investigate the presence of this enzyme on the outside of vascular cells (e.g. endothelial, smooth muscle).

In conclusion, we here describe the presence of an external NAD(P)H-oxidizing and superoxide-generating enzyme in human fibroblasts which displays several enzymic similarities to 15-lipoxygenases. External localization of lipoxygenases has not been described to date, therefore further structural characterization should aim to determine the identity of the enzyme responsible.

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