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# Cell-Surface NAD(P)H-Oxidase: Relationship to Trans-Plasma Membrane NADH-Oxidoreductase and a Potential Source of Circulating NADH-Oxidase

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## ABSTRACT

**The surface of mammalian cells faces an oxidizing environment that has the potential to damage proteins, lipids, and carbohydrates to which it is exposed. In contrast, the cytoplasm is reducing and its redox state is tightly regulated. Trans-plasma membrane oxidoreductases that shift electrons from cytosolic NADH to external electron acceptors such as oxygen are widely involved in cellular redox control. They reduce oxygen to water and may generate reactive oxygen species such as superoxide and hydrogen peroxide. In addition, external NAD(P)H-oxidases have been demonstrated on intact cells and as eluted proteins, but the relationship between trans-plasma membrane NADH-oxidoreductases and cell-surface NAD(P)H-oxidases is not known. To investigate further the relationship between plasma membrane NAD(P)H-oxidoreductases, and to gain insight into the physiological functions of these redox active membrane proteins, we have adapted a simple colorimetric assay for measuring the trans-plasma membrane NADH-oxidoreductase activity of viable cells to measure NAD(P)H-oxidase at the cell surface in real time. Using the cell-impermeable tetrazolium salt WST-1 in the presence of NADH or NADPH, but in the absence of an intermediate electron acceptor, we show that cell-surface NAD(P)H-oxidase is widely expressed on mammalian cells, being more abundant on rapidly proliferating cells than on resting neutrophils and spleen cells. The ratio of cofactor dependence of NAD(P)H-oxidase (NADH:NADPH) varied widely between different cells (0.7–5.2), suggesting a family of cell surface oxidases or that the activity of these enzymes may be modulated in various ways. Comparison of NAD(P)H-oxidase on the surface of viable cells with trans-membrane NADH-oxidoreductase, measured with WST-1 in the presence of 1-methoxy PMS, showed that cell-surface NAD(P)H-oxidase was differentially inhibited by the cell-impermeable thiol-blocking agent pCMBS, but was unaffected or stimulated by other thiol blocking agents. Capsaicin, which inhibits trans-plasma membrane NADH-oxidoreductase activity, stimulated surface NAD(P)H-oxidase. Metabolic inhibitors had little effect on surface NAD(P)H-oxidase activity but inhibited trans-plasma membrane activity. These results do not support the view the surface NAD(P)H-oxidase is a terminal oxidase for trans-plasma membrane NADH-oxidoreductase.** Antiox. Redox Signal. 2, 277–288.

## INTRODUCTION

THE EXTERNAL SURFACE OF THE PLASMA MEMBRANE plays an important role in mediating developmental processes, maintaining cellular homeostasis, ensuring the immunological integrity of the cell, and generating innate and acquired immune responses. Thus, complex intercellular communication and cellular interactions with the external environment, without which multicellular life would cease to exist, are an integral part of the evolutionary heritage of higher organisms. In addition to its individual identity at a particular point in time, the external surface of the cell is in a state of dynamic change, being continuously altered enzymatically by cell-surface proteases and other cell-surface and circulating enzymes, and by the

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processes of exocytosis, endocytosis, pinocytosis, and exfoliation.

The oxidizing environment of cellular fluids means that many cell-surface proteins must be continually reduced to maintain function, and this is achieved by a complex system of redox-related proteins and enzymes at the cell surface that include thioredoxin and protein disulfide isomerase, a major plasma membrane protein. In addition, it has been proposed (Morré *et al.*, 1999) that an NADH-oxidase at the cell surface plays an important role in maintaining the redox status of cell-surface proteins via its ability to act as a terminal oxidase for trans-plasma membrane NADH-oxidase. In this way, electrons are transferred from cytosolic NADH via membrane quinones to cell-surface NADH-oxidase, which is thought to be associated with protein disulfide isomerase. However, cell-surface NADH-oxidase is characterized by its inherent ability to oxidize NADH, a substrate not normally present at significant levels in body fluids. The fact that isolated cell-surface NADH-oxidase has inherent NADH as well as hydroquinone oxidase activity (Kishi *et al.*, 1999) suggests a complex role at the cell surface, rather than simply acting as a terminal oxidase for trans-plasma membrane NADH-oxidase.

Understanding the physiological role of cell-surface NADH-oxidase requires a robust cellular model where function can be studied with viable cells and where physiological substrates and products can be identified and monitored. To this end, we have developed a simple colorimetric method for measuring cell-surface NADH-oxidase on viable cells in real time, and we are using this assay to investigate the properties and functions of the enzyme.

The trans-plasma membrane oxidoreductase (PMOR) system of mammalian cells consists of at least two enzyme complexes, which are distinguished by their differential sensitivity to pCMBS and capsaicin (Brightman *et al.*, 1992; Vaillant *et al.*, 1996). These enzymes use cytosolic NADH to reduce extracellular substrates, including molecular oxygen to water. More commonly, cell-impermeable artificial electron acceptors such as ferricyanide and dichlorophenolindophenol (DCIP) have been used to measure ferricyanide-oxidoreductase (Goldenberg *et al.*, 1979), while we have shown that the cell-impermeable tetrazolium salt WST-1 is an efficient substrate for NADH-oxidoreductase (Berridge *et al.*, 1996; Berridge and Tan, 1998, 2000). Ferricyanide reduction by cells, and ferricyanide and DCIP reduction by isolated plasma membranes is strongly inhibited by pCMBS, but not by the vanilloid inhibitor capsaicin and its more potent analogue resiniferatoxin (Vaillant *et al.*, 1996). In contrast, an NADH-oxidase that is associated with isolated plasma membranes is inhibited by capsaicin and resiniferatoxin but not by pCMBS (Morré *et al.*, 1995; Vaillant *et al.*, 1996). We have shown that cellular reduction of WST-1 exhibits similar inhibitor characteristics to the plasma membrane NADH-oxidase described by Morré *et al.* (Berridge *et al.*, 1996; Berridge and Tan, 1998).

Superoxide production by trans-plasma membrane NAD(P)H-oxidase of activated phagocytic cells has been extensively studied (Babior *et al.*, 1973; Chanock *et al.*, 1994), and low-level superoxide production by other non-phagocytic cells, including fibroblasts and endothelial cells, has been described (Matsubara and Ziff, 1986; Meier *et al.*, 1989; Murrell *et al.*, 1990). In addition, O'Donnell and Azzi (1996) have described extracellular superoxide production by cultured human fibroblasts by adding NADH to cells and monitoring superoxide dismutase (SOD)-sensitive lucigenin or cytochrome *c* reduction. Interestingly, superoxide production was enhanced by adding arachidonic acid, linoleic acid, and (5S) hydroxyeicosatetraenoic acid (HETE) but not (15S)HETE, indicating 15-lipoxygenase-like activity. Conversely, purified rabbit reticulocyte 15-lipoxygenase was shown to oxidize NADH in the presence of arachidonic acid and to generate superoxide. This study indicates the presence of a cell-surface NAD(P)H-oxidase that may be able to utilize a variety of substrates to generate reducing electrons. Whether superoxide is an essential radical intermediate in this system, or a nonessential component of a pool of radicals that is efficiently removed by SOD, as has been previously shown in other systems (Winterbourn, 1981; Berridge and Tan, unpublished results), is not altogether clear.

The cell-impermeable tetrazolium salt WST-1, used in conjunction with an intermediate electron acceptor, was originally used to measure reduced pyridine nucleotides and to quantify viable cells (Ishiyama *et al.*, 1993). Commercialization of WST-1 as a cell proliferation reagent included a stable, proprietary, intermediate electron acceptor in a convenient, single-reagent product, and claimed that reduction was via a mitochondrial "succinate-tetrazolium reductase." The demonstration that WST-1 is reduced extracellularly by plasma membrane enzymes that are dependent on glycolytic metabolism (Berridge *et al.*, 1996; Berridge and Tan, 1998), raised questions about the mechanism of WST-1 reduction that required separating WST-1 from its accompanying intermediate electron acceptor.

In the present study, we have used WST-1 in the absence of an intermediate electron acceptor to demonstrate NAD(P)H-oxidase activity on the surface of viable cells and have also shown the existence of a cell-surface ferricyanide-reducing activity that is dependent on added NADH. The NAD(P)H:WST-1-oxidoreductase activity at the cell surface is compared with trans-plasma membrane NADH:WST-1-oxidoreductase measured in the presence of the intermediate electron acceptor, 1-methoxy PMS (but without added NADH), and with the NAD(P)H-oxidase of activated neutrophils which can also be efficiently measured with WST-1 in the presence or absence of an intermediate electron acceptor (Tan and Berridge, 2000).

## MATERIALS AND METHODS

### *Cells and cell culture*

The human tumor cell lines Jurkat, HL60, U937, A375, and Malme 3M were obtained from the American Type Culture Collection (Rockville, MD). HeLa cells were obtained from Dr. Antony Braithwaite, University of Otago. The osteosarcoma cell line, 143B and its $\rho^0$ derivative were from Dr. Mike Murphy, University of Otago, with permission from Professor Yau-Huei Wei, National Yang-Ming University, Taipei. P815 mastocytoma cells were from Dr. J Marbrook, University of Auckland, and the Lewis Lung carcinoma cell line LL-LCMV was from Dr. Ian Hermans, Malaghan Institute. Murine 32Dclone3 cells were from Dr. Steve Anderson, University of Colorado Health Sciences Center (Denver, CO) and 32Dcl3*bcr-abl* was derived by Jason Gush (Malaghan Institute, Wellington, NZ) by viral transfection of parental 32Dc13 cells. HeLa $\rho^0$ and P815 $\rho^0$ were derived by long-term culture with ethidium bromide and lack of mitochondrial DNA verified by PCR (Tan and Berridge, unpublished results). All cells were grown in RPMI-1640 medium (GIBCO-BRL, Grand Island, NY) supplemented with 5–10% fetal bovine serum (FBS), penicillin (25 $\mu$g/ml), and streptomycin (25 $\mu$g/ml). 32Dc13 cells were supplemented with 7.5% WEHI-3 conditioned medium as a source of IL-3. Cells were grown at 37°C in a humidified incubator maintained at 5% $CO_2$.

Human peripheral blood neutrophils were collected from venous blood of healthy donors with EDTA as an anticoagulant, and neutrophils isolated by centrifugation through Polymorphprep (Nycomed Pharma, Oslo, Norway) at 500 × *g* for 30 min at 20°C. Cells were washed twice in phosphate-buffered saline (PBS) and suspended at $10^7$/ml. Purity and viability were greater than 95%.

Spleen cells were obtained from adult C57-B16 mice by standard procedures. Dispersed cells were fractionated on Isopaque-Ficoll ($\rho$ = 1.09) at 2,000 × *g* for 15 min at 20°C and washed in PBS prior to use.

### *Materials*

WST-1 and 1-methoxy phenazine methosulfate (PMS) were from Dojindo Laboratories (Kumamoto, Japan). NADH, NADPH, and the WST-1 cell proliferation reagent were from Boehringer, Mannheim (Mannheim, Germany). 2-Deoxy-D-glucose was from Fluka (Buchs, Switzerland). Unless otherwise stated, all other chemicals and reagents were from Sigma Chemical Company (St. Louis, MO).

### *Trans-plasma membrane WST-1 reduction*

WST-1 reduction across the plasma membrane of viable cells was determined either by

using the commercial WST-1 cell proliferation reagent supplied by Boehringer Mannheim or by mixing 4.5 ml of 5.5 m*M* WST-1 and 0.5 ml of 2 m*M* 1-methoxy PMS, and adding 8 $\mu$l or 10 $\mu$l of this reagent mixture to 0.1 ml of cells (2–3 $\times$ $10^5$/ml) suspended in RPMI-1640 medium containing 7% FBS or in Hank's balanced salt solution (HBSS). Cells were incubated for times up to 45 min at 37°C, and WST-1 formazan production was read at intervals in a BioRad ELISA plate reader or at the desired times in a Titertek Multiscan reader at 450 nm.

*Ferricyanide reduction*

Cells ($2 \times 10^6$/ml) were suspended in PBS containing 10 m*M* glucose and 1 m*M* sodium ferricyanide was added. The reaction mixture was incubated in a BioRad enzyme-linked immunosorbent assay (ELISA) plate reader at 37°C for 60 min, after which the plate was centrifuged and the supernatant collected and assayed for ferrocyanide using the bathophenanthroline-disulfonic acid (BPS) assay of Avron and Shavit (1963). Briefly, samples containing up to 0.1 $\mu$mol ferricyanide were diluted to 0.7 ml with water and 0.3 ml of reagent containing 3 *M* sodium acetate, 0.2 *M* citric acid, 3.3 m*M* ferric chloride, and 3.3 mg/ml BPS added. After 5 min, the absorbance at 535 nm was measured in a Unicam Heλios $\gamma$ spectrophotometer, and the rate of ferrocyanide production (ferricyanide reduction) was calculated using an extinction coefficient of 10,500 $M^{-1}cm^{-1}$ for 1,10-phenanthroline ferrocyanide. In all experiments, ferricyanide reduction in the absence of cells was subtracted as a control.

*Cell-surface WST-1 reduction*

Initially, WST-1 reduction at the cell surface was measured spectrophotometrically at 450 nm (1-cm path length) using either a Carey scanning spectrophotometer or a Unicam Heλios $\gamma$ spectrophotometer and a reaction volume of 1 ml or 0.5 ml, respectively. Later experiments were adapted to microplate format involving a 0.1-ml reaction volume (about 0.3-cm path length). Plates were shaken at regular intervals and absorbance read in a BioRad ELISA plate reader. In general, cells were used at 5–10 $\times$ $10^5$/ml in HBSS and equilibrated with 200 $\mu$*M* NADH or NADPH for 5 min prior to adding WST-1 to 0.45 m*M*. In all experiments, WST-1 reduction was read against controls without added cells and buffer controls were included with cells and WST-1 but without added NAD(P)H.

*Elution of NADH-oxidase and measurement of eluted and serum WST-1-reducing activity*

Packed cells were treated with an equal volume of 0.1 *M* sodium acetate pH 5 for 1 hr at 37°C according to the method of del Castillo-Olivares *et al.* (1998). Eluted enzyme and WST-1-reducing activity in serum were determined spectrophotometrically at 450 nm (1-cm path length) using either a Carey scanning spectrophotometer or a Unicam Heλios $\gamma$ spectrophotometer and a reaction volume of 1 ml or 0.5 ml, respectively. Eluted enzyme or serum was diluted 1:10 in PBS or HBSS and equilibrated with 200 $\mu$*M* NADH. The reaction was initiated by adding WST-1 to 0.45 m*M*.

## RESULTS

Following procedures originally described by Ishiyama *et al.* (1993), we have confirmed that the rate constant of WST-1 reduction by cells is low in the absence of an intermediate electron acceptor. In addition, we observed that adding NADH rather than 1-methoxy PMS to cells results in WST-1 reduction. In this situation, NADH is unlikely to be acting as an intermediate electron acceptor because, in the absence of cells, it is rapidly oxidized chemically in the presence of WST-1 and an intermediate electron acceptor (Ishiyama *et al.*, 1993; Berridge *et al.*, 1996). These results indicate the presence of an NADH:WST-1-oxidoreductase at the cell surface.

Dependence of WST-1 reduction on added NADH for several different cell types is shown in Fig. 1A. Initial rates of WST-1 reduction were directly correlated with added NADH and with cell concentration (Fig. 1B). Table 1 compares NADH-dependent WST-1 reduction with trans-plasma membrane WST-1 reduction for

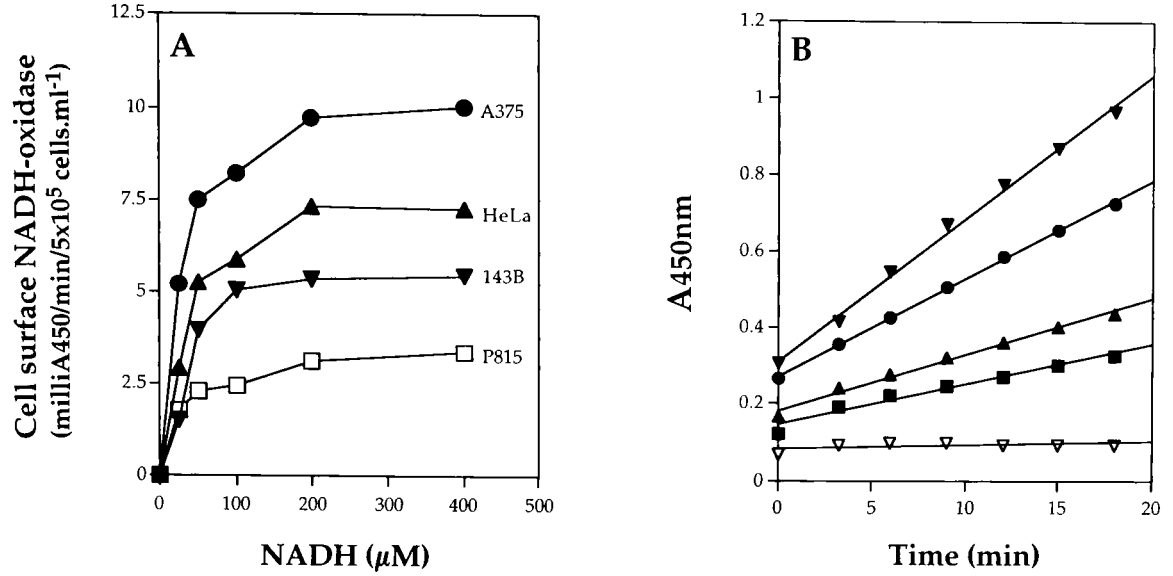


**FIG. 1. NADH-dependent WST-1 reduction at the cell surface.** (**A**) The human (filled symbols) and murine (empty symbol) cells types indicated were equilibrated by incubation with increasing concentrations of NADH for 5 min prior to adding WST-1 to 0.45 m*M* and measuring the increased absorbance at 450 nm over the linear range of the reaction. (**B**) A375 human melanoma cells were preincubated with 200 $\mu M$ NADH for 5 min prior to adding WST-1 to 0.45 m*M* and measuring the rate of reaction at the times indicated. Cell concentrations were: (▼) $10^6$/ml, (●) $5 \times 10^5$/ml, (▲) $2.5 \times 10^5$/ml, (■) $1.25 \times 10^5$/ml, (▽) no added cells.

several human tumor cell lines. With Jurkat, HL60, and 143B cells, levels of NADH-dependent WST-1 reduction at the cell surface were similar as were levels of trans-plasma membrane WST-1 reduction. With HeLa cells, both trans-plasma membrane WST-1 reduction and NADH-dependent reduction of WST-1 at the cell surface were elevated. In contrast, 143B$\rho^0$ cells exhibited high levels of trans-plasma membrane NADH-oxidase activity, but NADH-dependent reduction of WST-1 at the cell surface was not elevated. Table 1 also shows that with each cell type, cellular reduction of ferricyanide increased following addition of NADH to cells. A simple relationship between cellular ferricyanide reduction in the presence and absence of NADH was not evident.

NADH-dependent WST-1 reduction was also determined with nondividing human peripheral blood neutrophils and with murine spleen cells, and following activation of neutrophils with phorbol myristate acetate (PMA). Table 2 shows that nondividing cells exhibited low levels of NADH-dependent WST-1 reduc-

TABLE 1. REDUCTION OF WST-1 AND FERRICYANIDE BY TRANS-PLASMA MEMBRANE AND CELL-SURFACE NADH-OXIDOREDUCTASES

| | *WST-1 reduction (milliA450/min per 3 × $10^5$ cells.$ml^{-1}$)* | | *Ferricyanide reduction (pmol/sec per $10^6$ cells)* | | |
|---|---|---|---|---|---|
| *Cell line* | *Trans-PM (−NADH/+IEA)* | *Cell surface (+NADH/−IEA)* | *Trans-PM (−NADH)* | *t-PM+ surface (+NADH)* | *Cell surface (Δ)* |
| Jurkat T-lymphoblastic | 19.6 ± 0.2 | 4.18 ± 0.39 | 17.7 ± 0.9 | 22.8 ± 0.8 | 5.1 ± 0.2 |
| HL60 myelomonocytic | 19.0 ± 0.4 | 4.62 ± 0.18 | 16.5 ± 0.9 | 21.0 ± 1.6 | 4.5 ± 0.3 |
| HeLa cervical carcinoma | 51.7 ± 1.4 | 7.20 ± 0.32 | 32.5 ± 1.8 | 40.7 ± 2.8 | 8.2 ± 0.5 |
| 143B osteosarcoma | 18.9 ± 0.4 | 4.84 ± 0.05 | 26.2 ± 1.5 | 39.2 ± 2.7 | 13.0 ± 0.8 |
| 143B$\rho^0$ | 42.8 ± 0.7 | 4.62 ± 0.32 | 17.6 ± 1.1 | 23.8 ± 1.6 | 6.2 ± 0.4 |

TABLE 2. REDUCTION OF WST-1 BY CELL-SURFACE AND TRANS-PLASMA MEMBRANE NADH-OXIDOREDUCTASES ON HUMAN AND MURINE CELLS

| | *Initial rate WST-1 reduction (milliA450/min per $5 \times 10^5$ cells·ml$^{-1}$)* | |
|---|---|---|
| *Cell type* | *Cell surface* | *Trans-plasma membrane* |
| Human | | |
| Jurkat T-lymphoblastic | 5.07 ± 0.72 | 36.8 ± 2.2 |
| HL60 myelomonocytic | 4.49 ± 0.16 | 30.8 ± 1.2 |
| HeLa cervical carcinoma | 7.24 ± 0.47 | 65.3 ± 8.0 |
| 143B osteosarcoma | 4.60 ± 0.29 | 44.3 ± 7.3 |
| U937 macrophage | 3.62 ± 0.27 | 23.8 ± 3.2 |
| A375 melanoma | 8.53 ± 0.95 | 27.0 ± 4.7 |
| Malme 3M melanoma | 10.59 ± 0.30 | 44.7 ± 4.3 |
| Neutrophils (resting) | 1.45 ± 0.43 | 0.7 ± 0.3 |
| Neutrophils (PMA-activated) | 5.25 ± 0.51 | 33.3 ± 4.5 |
| Murine | | |
| 32Dcl3 IL-3-dependent | 3.96 ± 0.30 | 26.5 ± 3.7 |
| 32Dcl3*bcr-abl* (transformed) | 2.83 ± 0.21 | 22.5 ± 2.0 |
| P815 mastocytoma | 4.64 ± 0.25 | 21.7 ± 2.5 |
| LL-LCMV lung carcinoma | 7.31 ± 1.62 | 63.2 ± 2.3 |
| Spleen (unstimulated) | 0.79 ± 0.03 | 0.8 ± 0.2 |

tion. Activation of neutrophils increased cell surface WST-1 reduction by about four-fold. These results are similar to those observed with trans-plasma membrane WST-1-reducing activity, although in this situation background levels were lower, resulting in a much greater percentage increase following cell activation. All other human and murine cell lines tested also reduced WST-1 in the presence of NADH, with human melanoma cells showing the highest activity. It is noteworthy that murine 32D cells transformed with the *bcr-abl* oncogene showed lower levels of surface WST-1-reducing activity than parental 32D cells.

In addition to NADH, the ability of NADPH to facilitate WST-1 reduction at the cell surface was determined. Table 3 shows that NADPH also served as a cofactor for WST-1 reduction with each of the human and murine cells tested. The ratio of cofactor dependence (NADH: NADPH) varied considerably between the different cell types from 0.7 to 5.2, with 143B osteosarcoma cells and melanoma cell lines exhibiting a preference for NADH and murine mastocytoma cells preferring NADPH. Several cell lines (HeLa and 32D) and neutrophils showed equal ability to utilize NADH or NADPH. In general, buffer controls gave low WST-1 reduction, whereas WST-1 reduction by NADH or NADPH in the absence of cells was not significantly above background.

To investigate further the properties of NADH-dependent WST-1-reducion at the cell surface, inhibitor studies were carried out, initially using Jurkat and 143B cells. Table 4 shows that the cell-impermeable thiol (SH) blocking agent pCMBS (20 $\mu M$) inhibited NADH-dependent WST-1 reduction at the cell surface by 70–80% while having little effect on trans-plasma membrane WST-1 reduction. In contrast, the cell-permeable thiol blocking agent, *N*-ethylmaleamide (20 $\mu M$), stimulated WST-1 reduction by up to 83% while strongly inhibiting trans-plasma membrane WST-1 reduction by up to 97%. Similar results were obtained with iodoacetamide (5 m$M$), except that the stimulatory effect on NADH-dependent WST-1 reduction was less pronounced or absent. The vanilloid inhibitor capsaicin (100 $\mu M$), which is known to inhibit plasma membrane NADH-oxidase (Morré *et al.*, 1995) and trans-plasma membrane WST-1-reducing activity (Berridge and Tan, 1998), stimulated NADH-dependent WST-1 reduction by Jurkat and 143B cells by 24% and 46%, respectively, while inhibiting trans-plasma membrane WST-1 reduction as expected. Retinoic acid (20 $\mu M$), another known inhibitor of plasma membrane NADH-

TABLE 3. CAFACTOR-DEPENDENCE OF CELL-SURFACE NAD(P)H-OXIDASE ON DIFFERENT CELLS

| | *Initial rate WST-1 reduction (milliA450/min per $5 \times 10^5$ cells·ml$^{-1}$)* | | | | |
|---|---|---|---|---|---|
| | *Cell-surface NAD(P)H-oxidase* | | | | |
| *Cell type* | *Buffer* | *NADH* | *NADPH* | *Ratio* | *Trans-plasma membrane NADH-oxidase* |
| Human | | | | | |
| HeLa cervical carcinoma | 0.24 ± 0.05 | 7.81 ± 0.56 | 8.25 ± 1.04 | 0.95 | 59.8 ± 2.0 |
| HeLa$\rho^0$ | 0.65 ± 0.02 | 14.01 ± 1.78 | 13.11 ± 1.23 | 1.07 | 104.3 ± 0.5 |
| 143B osteosarcoma | 0.30 ± 0.01 | 4.75 ± 0.04 | 0.91 ± 0.05 | 5.22 | 40.0 ± 0.2 |
| 143B$\rho^0$ | 0.23 ± 0.01 | 5.06 ± 0.29 | 1.89 ± 0.01 | 2.68 | 68.0 ± 6.8 |
| U937 macrophage | 0.23 ± 0.01 | 3.35 ± 0.31 | 1.13 ± 0.15 | 2.96 | 20.7 ± 0.5 |
| A375 melanoma | 0.17 ± 0.02 | 11.24 ± 2.10 | 2.40 ± 0.14 | 4.68 | 22.3 ± 1.0 |
| Malme 3M melanoma | 0.78 ± 0.18 | 9.82 ± 0.65 | 2.95 ± 0.41 | 3.33 | 44.7 ± 0.5 |
| Neutrophils (resting) | 0.34 ± 0.00 | 1.36 ± 0.07 | 1.30 ± 0.01 | 1.06 | NA |
| Murine | | | | | |
| 32Dcl3 | 0.22 ± 0.01 | 3.98 ± 0.53 | 3.87 ± 0.11 | 1.03 | 22.7 ± 0.7 |
| 32Dcl3*bcr-abl* | 0.15 ± 0.02 | 2.83 ± 0.24 | 1.05 ± 0.08 | 2.70 | 18.0 ± 0.2 |
| P815 | 0.22 ± 0.01 | 4.66 ± 0.92 | 6.78 ± 0.74 | 0.69 | 16.7 ± 3.5 |
| P815$\rho^0$ | 0.13 ± 0.09 | 4.62 ± 0.58 | 5.69 ± 0.08 | 0.81 | 33.7 ± 0.3 |
| No cells | 0.03 ± 0.02 | 0.01 ± 0.12 | 0.12 ± 0.04 | NA | 0.5 ± 0.1 |

oxidase (Morré and Brightman, 1991), weakly inhibited trans-plasma membrane WST-1 reduction but stimulated NADH-dependent WST-1 reduction by 143B cells by about 40%.

The effects of metabolic inhibitors on WST-1 reduction were also investigated. The glycolytic inhibitor, 2-deoxyglucose (5 m*M*), had little effect on NADH-dependent WST-1 reduction at the cell surface, but inhibited trans-plasma membrane WST-1 reduction by 29–36% consistent with rapid depletion of glycolytic NADH which is the most likely reductant in this system. Likewise, the respiratory inhibitor cyanide (1 m*M*) had little effect on NADH-dependent WST-1 reduction at the cell surface but stimulated trans-plasma membrane WST-1 reduction by 60–70%. This can be explained by cyanide having a sparing effect on cytosolic NADH utilization in the absence of mitochondrial respiration.

Table 4 also shows that both NADH-dependent cell-surface and trans-plasma membrane WST-1 reduction were extensively inhibited by SOD (20 $\mu$g/ml), indicating direct or indirect involvement of superoxide in WST-1 reduction. Similar inhibitor results were obtained with several other cell lines including 143B$\rho^0$, HeLa, 32D, and LL-LCMV cells, and with primary

TABLE 4. EFFECT OF INHIBITORS ON TRANS-PLASMA MEMBRANE AND CELL-SURFACE NADH-OXIDASE ACTIVITY

| | *NADH-oxidase activity (% control WST-1 reduction)* | | | |
|---|---|---|---|---|
| | *Jurkat* | | *143B* | |
| *Inhibitor* | *Trans-PM* | *cell surface* | *Trans-PM* | *cell surface* |
| pCMBS (20 $\mu M$) | 104 ± 2 | 30 ± 1 | 102 ± 1 | 19 ± 1 |
| *N*-Ethylmaleamide (20 $\mu M$) | 19 ± 1 | 120 ± 7 | 3 ± 1 | 183 ± 19 |
| Iodoacetamide (5 m*M*) | 18 ± 0 | 102 ± 1 | 11 ± 0 | 125 ± 5 |
| Capsaicin (100 $\mu M$) | 42 ± 1 | 124 ± 2 | 63 ± 2 | 146 ± 6 |
| 2-deoxyglucose (5 m*M*) | 64 ± 2 | 97 ± 1 | 71 ± 1 | 103 ± 2 |
| Cyanide (1 m*M*) | 160 ± 3 | 119 ± 3 | 170 ± 3 | 126 ± 2 |
| Retinoic acid (20 $\mu M$) | ND | ND | 76 ± 3 | 138 ± 7 |
| SOD (20 $\mu$g/ml) | ND | ND | 21 ± 0 | 18 ± 1 |

spleen cells (Berridge and Tan, 2000), indicating that the results, particularly those with the thiol blocking agents and capsaicin were not restricted to Jurkat and 143B cells.

Inhibitor studies were also conducted with the cell-surface NAD(P)H-dependent WST-1 reducing activity of human peripheral blood neutrophils (Table 5). As with other cell types, both NADH and NADPH-dependent WST-1 reduction by resting neutrophils was inhibited by pCMBS but not by capsaicin. The NADPH-oxidase inhibitor diphenyleneiodinium (DPI, 20 $\mu M$) had little effect on NADH-dependent WST-1 reduction, but a small (27%) inhibitory effect was observed with NADPH-dependent WST-1 reduction, perhaps reflecting a small contribution of respiratory burst NADPH-oxidase to this activity. In other studies with HeLa cells, we have also observed small titratable inhibitory effects of DPI on NADPH-dependent WST-1 reduction but not with NADH-dependent WST-1 reduction (Tan and Berridge, unpublished results). Catalase did not affect either NADH or NADPH-dependent WST-1 reduction, whereas SOD inhibited both by about 70%.

Initial attempts to elute cell-surface NAD(P)H-reducing enzyme from the surface of several different cell types is shown in Table 6. Treatment of cells with sodium acetate, pH 5, resulted in WST-1-reducing activity being recovered in the cell wash, with human tumor cell lines yielding three to four times more activity than non-transformed 32D cells. Whether these results point to cell-surface WST-1-reducing activity of tumor cells being less tightly associated with the plasma membrane of these cells awaits more detailed analysis. However, it is worth noting that WST-1-reducing activity in the serum of cancer patients is elevated relative to healthy controls, and that this activity is being investigated as a generic serum test for cancer (Berridge and Tan, 1999).

## DISCUSSION

In this study, we have demonstrated the presence of an NAD(P)H-dependent oxidoreductase at the surface of a wide variety of cells. This enzyme, which can be eluted from the cell surface at pH 5, and which may be responsible for increased NADH-oxidase activity in the serum of cancer patients, reduces the cell-impermeable tetrazolium salt WST-1 in the presence of NADH or NADPH, neither of which readily cross the plasma membrane. Although NAD(P)H-dependent WST-1-reducing activity was observed with all nontransformed, transformed, and tumor cell lines investigated, low levels of activity were also observed on non-dividing cells such as human peripheral blood neutrophils and freshly prepared resting mouse spleen cells. With neutrophils, this activity increased several-fold following activation with the phorbol ester, PMA.

A plasma membrane NADH-oxidase activity that is inhibited by capsaicin has previously been described (Morré *et al.*, 1995, 1996). A similar activity was shown to be eluted from the

TABLE 5. EFFECT OF INHIBITORS ON CELL-SURFACE NAD(P)H-OXIDASE ACTIVITY OF HUMAN PERIPHERAL BLOOD NEUTROPHILS

| | *NAD(P)H-oxidase activity ($\Delta OD_{450}$ at 45 min, % control WST-1 reduction)* | |
|---|---|---|
| *Inhibitor* | *NADH* | *NADPH* |
| Control | 0.434 ± 0.039 (100) | 0.467 ± 0.016 (100) |
| pCMBS (20 $\mu M$) | 0.178 ± 0.131 (41) | 0.258 ± 0.012 (55) |
| Capsaicin (100 $\mu M$) | 0.430 ± 0.012 (99) | 0.423 ± 0.009 (91) |
| Diphenyleneiodinium (20 $\mu M$) | 0.404 ± 0.015 (93) | 0.341 ± 0.020 (73) |
| Catalase (20 $\mu$g/ml) | 0.486 ± 0.023 (112) | 0.466 ± 0.020 (100) |
| SOD (20 $\mu$g/ml) | 0.123 ± 0.004 (28) | 0.138 ± 0.006 (30) |

TABLE 6. ELUTION OF NADH-OXIDASE FROM THE CELL SURFACE

| Cell line | *Cell-surface NADH-oxidase (milliA450/min per $5 \times 10^5$ cells·ml$^{-1}$)* | *Eluted NADH-oxidase (specific activity, arbitrary units)* |
|---|---|---|
| Jurkat | 5.11 ± 0.72 | 1.21 ± 0.06 |
| HeLa | 7.24 ± 0.47 | 1.58 ± 0.11 |
| 143B | 4.60 ± 0.29 | 1.55 ± 0.03 |
| 143B$\rho^0$ | 4.64 ± 0.37 | 1.52 ± 0.05 |
| 32Dcl3 | 3.96 ± 0.30 | 0.40 ± 0.03 |

surface of HeLa cells and to be present in the serum of cancer patients (Chueh *et al.*, 1997; del Castillo-Olivares *et al.*, 1998). In these studies, spectrophotometric measurement of NADH oxidation was routinely used to measure plasma membrane NADH-oxidase activity, and the capsaicin-inhibitable activity was shown to be tumor specific. Comparison of these results with those described in the present study show several fundamental differences. First, the only activity that we have observed to be consistently inhibited by capsaicin and its analogues is the coupled trans-plasma membrane WST-1-reducing activity that is dependent on a cytosolic reductant, most likely, NADH. Neither the cell-surface WST-1-reducing activity that is dependent on adding NADH to cells, nor the activity present in the serum of cancer patients was inhibited by capsaicin. Rather, these activities were consistently stimulated by capsaicin and inhibited by the thiol-blocking agent pCMBS. Because the tetrazolium dye WST-1 is probably reduced in solution or at a site removed from the NADH-binding site of the NADH-oxidase enzyme, it is conceivable that capsaicin may differentially affect NADH oxidation and WST-1 reduction, even though NADH oxidation is required for WST-1 reduction.

Thus, it is possible that capsaicin could act as a catalyst to facilitate WST-1 reduction while inhibiting NADH oxidation at a site proximal to the NADH-binding site of the enzyme. Alternatively, different cell-surface enzymes might be involved.

Second, we have not observed significant qualitative differences between nontransformed and transformed cells regarding sensitivity of NADH-dependent WST-1 reduction to capsaicin or other sulfhydryl or metabolic inhibitors. Thus, growth factor-dependent (nontransformed) 32D cells exhibited a similar inhibitor profile to transformed cells and tumor cell lines (Berridge and Tan, 2000; unpublished results), whereas WST-1 reduction by neutrophils remained unaffected by capsaicin (Table 5). These results appear to differ from those of Morré *et al.* 1995, 1996), who described a capsaicin-inhibitable NADH-oxidase activity associated with the plasma membrane of transformed but not nontransformed cells. They also provided evidence that capsaicin differentially affected the growth of tumor cells and nontumor cell lines (Morré *et al.*, 1995). It is interesting to note that the ability of capsaicin to inhibit the NADH-oxidase activity associated with the plasma membrane of tumor cells or present in the serum and urine of cancer patients has not always given consistent results. Thus, in some situations, capsaicin was shown to stimulate NADH-oxidase activity and this appeared to be related to the redox state of the enzyme (Yantiri *et al.*, 1998; Morré *et al.*, 1999). In addition, the ability of capsaicin to inhibit routinely trans-plasma membrane NADH:WST-1-reducing activity, yet stimulate or have no effect on surface and serum NADH:WST-1-reducing activity, suggests that more than one enzyme or mechanism of action may be involved.

In the present study, we have used pyridine nucleotide cofactors to provide reducing electrons for WST-1 reduction at the cell surface. Because pyridine nucleotides do not occur in extracellular body fluids at the concentration needed to facilitate WST-1 reduction, other cofactors or substrates must serve the physiological function of this enzyme. Recently, O'Donnell and Azzi (1996) described an NAD(P)H-oxidase

at the surface of human fibroblasts that exhibited characteristics of a 15-lipoxygenase, in that in the presence of NADH lucigenin reduction was stimulated by arachidonic acid and by (5S)HETE but not (15S)HETE, and was inhibited by SOD, and interestingly, was inhibited by pCMBS but not by *N*-ethylmaleamide. These properties bear certain similarities to those of the cell-surface NAD(P)H:WST-1 oxidoreductase described in the present study and to the surface NADH-oxidase described by Morré. However, the correlation of SOD-sensitive lucigenin reduction on human fibroblasts with SOD-sensitive cytochrome *c* reduction is not consistent with the present studies in which we have been unable to detect significant levels of NADH-dependent cytochrome *c* reduction. It is possible, however, that SOD-sensitive NADH-dependent lucigenin and cytochrome *c* reduction are unrelated enzyme activities. In preliminary studies, we have observed small (17–26%) increases in NADH-dependent WST-1 reduction with HeLa and P815 cells in the presence of arachidonic acid (20 $\mu M$).

These studies need to be repeated with arachidonate and other serum polyunsaturated fatty acids, and the dependence of WST-1 reduction on cofactors and other serum factors investigated. Arachidonic acid is a precursor for hydroxyperoxytetraenoic acid (HPETE) precursors, for hydroxytetraenoic (HETE) acids and for many physiologically active leukotrienes and lipoxins (Samuelsson *et al.*, 1987). Many of these compounds have potent pro- and anti-inflammatory activity or exhibit vasoactivity. Thus, a potential role for cell-surface NADH-oxidase in regulating arachidonic acid metabolites is possible and this would depend on the cell type, lipid environment, and physiological status of the tissue. Although lipoxygenases have been thought to be associated with intracellular membranes and with the cytosol, there does not appear to have been any systematic investigation of the possible presence of lipoxygenases at the surface of the plasma membrane. Nevertheless, phospholipase $A_2$, which generates arachidonic acid from membrane phospholipids, is a major secretory component of inflammatory cells and is a potent constituent of venomous secretions and stings. Thus, it is likely that serum and cell-surface mechanisms exist that counteract the pro-inflammatory actions of arachidonate and its highly bioactive metabolites. A potential role for cell-surface NAD(P)H-oxidase and/or NAD(P)H:WST-1 oxidoreductase in fatty acid metabolism is therefore possible.

The cell-surface NADH-oxidase described by Morré has been linked with dithiol reduction and dithiol interchange, and has been proposed to be the terminal oxidase of trans-plasma membrane NADH-oxidase (Morré *et al.*, 1999). Our inhibitor results do not support a terminal oxidase function for the NAD(P)H-dependent WST-1-reducing activity described here, particularly regarding the differential effects of cell-impermeable pCMBS on cell surface and trans-plasma membrane WST-1-reducing activity. In addition, the dissociation of trans-plasma membrane and surface NADH-oxidase activities in parental and $\rho^0$ 143B osteosarcoma cells suggests distinct activities. However, it would be interesting to determine whether WST-1 reduction affects the redox status of thiol-regulated proteins at the cell surface or thioredoxin, a key thiol regulatory protein known to be present on the cell surface.

It is possible that cell-surface NADH-oxidase might be promiscuous with respect to its oxidoreductase potential, being capable of using a number of different electron donors and transferring electrons to a variety of targets, some of which may be transient radical species. If the functional roles of cell-surface NADH-oxidase are to be fully understood, it is important that molecular, biochemical, and cellular approaches are used and that functional assays are employed to study appropriate redox reactions at the cell surface and electron transport across the plasma membrane. The results presented here suggest that plasma membrane oxidoreductases may be a more complex system of regulatory redox controls than previous results suggest.

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## ABBREVIATIONS

BPS, bathophenanthroline-disulfonic acid; CEA, carcinoembryonic antigen; DCIP, dichlorophenolindophenol; DPI, diphenyleneiodinium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroxyperoxyeicosatetraenoic acid; LL, Lewis lung; PBS, phosphate-buffered saline; pCMBS, *p*-chloromercuriphenylsulfonic acid; PM, plasma membrane; PMA, phorbol myristate acetate; PMOR, trans-plasma membrane oxidoreductase; PMS, phenazine methosulfate; SH, thiol; SOD, superoxide dismutase; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt.

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