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Forum Review Article

High-Capacity Redox Control at the Plasma Membrane of Mammalian Cells: Trans-Membrane, Cell Surface, and Serum NADH-Oxidases

MICHAEL V. BERRIDGE and AN S. TAN

ABSTRACT

The high capacity of proliferating mammalian cells to transfer electrons from cytosolic NADH to extracellular electron acceptors like oxygen is poorly understood and not widely recognized. Nevertheless, trans-plasma membrane electron transport (plasma membrane redox control) probably ranks alongside the Na^+/H^+ antiport system (pH control) and glucose transport in facilitating cellular responses to physiological stimuli. These plasma membrane transport systems are acutely responsive to receptor ligation by growth factors, polypeptide hormones, and other cell activators. A novel tetrazolium-based cell proliferation assay that we have shown to measure an NADH-oxidoreductase component of the trans-plasma membrane electron transport system has allowed direct comparisons with NADH:ferricyanide-oxidoreductase and respiratory burst NADPH-oxidoreductase. In addition, an NAD(P)H-oxidase at the cell surface and an NADH-oxidase activity in body fluids can be measured by modifying the basic cell proliferation assay. As determined by reduction of the cell-impermeable tetrazolium reagent, WST-1, electron transfer across the plasma membrane of dividing cells can exceed that of fully activated human peripheral blood neutrophils. Cellular reduction of WST-1 is dependent on the presence of an intermediate electron acceptor and is inhibited by superoxide dismutase (SOD) and by oxygen, implying indirect involvement of superoxide in WST-1 reduction. Cell-surface NAD(P)H-oxidase and serum NADH-oxidase are shown to be distinct from trans-plasma membrane NADH-oxidoreductase by their differential sensitivity to capsaicin and pCMBS. The glycolytic metabolism of cancer cells may be linked to changes in trans-plasma membrane NADH:WST-1-oxidoreductase activity and to increased serum NADH-oxidase in cancer. Antiox. Redox Signal. 2, 231–242.

INTRODUCTION

TRANSPORT SYSTEMS traversing the plasma membrane facilitate cellular responses to physiological stimuli such as growth factors and polypeptide hormones acting through their cognate cell-surface receptors. Thus, in addition to receptor signaling to the nucleus, which leads to gene transcription, activation of cell-surface receptors also results in acute changes in plasma membrane transport systems. These changes, which can occur within minutes of receptor ligation, include increased glucose transport (Hamilton *et al.*, 1988; Merrall *et al.*, 1993; Berridge and Tan, 1995; McCoy *et al.*, 1997) rapid changes in Na^+/H^+ exchange (pH control) (Mills *et al.*, 1985; Vairo *et al.*, 1990; Wada *et al.*, 1993), and increased electron transport across the plasma membrane (redox control) (Morré and Brightman, 1991; Brightman *et al.*, 1992). Similarly, cell transformation involves functional changes in plasma membrane

Malaghan Institute of Medical Research, Wellington School of Medicine, Wellington South, New Zealand.

transport systems (Morré *et al.*, 1995a; Zurbriggen and Dreyer, 1996; Yamagata and Tannock, 1996; Ahmed and Berridge, 1997, 1998) that, in some cases, can be readily distinguished from changes in transporter expression. Regulation of these key transport functions at the plasma membrane has frequently been overlooked in the quest to define linear signaling pathways to the nucleus that regulate gene expression. Nevertheless, proximal control of glucose transport, Na^+/H^+ exchange, and plasma membrane electron transport is probably facilitated by signaling pathways originating from activated cell-surface receptors or, in the case of oncogenes, from constitutively activated receptors or components of signaling pathways. These pathways could be either unique or held in common with other pathways, or could diverge from a common phosphorylation or G-protein-linked event at the cytoplasmic domain of the receptor. A generalized scheme of coordinate regulation of plasma membrane transport systems by receptor activation is shown in Fig. 1.

Electron transport across the plasma membrane is a widespread if not universal property of living cells (Crane *et al.*, 1985; Asard *et al.*, 1998), a fact not always appreciated in the scientific literature (Schrenzel *et al.*, 1998). Specialized electron transport systems like the respiratory burst NADPH-oxidase of phagocytic cells are also known, but these are usually restricted in their cellular distribution and expression. The plasma membrane oxidoreductase (PMOR) system of mammalian cells is a multienzyme complex that transfers electrons from cytosolic NADH to extracellular electron acceptors. Although molecular oxygen is probably the major electron acceptor and water the product, these transport systems have mostly been studied using artificial acceptors like ferricyanide and dichlorophenolindophenol (DCIP), or in the case of biochemical investigations, oxidation of the reduced pyridine nucleotide cofactor, NADH. At least two distinct enzyme activities have been described; NADH:ferricyanide-oxidoreductase and NADH-oxidase (Brightman *et al.*, 1992). Although NADH:ferricyanide-oxidoreductase has been investigated both with viable cells and with isolated plasma membranes, most investigations of NADH-oxidase have involved purified plasma membranes. Cyanide-resistant oxygen consumption by whole cells has been shown to be distinct from ferricyanide-reducing activity (Larm *et al.*, 1994).

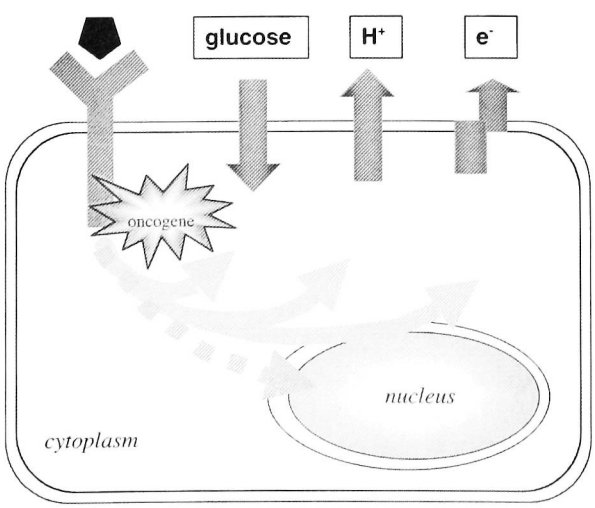

FIG. 1. Acute regulation of plasma membrane transport systems.

Recently, we discovered that a new sulfonated tetrazolium salt, marketed as a cell proliferation reagent that measures mitochondrial succinate dehydrogenase, and containing a stable, proprietary intermediate electron acceptor, was in fact reduced outside the cell by a trans-plasma membrane NADH:WST-1 oxidoreductase (Berridge *et al.*, 1996; Berridge and Tan, 1998). The availability of a simple, microplate-based colorimetric assay for measuring trans-plasma membrane NADH-oxidoreductase of viable cells in real time has provided new opportunities for investigating the functional role of this enzyme. Furthermore, by identifying the proprietary intermediate electron acceptor and separating it from the tetrazolium salt, we have been able to demonstrate a cell-surface NAD(P)H-oxidase and a serum NADH-oxidase with similar properties. The serum NADH-oxidase is elevated in patients with advanced cancer and is being investigated as a generic cancer test (Berridge and Tan, 1999).

This review will focus primarily on the cell-impermeable tetrazolium salt, WST-1, and its use in measuring plasma membrane NADH-oxidoreductases of viable cells. This assay will be compared with other biochemical method-

ologies and with NADH-ferricyanide reductase. The results will be discussed with particular reference to possible physiological roles of plasma membrane oxidoreductases and changes in these enzymes that may be related to cancer.

USE OF THE TETRAZOLIUM SALT, WST-1, AS AN EXTRACELLULAR ELECTRON ACCEPTOR

The tetrazolium salt, WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium], was originally developed to yield a water-soluble formazan when used in conjunction with a stable intermediate electron acceptor, and was initially used to measure viable cells, NADH, and redox-coupled reactions such as lactate dehydrogenase (Ishiyama *et al.*, 1993, 1995). Applications of WST-1 as a cell proliferation reagent appear to have overlooked the fact that added sulfonate groups not only greatly improve solubility, but also generate products that do not readily cross the plasma membrane. This fact was appreciated in the early 20th century when disulfonated indigo dyes were used to distinguish between intracellular and extracellular reductases in a rat tumor model (Voegtlin *et al.*, 1925). Nevertheless, the dogma that tetrazolium salts are reduced by active mitochondria (Mosmann, 1983) was also applied to WST-1, despite warning signals that most reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Berridge and Tan, 1993; Berridge *et al.*, 1996) and [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sul fophenyl)-2*H*-tetrazolium (MTS) (Goodwin *et al.*, 1996) was not mitochondrially associated. The observation that superoxide dismutase (SOD) extensively inhibited cellular WST-1 reduction by proliferating cells (Berridge *et al.*, 1996; Berridge and Tan, 1999) provided the first clear indication that a trans-plasma membrane NADH-oxidase was responsible for WST-1 reduction rather than an intracellular oxidoreductase. In addition, WST-1 formazan accumulated in the culture medium, none being associated with cell pellets at any time following WST-1 addition. In some experiments, localized color development could be observed at the cell surface, but this dissipated rapidly. In other experiments carried out in collaboration with Dr. Mike Davies (Heart Research Institute, Sydney), electron paramagnetic resonance (EPR) studies showed a buildup of 1-methoxy phenazine methosulfate (PMS) radicals in the culture medium of mouse J774 macrophage cells over a period of 30 min (unpublished results). This provides a mechanistic basis for involvement of this intermediate electron acceptor in WST-1 reduction.

WST-1 is marketed as a cell proliferation reagent containing a stable intermediate electron acceptor, but the nature of the intermediate electron acceptor was not made available by the manufacturer. To study the mechanism of WST-1 reduction and to develop WST-1 further as a cellular redox reagent, we have used WST-1 chemical and the intermediate electron acceptor originally described by Ishiyama *et al.* (1993). This combination gives results similar to those obtained with the commercial WST-1 cell proliferation reagent.

The reagents used to measure trans-plasma membrane NADH-oxidase, cell-surface NAD(P)H-oxidase, eluted and serum NAD(P)H-oxidase, respiratory burst NADPH-oxidase and NADH, and NADPH are summarized in Table 1.

TRANS-PLASMA MEMBRANE ELECTRON TRANSPORT

Reduction of WST-1 reagent in real time has been demonstrated in all viable cells investigated. Initial rates of WST-1 reduction are highest in rapidly proliferating cells and lowest in resting cells such as mouse spleen cells and resting human peripheral blood neutrophils (Table 2). Treatment of spleen cells with concanavalin A (Con A) (5 μg/ml, 16 hr) increased WST-1 reduction 15-fold while activation of neutrophils with PMA (100 ng/ml) increased WST-1 reduction by 50-fold. It should be noted that ConA treatment of spleen cells results in a proliferative stimulus, whereas PMA activation of neutrophils triggers a respiratory burst in the absence of proliferation. With rapidly proliferating cells, there appears to be no correlation

TABLE 1. NAD(P)H-OXIDASE MEASUREMENT USING THE CELL-IMPERMEABLE TETRAZOLIUM SALT, WST-1

	Assay components		
Activity measured	*WST-1 (450 μM)*	*1-methoxy PMS (20 μM)*	*NADH/NADPH (200 μM)*
Trans-plasma membrane NADH-oxidase	+	+	−[a]
Cell surface NAD(P)H-oxidase	+	−	+
Eluted/serum NAD(P)H-oxidase	+	−	+
Respiratory burst NADPH-oxidase	+	−	−[a]
NADH and NADPH	+	+	+

[a]Cell assay: cofactor present in the cytosol.

between cell doubling time and WST-1 reduction because Jurkat and 32D cells, which have a doubling time of 13–15 hr, reduced WST-1 at a lower initial rate than HeLa and 143B cells that have a longer doubling time of 16–20 hr.

It is notable that many rapidly proliferating cells reduce WST-1 at about the same rate as fully activated human peripheral blood neutrophils and, in some cases (*e.g.*, HeLa, Lewis lung carcinoma and 143Bρ^0 cells), initial rates of WST-1 reduction were double those of activated neutrophils. Fully activated human peripheral blood neutrophils generate extracellular superoxide at about 8–10 nmol superoxide/min per 10^6 cells as determined by ferricytochrome *c* reduction, and we have shown that WST-1 is a more efficient electron acceptor in this system than ferricytochrome *c* (Tan and Berridge, 2000).

Although WST-1 reduction was strongly inhibited by SOD with all cells tested (Berridge and Tan, 1998 and unpublished data), comparisons based on superoxide production would be compromised because superoxide involvement in WST-1 reduction appears to be indirect (*i.e.*, inhibited by oxygen, unpublished results). From the data presented in Table 2, it can be calculated that the electron flux across the plasma membrane of rapidly proliferating cells, in terms of the 2-electron reduction of WST-1, is $1-2 \times 10^8$ electrons/second per cell. Direct comparisons with neutrophils are justified because WST-1 is reduced to about the same extent whether or not 1-methoxy PMS is employed (Tan and Berridge, 2000).

Ferricyanide reduction was also determined for several of the human cell lines shown in Table 2. Ferricyanide reduction was highest with HeLa cells and lowest with HL60 cells. WST-1 reduction was also high in HeLa cells but, interestingly, the ρ^0 derivative of 143B showed elevated WST-1 reduction compared with the parental cell line and reduced ferricyanide reduction. These results contrast with a previous study of a ρ^0 derivative of human T-lymphoblastic Namalwa cells in which plasma membrane ferricyanide reductase activity was elevated with little change in cyanide-resistant oxygen consumption (Larm *et al.*, 1994).

Cellular reduction of WST-1 can be clearly distinguished from ferricyanide reduction, not only by its differential sensitivity to SOD but also by differences in sensitivity to the cell-impermeable sulfhydryl blocking reagent, p-chloromercuriphenylsulfonic acid (pCMBS), the vanilloid inhibitor, capsaicin, and its potent analogue, resiniferatoxin (Berridge and Tan, 1998). Table 3 summarizes the effects of these and other metabolic and respiratory inhibitors on WST-1 reduction by human and murine cell lines and spleen cells. The results show that 2-deoxyglucose (5 m*M*), a competitive inhibitor of glucose transport and consequently glycolysis, inhibited trans-plasma membrane WST-1 reduction by 25–36% (out of a maximum of 50% inhibition possible because of the presence of 5 m*M* glucose in the culture medium). In contrast, the respiratory inhibitor, cyanide, promoted WST-1 reduction in cells with functional mitochondria. More detailed analysis of the effects of these and other inhibitors has been published (Berridge and Tan, 1998). The results are consistent with WST-1 reduction being depen-

TABLE 2. CELLULAR REDUCTION OF WST-1 AND FERRICYANIDE

Cell type	WST-1 reduction (milliA450/min)		Ferricyanide reduction (pmol/sec per 10^6 cells)
	Trans-plasma membrane	Cell surface	
Human			
Jurkat T-lymphoblastic	22.1 ± 1.3	5.07 ± 0.72	23.8 ± 0.2
HL60 myelomonocytic	18.5 ± 0.7	4.49 ± 0.16	11.5 ± 0.8
HeLa cervical carcinoma	39.2 ± 4.8	7.24 ± 0.47	34.4 ± 1.0
143B osteosarcoma	26.6 ± 4.4	4.60 ± 0.29	31.0 ± 0.8
143Bρ^0 osteosarcoma	45.6 ± 5.8	4.64 ± 0.37	19.3 ± 1.0
U937 macrophage	14.3 ± 1.9	3.62 ± 0.27	
A375 melanoma	16.2 ± 2.8	8.53 ± 0.95	
Malme 3M melanoma	26.8 ± 2.6	10.59 ± 0.30	
Neutrophils (resting)	0.4 ± 0.2	1.45 ± 0.43	
Neutrophils (PMA activated)	20.0 ± 2.7	5.25 ± 0.51	
Murine			
32Dcl3 IL-3-dependent	15.9 ± 2.2	3.96 ± 0.30	
32Dcl3bcr-abl (transformed)	13.5 ± 1.2	2.83 ± 0.21	
P815 mastocytoma	13.0 ± 1.5	4.64 ± 0.25	
LL-LCMV lung carcinoma	37.9 ± 1.4	7.31 ± 1.62	
Spleen (unstimulated)	0.5 ± 0.1	0.79 ± 0.03	
Spleen (ConA stimulated)	7.5 ± 0.2	ND	

Results are the average ± SEM of between 2–6 experiments except for spleen cells and the ferricyanide assay where results are the average ± SEM of duplicates in one experiment. WST-1 reduction was determined using a microplate assay (0.1 ml) with cells at 2.5–3 × 10^5/ml for the trans-plasma membrane assay and 5 × 10^5/ml for the surface oxidase assay. Ferricyanide reduction and neutrophil WST-1 reduction were measured spectrophotometrically at 535 nm and 450 nm, respectively (Berridge and Tan, 1998, 2000).

dent on glycolytic NADH production with a sparing action of respiratory inhibitors on cytoplasmic NADH levels. Interestingly, WST-1 reduction by 143Bρ^0 cells was inhibited by cyanide, and this is explained by inhibitory effects on glycolytic and other cytosolic enzymes. These inhibitor studies support the cell studies described in Table 2, and, taken together, indicate that WST-1 reduction is dependent on glycolytic metabolism.

Capsaicin has been shown to inhibit preferentially the NADH-oxidase activity of isolated plasma membranes from transformed cells and the growth of transformed cells in culture, but failed to inhibit these activities in nontransformed cells (Morré *et al.*, 1995a, 1996a). However, capsaicin also inhibits mitochondrial NADH-coenzyme Q oxidoreductase (Shimomura *et al.*, 1989), raising questions about the validity of attributing growth inhibition to surface NADH-oxidase inhibition in long-term cell growth assays conducted over several days. In this respect, we have shown that capsaicin inhibits short-term WST-1 reduction over 2 hr to a similar extent in non-transformed growth factor-dependent cell lines (*e.g.*, 32D) and transformed cell lines (*e.g.*, HeLa, 143B, Jurkat, and Lewis lung) (Table 3). Furthermore, resiniferatoxin inhibition of WST-1 reduction cannot be distinguished between nontransformed cells (*e.g.*, 32D cells, thymocytes, and spleen cells), and transformed cells (K562, J774, RAW 264.7, and P388D1), 50% inhibition being observed at about 20μg/ml (Tan and Berridge, unpublished results). It is possible that different methods of assaying plasma membrane NADH-oxidase (WST-1 reduction by viable cells versus NADH oxidation by isolated plasma membranes) may yield different results. It is interesting to note that intracellular metabolic effects of capsaicin and resiniferatoxin have been demonstrated previously using the membrane permeable tetrazolium salt, MTT (Berridge and Tan, 1998), which is reduced intracellularly by non-mitochondrial enzymes (Berridge and Tan, 1993) and is readily reduced by ρ^0 cells devoid of active mitochondria (Berridge and Tan, unpublished results).

The cell-impermeable thiol-blocking agent pCMBS had little effect on most trans-plasma membrane WST-1 reduction. In contrast, other

TABLE 3. EFFECT OF INHIBITORS ON TRANS-PLASMA MEMBRANE AND SURFACE WST-1 REDUCTION BY DIFFERENT CELLS

	WST-1 reduction (% control)											
	pCMBS (20 μM)		*N-ethylmaleamide (20 μM)*		*Iodoacetamide (5 mM)*		*Capsaicin (100 μM)*		*2-Deoxyglucose (5 mM)*		*Cyanide (5 mM)*	
Cell line	*t-PM*	*surface*	*t-PM*	*surface*	*t-PM*	*surface*	*t-PM*	*surface*	*t-PM*	*surface*	*t-PM*	*surface*
Experiment 1												
Jurkat	104	30	19	120	18	102	42	124	64	97	160	110
143B	102	19	3	183	11	121	63	146	71	103	170	126
143Bρ^0	96	23	10	140	18	129	54	129	75	104	76	112
Experiment 2												
HeLa	90	29	3	128			62	112				
32Dc123	93	14	ND				66	155				
LL-LCMV	62	10	8	175			67	104				
Spleen	94	3	4	103			ND					

ND, Not determined; t-PM, trans-plasma membrane. For clarity, standard errors have been omitted and were less than ± 5%.

cell-permeable thiol-blocking agents (*N*-ethylmaleamide [NEM] and iodoacetamide) extensively inhibited WST-1 reduction in all cells, consistent with their widespread effects on cellular enzymes.

The characteristics of cellular reduction of WST-1 suggest a trans-plasma membrane NADH:WST-1-oxidoreductase activity. Involvement of cytosolic NADH is circumstantial and a contribution of other cytoplasmic reducing agents cannot be formally excluded. The relationship of the putative NADH:WST-1-oxidoreductase to plasma membrane electron transport activities described by others is not altogether clear. An NADH-coenzyme Q oxidoreductase that appears to be related to NADH-cytochrome b_5 reductase has been purified from liver (Villalba *et al.*, 1995) and this enzyme may initiate electron flow from cytosolic NADH to membrane coenzyme Q. Furthermore, a cell-surface NADH-oxidase with hydroquinone oxidase activity has also been described (Kishi *et al.*, 1999) that could potentially be involved in WST-1 reduction at the cell surface. This enzyme, which can be eluted from HeLa cells, was inhibited by capsaicin and by the anti-tumor sulfonylurea drug, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984) suggesting its involvement as a terminal oxidase in trans-plasma membrane electron transport.

CELL-SURFACE NAD(P)H-OXIDASE

When WST-1 is added to cells in the absence of an intermediate electron acceptor, little reduction is observed. However, addition of NADH results in concentration-dependent WST-1 reduction that is not observed in the absence of cells (Berridge and Tan, 2000). In this situation, NADH is not simply acting as an alternative weak intermediate electron acceptor because the characteristics of reduction are quite distinct (Tables 2 and 3). Interestingly, NADH also promoted cellular reduction of ferricyanide (Berridge and Tan, 2000), although, in this case, background reduction of ferricyanide by NADH, in the absence of cells, was significant and was subtracted.

Cell-surface NADH-oxidase measured by WST-1 reduction in the presence of NADH was present on all dividing and non-dividing cells investigated with highest levels on human melanoma (Malme 3M and A375) and carcinoma (HeLa and Lewis lung) cell lines (Table 2). Most other tumor cell lines and growth factor-dependent cell lines were grouped at around 3–5 milliA450/min. Non-dividing murine spleen cells and human peripheral blood neutrophils showed the lowest levels of surface NADH-oxidase.

The inhibitor studies presented in Table 3 show that cell-surface NADH-oxidase exhibits a distinct pattern of inhibition compared with trans-plasma membrane WST-1-reducing activity. pCMBS extensively inhibited surface NADH-oxidase activity in all cells tested, whereas capsaicin stimulated activity in most cells. Surprisingly, thiol-blocking agents that readily cross the plasma membrane (*N*-ethylmaleamide and iodoacetamide) either did not affect or stimulated surface WST-1 reduction. These results are difficult to reconcile with pCMBS inhibition and suggest a complex hierarchy of availability of sulfhydryl groups on key cell-surface and cytosolic enzymes. In addition, the metabolic inhibitors, 2-deoxyglucose and cyanide failed to significantly affect surface NADH-oxidase activity, indicating the absence of a direct link with cell metabolism. With pCMBS, NEM, and capsaicin, similar results were obtained with a variety of different transformed and nontransformed cell lines and with primary spleen cells, indicating that the distinct inhibitor profile is reproducible and common to many different cell types. Of all inhibitors investigated, only SOD routinely inhibited both cell-surface and trans-plasma membrane NADH-oxidase activity. These results do not support the view that cell-surface NADH-oxidase activity detected with WST-1 is the terminal oxidase of trans-plasma membrane NADH-oxidase. Rather, it appears that a distinct cell-surface oxidase activity is being measured. Confirmation of this may have to await purification and molecular cloning of the donor oxidase of trans-plasma membrane NADH-oxidase and the surface NADH-oxidase measured by adding NADH and WST-1 to cells.

Initial attempts to elute surface NADH-oxidase from a variety of different cells by brief treatment at pH 3.3 failed to generate WST-1-reducing activity in the cell supernatant following neutralization. However, washing cells with 0.1 *M* sodium acetate at pH 5 (del Castillo-Olivares *et al.*, 1998) resulted in the release of NADH-oxidase from several human tumor cell lines (Berridge and Tan, 2000). In contrast, surface NADH-oxidase was poorly released from non-transformed 32Dcl3 and 32Dcl23 cells. Eluted NADH-oxidase showed similar inhibitor characteristics to surface NADH-oxidase with pCMBS and capsaicin (Tan and Berridge, unpublished results). Polyacrylamide gel electrophoresis of eluted protein showed a major 35-kDa band that correlated closely with the level of WST-1-reducing activity measured in the eluates.

Cell-surface NADH-oxidase has previously been eluted from HeLa cells and this activity purified and characterized (Morré *et al.*, 1995b; del Castillo-Olivares *et al.*, 1998). The enzyme was shown to be sensitive to capsaicin and to the anticancer sulfonylurea LY181984 as determined by NADH oxidation. These results differ from those obtained using WST-1 to measure NADH-oxidase activity. Although we have been unable to obtain LY181984, differences in sensitivity to capsaicin suggest that different enzymes or possibly different activation states or protein associations may be involved. Molecular characterization of purified protein is needed to resolve these differences.

In addition to demonstrating surface NADH-oxidase on dividing cells, human peripheral blood neutrophils were also shown to express surface NADH-oxidase activity both in their resting state (Table 2) and following activation with phorbol myristate acetate (PMA). Thus, terminally differentiated neutrophils express low levels of surface NADH-oxidase, and this activity shows a similar inhibitor response profile to that observed with dividing cells, *i.e.*, inhibition by pCMBS, insensitivity to capsaicin, and inhibition by SOD. Neither diphenylene iodinium (DPI), a specific inhibitor of flavoprotein centers, nor catalase had any effect on the surface NADH-oxidase activity of neutrophils.

Finally, the surface NADH-oxidase detected with WST-1 was also able to use NADPH as a cofactor for WST-1 reduction, and this was demonstrated with all cell types mentioned in Table 2 (Berridge and Tan, 2000). Interestingly, the initial rate of WST-1 reduction varied with the different cofactors. With HeLa, 32D cells, and neutrophils, the NADH:NADPH ratio was near unity; with P815, the NADH:NADPH ratio was <1, whereas other cell lines exhibited ratios of 2.7–5.2. Again, different cofactor requirements suggest different enzyme proteins or perhaps different modifications of a single enzyme protein. Biochemical characterisation of eluted NADH-oxidase should help resolve this issue.

A cell-surface NAD(P)H-oxidase on cultured human fibroblasts that generates superoxide following addition of NADH or NADPH (SOD-sensitive lucigenin chemiluminescence and cytochrome *c* reduction) has been described (O'Donnell and Azzi, 1996). This activity, which produced superoxide at about the same rate as activated monocytes (2 nmol/min per 10^6 cells), was completely abolished with pCMBS (100 μM) but was not affected by NEM (60 μM) or DPI (20 μM), characteristics that are similar to those observed with WST-1. Evidence was presented supporting a lipid-metabolizing role for the enzyme, and recombinant rabbit 15-lipoxygenase was shown to produce superoxide and to oxidize NADH.

NADH-OXIDASE IN SERUM: A POTENTIAL TEST FOR CANCER

NADH-oxidase has been detected in culture medium conditioned by HeLa cells, and this activity was shown to be similar to that at the cell surface and to eluted enzyme (Morré *et al.*, 1996b; del Castillo-Olivares *et al.*, 1998). A capsaicin-sensitive NADH-oxidase activity has also been demonstrated in the sera of cancer patients (Morré *et al.*, 1997) and this activity purified as a heat and protease-resistant 33.5-kDa protein (Chueh *et al.*, 1997). To investigate whether serum NADH-oxidase activity could be detected using WST-1, we analyzed sera from mice bearing Lewis lung tumors. Figure 2 shows that in this model tumor size correlated with serum NADH-oxidase activity. The increase was absolute and, unlike the Morré as-

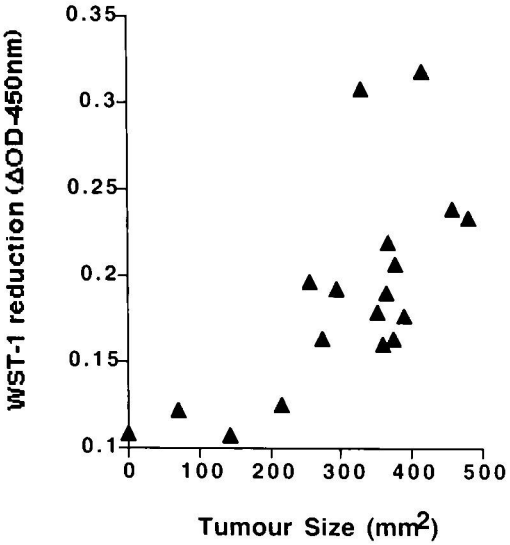

FIG. 2. Groups of 3–4 C57BL6 mice were inoculated subcutaneously with 10^6 Lewis lung carcinoma cells. After 21 days, tumor size was measured as the product of opposing diameters and serum NADH-oxidase activity determined using WST-1 (450 μM) and NADH (200 μM) (Berridge and Tan, 2000). Absorbance was measured at 450 nm in a spectrophotometer.

say system, capsaicin was not used to define the activity. These results led us to investigate WST-1 reduction by serum of patients with advanced colon cancer. In a retrospective study, all 8 patients with advanced colon cancer with liver metastases scored positive ($p < 0.01$) whereas serum from a patient with a small primary tumor failed to reach significance. Another patient with primary liver cancer also scored positively. WST-1-reducing activity was titratable, but as with the cell studies described above, WST-1 reduction was stimulated by resiniferatoxin and by capsaicin. Comparison of WST-1-reducing activity with CEA tests conducted previously showed a high degree of correlation with only the primary liver cancer scoring negative in the CEA test. Subsequent investigation of 58 patients with advanced colon cancer with liver metastases confirmed the initial study showing 83% sensitivity compared with 90% for the CEA test and a combined sensitivity of 93%. A prospective study of cancer patients is presently ongoing.

SUMMARY AND CONCLUSIONS

As shown by reduction of the cell-impermeable tetrazolium salt WST-1 in the presence of an intermediate electron acceptor, a trans-

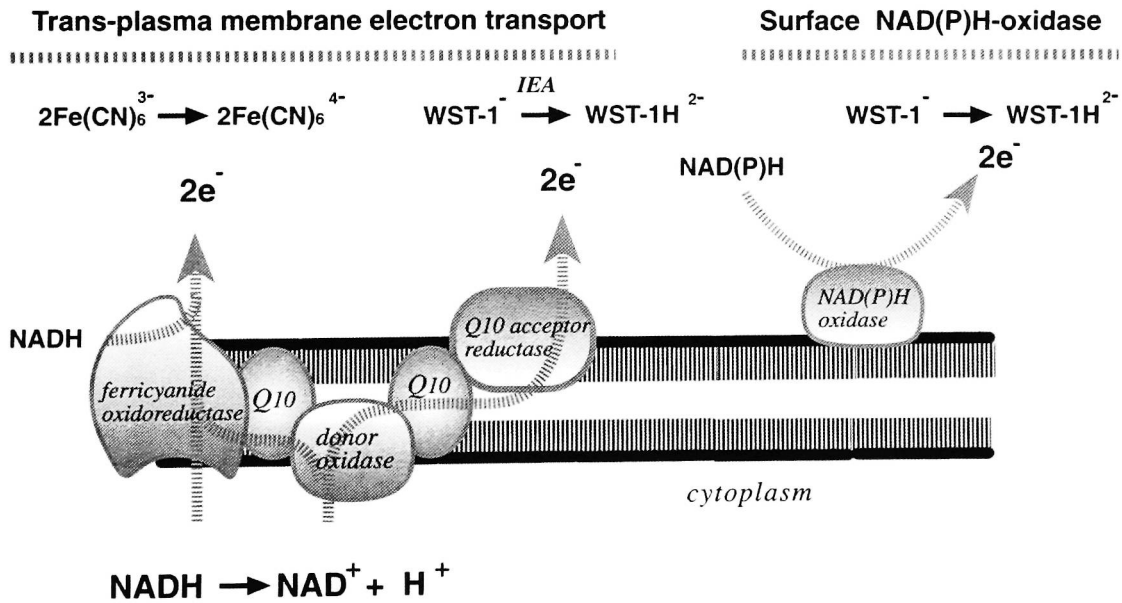

FIG. 3. Model of mammalian plasma membrane electron transport systems. Trans-plasma membrane electron transport is depicted as two independent or overlapping multicomponent enzyme systems identified by ferricyanide and WST-1 reduction. Ubiquinone (coenzyme Q10) is involved as an electron acceptor/donor in the electron transport chain, whereas external NADH can promote ferricyanide reduction (Berridge and Tan, 2000). No equivalent external interaction of NADH with NADH:WST-1 oxidoreductase can be measured because NADH rapidly reduces WST-1 chemically in the presence of intermediate electron acceptor. Surface NAD(P)H-oxidase is measured in the absence of an intermediate electron acceptor by adding NADH or NADPH.

plasma membrane electron transport activity is shown to continuously shift electrons across the plasma membrane of rapidly dividing mammalian cells. At best this electron flux occurs at about twice the rate of fully activated human peripheral blood neutrophils (*i.e.*, 200 million electrons/sec per cell). In transformed cells and tumor cell lines, the electron flux was equal to or greater than that of growth factor-dependent cell lines and activated spleen cells, whereas electron transport in resting cells was an order of magnitude lower. WST-1 reduction was shown to be distinct from ferricyanide reduction and to generate electrons at a 15–20 times greater rate, assuming similar efficiency of electron capture. NADH:WST-1-oxidoreductase shares properties with plasma membrane NADH-oxidases described by others.

In addition, we have used WST-1 to describe an active NAD(P)H-oxidase on the surface of viable cells and, as with trans-plasma membrane WST-1 reduction, have shown that this activity is greatest with proliferating cells and least with non-dividing spleen cells and neutrophils. Cell-surface NAD(P)H-oxidase generates about 10% of the reducing electrons of the trans-plasma membrane activity. A model of plasma membrane electron transport systems leading to WST-1 and ferricyanide reduction is shown in Fig. 3. Cell-surface NADH-oxidase is readily released from tumor cell lines, and increased WST-1-reducing activity is evident in the serum of mice bearing tumors and in the serum of advanced colon cancer patients.

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ABBREVIATIONS

CEA, carcinoembryonic antigen; ConA, concanavalin A; DCIP, dichlorophenolindophenol; DPI, diphenylene iodinium; EPR, electron paramagnetic resonance; IEA, intermediate electron acceptor; LY181984, *N*-(4-methyl-phenylsulfonyl)-*N'*-(4-chlorophenyl) urea; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NEM, *N*-ethylmaleamide; pCMBS, *p*-chloromercuriphenylsulfonic acid; PMOR, plasma membrane NADH-oxidoreductase; PMA, phorbol myristate acetate; PMS, phenazine methosulfate; SOD, superoxide dismutase; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl) -2*H*-tetrazolium, monosodium salt.

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Address reprint requests to:
Dr. M.V. Berridge
Malaghan Institute of Medical Research
Wellington School of Medicine
PO Box 7060
Wellington South, New Zealand

E-mail: mberridge@malaghan.org.nz

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