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Differential Response of the NADH Oxidase of Plasma Membranes of Rat Liver and Hepatoma and HeLa Cells to Thiol Reagents

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NADH oxidase activity of plasma membranes from rat hepatoma and HeLa cells responded to thiol reagents in a manner different from that of plasma membranes of liver. Specifically, the NADH oxidase activity of plasma membranes of HeLa cells was inhibited by submicromolar concentrations of the thiol reagents *p*-chloromercuribenzoate (PCMB), *N*-ethylmaleimide (NEM), or 5,5′-dithiobis-(2-nitrophenylbenzoic acid) (DTNB), whereas that of the rat liver plasma membranes was unaffected or stimulated over a wide range of concentrations extending into the millimolar range. With some hepatoma preparations, the NADH oxidase activity of hepatoma plasma membranes was stimulated rather than inhibited by PCMB, whereas with all preparations of hepatoma plasma membranes, NEM and DTNB stimulated the activity. In contrast, NADH oxidase activity of rat liver plasma membrane was largely unaffected over the same range of PCMB concentrations that either stimulated or inhibited with rat hepatoma or HeLa cell plasma membranes. Dithiothreitol and glutathione stimulated NADH oxidase activity of plasma membranes of rat liver and hepatoma but inhibited that of HeLa plasma membranes. The findings demonstrate a difference between the NADH oxidase activity of normal rat liver plasma membranes of rat hepatoma and HeLa cell plasma membranes in addition to the differential response to growth factors and hormones reported previously (Bruno *et al.*, 1992). Results are consistent with a structural modification of a NADH oxidase activity involving thiol groups present in plasma membranes of rat hepatoma and HeLa cells but absent or inaccessible with plasma membranes of rat liver.

KEY WORDS: NADH oxidase; plasma membrane; cancer; thiols; growth factors; rat liver; hepatoma; HeLa cells.

INTRODUCTION

The oxidation of NADH at the animal plasma membrane involves several enzymatic systems. One is the standard system in which electrons are transferred to ferricyanide and other artificial electron acceptors (Crane *et al.*, 1985). Another is the hormone- and growth-factor stimulated oxidation of NADH (Brightman *et al.*, 1992; Morré and Brightman, 1991) that is constitutively activated in the plasma membranes from transformed cells and tissues (Morré *et al.*, 1991; Bruno *et al.*, 1992). Several correlative studies have produced evidence for the involvement of this growth factor-responsive NADH oxidase in the control of cell proliferation (Morré and Crane, 1990). The activity in transformed cells and tissues was distinguished from that of liver in that the growth factor- and hormone-responsiveness was lost in plasma membranes of transformed liver tissues. These studies were with hyperplastic nodules of liver induced by the liver carcinogen, 2-acetylaminofluorene (Morré *et al.*, 1991) and transplanted rat hepatomas (Bruno *et al.*, 1992).

The NADH oxidase activity of liver plasma membranes is unique among oxidoreductase activities

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not only in its response to growth factors and hormones but, also, in its response to inhibitors and activators other than growth factors and hormones (Morré and Crane, 1990; Morré *et al.*, 1988, 1992). In this regard it differs from the more usual NADH-ferricyanide oxidoreductase activity also found with the mammalian plasma membrane (Crane *et al.*, 1985). In order to further characterize this unusual NADH oxidase activity, we extended our studies to include responses to thiol reagents.

MATERIALS AND METHODS

Purification of Plasma Membranes from Rat Liver

The 5000 × *g* pellet from the preparation of Golgi apparatus (Morré, 1971) was the starting material. The fluffy layer which contains the Golgi apparatus fraction was mixed, withdrawn and excluded from the plasma membrane preparations. Cold 1 mM $NaHCO_3$ (5 ml) was added to each tube and the friable yellow-brown upper part of the pellet was resuspended with a pen-brush, leaving the reddish tightly packed bottom part of the pellet undisturbed. The resuspended material was homogenized in aliquots of 5 ml each in a 30-ml stainless steel (Duragrind) homogenizer 20 times by hand. The homogenates were combined, diluted with cold 1 mM $NaHCO_3$ (1 : 1 dilution), and centrifuged at 6000 × *g* in a HB4 rotor for 15 min. The supernatant was discarded and the pellet was used for the two-phase separation (Morré and Morré, 1989).

The two-phase system contained 6.4% (w/w) Dextran T-500 (Pharmacia), 6.4% (w/w) polyethylene glycol 3350 (Fisher), and 5 mM potassium phosphate buffer (pH 7.2) (Morré and Morré, 1989). The homogenate (1 g) was added to the two-phase system and the weight of the system was brought to 8 g with distilled water. The tubes were inverted vigorously for 40 times in the cold (4°C). The phases were separated by centrifugation at 750 rpm (150 × *g*) in a Sorvall HB4 rotor for 5 min. The upper phases were carefully withdrawn with a pasteur pipette, divided in half, and transferred into 40-ml plastic centrifuge tubes, diluted with cold 1 mM $NaHCO_3$ and centrifuged at 10,000 × *g* in a HB4 rotor for 30 min. Plasma membrane pellets were resuspended in 50 mM Tris-Mes buffer (pH 7.2) and stored at −70°C. Proteins were determined using the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985) with bovine serum albumin as standard. Yields were approximately 3–5 mg per 10 g liver and purity greater than 80% (Navas *et al.*, 1989).

Purification of Hepatoma Plasma Membranes

For isolation of hepatoma plasma membranes (Bruno *et al.*, 1992; Navas *et al.*, 1989) male Fischer 344 rats weighing 100–125 g were purchased from Harlan Animal Supply (Indianapolis, Indiana). Hepatocellular carcinomas induced initially with 2-acetylaminofluorene and designated RLT-28 were propagated *in vitro* in syngeneic recipients as described by Kloppel and Morré (1980). In brief, the tumors were harvested immediately after the animal was killed, cleaned in basic salt solution, and trimmed to remove any capsular or necrotic material. Approximately two pieces (1 mm × 1 mm × 7 mm) were subcutaneously injected with a cancer-implant needle (Popper and Sons, New Hyde Park, New York) on the left mid-lateral surface. The animals were fed *ad libitum* and killed after about 3–4 weeks.

Purification of Plasma Membranes from HeLa Cells

HeLa cells were grown as suspension cultures in minimum essential medium (MEM) supplemented with horse serum, diferric transferrin, and heparin. Cells were collected by centrifugation for 6 to 15 min at 1,000–3,000 rpm. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10 to 30 min to swell the cells. Homogenization was with a Polytron homogenizer for 30 to 40 sec at 10,500 rpm using a PT-PA 3012/23 or ST-probe in 7–8 ml aliquots. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at 1,000 rpm (175 × *g*) to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at 1.4×16^6 *g* min (e.g., 1 h at 23,500 *g*) to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of approximately 1 ml per pellet from 5×10^8 cells. The resuspended membranes were then loaded onto the two-phase system constituted on a weight basis as described above for rat liver. The upper phase, enriched in plasma membranes, was diluted 5-fold with 1 mM $NaHCO_3$ and the membranes were collected by centrifugation. The purity of the

plasma membrane was determined to be >90% by electron microscope morphometry. The yield was 20 mg plasma membrane protein from 10^{10} cells.

Spectrophotometric Assay

NADH oxidase activity was determined from the disappearance of NADH measured at 340 nm. The reaction mixture contained in a final volume of 2.5 ml, 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN to inhibit any contaminating mitochondrial oxidase activity, 100–200 μg plasma membrane protein, and 150 μM NADH. Assay was at 37°C with constant stirring using a Hitachi U3210 spectrophotometer with continuous recording over two 5-min intervals once a steady-state rate was reached. A millimolar extinction coefficient of 6.22 was used to determine specific activity of the NADH oxidase activity.

Activity Characterization

For determination of pH optima, pH was varied in increments of 0.5 pH units between pH 5.0 and 8.0 and in increments of 0.2 pH units between pH 6.8 and 7.4. The K_m's for NADH were determined from double reciprocal (Lineweaver–Burk) analyses of kinetic determinations over the range 15–240 μM NADH (0, 15, 30, 60, 120, and 240 μM). Minus substrate blank rates measured over the same time increments were subtracted. Ion requirements were evaluated from the response to Mg^{2+}, Mn^{2+}, Na^{+}, K^{+}, and Ca^{2+} added over the concentration range 10 nM to 100 mM. Response to GTP was determined serially over the concentration range 1 nM to 10 mM. Results are based on a minimum of three experiments. Standard deviations were calculated among individual experiments.

Thiol Determinations

Thiol determinations were according to the Ellman procedure (Ellman, 1958).

Statistical Analysis

Statistical significance comparing the NADH oxidase activity of plasma membranes in response to thiol reagents and that of untreated membrane was determined using the Student's two-tailed t-test.

RESULTS

The plasma membrane preparations were characterized extensively based on both morphological and enzymatic criteria. Analyses of marker enzyme activities summarized in Table I show the expected enrichments in a plasma membrane marker activity 5′-nucleotidase compared to total homogenate (Navas *et al.*, 1989). Also compared to total homogenate, the plasma membranes were depleted in the mitochondrial marker succinate-INT-reductase. Mitochondrial activities were near the limit of detection for HeLa plasma membranes, low for liver plasma membranes, and somewhat higher for hepatoma plasma membranes. Also depleted relative to total homogenate were the endoplasmic reticulum markers glucose-6-phosphatase and NADPH-cytochrome *c* oxido-reductase. NADPH-cytochrome *c*

Table I. Activities of Marker Enzymes Determined for Plasma Membrane (PM) Preparations Utilized Compared to Starting Total Homogenates (TH)[a]

	Specific activity					
	Rat liver		Rat hepatoma		HeLa cells	
Marker	TH	PM	TH	PM	TH	PM
5′-Nucleotidase	3.4 ± 0.2	45 ± 15	4.5 ± 3.5	16 ± 7	12 ± 4	80 ± 10
Succinate-INT-reductase	7.2 ± 3.4	0.8 ± 0.4	7.9 ± 5.1	2.6 ± 2.0	1.4 ± 2.1	0.7 ± 0.5
NADPH-cyt *c* reductase	26.9 ± 12.4	7.2 ± 0.6	8.0 ± 1.3	3.9 ± 3.9	13.5 ± 4.5	6.0 ± 3.7
Glucose-6-phosphatase	20.4 ± 5.2	0.6 ± 0.6	32 ± 10	2.0 ± 2.0	Not determined	
Galactosyl transferase	1.9 ± 1.2	3.1 ± 1.0	6.5 ± 0.9	2.6 ± 0.1	3.6 ± 1.9	0.3 ± 0.1

[a] Results are means of results from 3 to 5 preparations analyzed at random during the study ± standard deviations among the preparations. Plasma membrane preparations also were monitored by electron microscopy and for the presence of cytochromes. Preparations containing more than 20% mitochondria + endoplasmic reticulum were discarded prior to analysis. Units of specific activity are μmol/min/mg protein for 5′-nucleotidase, succinic-INT-reductase, and glucose-6-phosphatase, nmol/h/mg protein for galactosyl transferase, and nmol/min/mg protein for NADPH-cytochrome *c* reductase.

Table II. Characteristics of NADH Oxidase Activity Comparing Liver, Hepatoma, and HeLa Plasma Membranes

Characteristic	Source of plasma membrane		
	Liver	Hepatoma	HeLa
pH optimum	7.0 ± 0.1	7.0 ± 0.1	7.0 ± 0.1
K_m for NADH (μM)	12 ± 8	22 ± 10	15 ± 10
Ion requirements	None[a]	None	None
GTP stimulation (μM)	0.01	10	>100
Specific activity (nmol/min/mg protein)	1.8 ± 0.3	2.8 ± 0.3	0.4 ± 0.05

[a] < 20% stimulation, $Mg^{2+} \leq Ca^{2+} \leq Mn^{2+}$.

oxido-reductase, however, is present in highly purified plasma membranes (Crane *et al.*, 1985) and therefore yields an overestimate of the endoplasmic reticulum contamination of isolated plasma membranes. Galactosyl transferase also was not enriched in the plasma membrane fractions, suggesting little or no contamination of any of the plasma membranes by Golgi apparatus.

With rat liver where purified reference fractions were available, the contamination by endoplasmic reticulum was estimated to be 2.5%, that of mitochondria 4%, and that of Golgi apparatus about 1.5%. These estimates agree with estimates based on morphometric analyses by electron microscopy which showed the preparations to be 90 ± 4% plasma membranes. Major contaminants were restricted to mitochondria (4%) and endoplasmic reticulum (3%). The yield of plasma membrane was estimated to average between 15 and 20% based on recovery of marker enzymes.

The NADH oxidase activities of hepatoma and HeLa cell plasma membranes were characterized and shown to have steady-state characteristics similar to those of liver (Table II). The pH optima (7.0) and K_m's for NADH were similar for the activities from all three plasma membrane sources. Specific activities on a protein basis differed, however. Those of hepatoma plasma membranes were greater than for liver and those of HeLa cells were lower than for liver. The specific activity of NADH oxidation by HeLa plasma membranes was similar to that of human tumor xenografts produced in immunosuppressed mice (not shown). All three activities were resistant to cyanide, and assays were in the presence of 1 mM potassium cyanide. The NADH oxidase activities were little affected by divalent ions, and the responses of all three sources of plasma membranes to divalent ions were similar. The stimulation was < 20% at a near optimum concentration of 0.01 mM for Mg^{2+}, Ca^{2+}, or Mn^{2+}.

The NADH oxidase activity of rat liver plasma membrane has been reported to be enhanced by nanomolar concentrations of guanine nucleotides (Morré *et al.*, 1993). Considerably more GTP was required to stimulate the NADH oxidase activity of hepatoma plasma membranes than for liver plasma membranes. The NADH oxidase activity of HeLa cell plasma membranes was not stimulated even at 100 μM GTP.

A characteristic that distinguishes the NADH oxidase activities of liver plasma membranes from those of hepatoma and HeLa cell plasma membranes was observed in the response to various thiol reagents (Table III). Generally the NADH oxidase activity of plasma membranes of rat liver was unresponsive except at very high concentrations to thiol reagents. With hepatoma and HeLa cell plasma membranes, the NADH oxidase was either inhibited or stimulated by the thiol reagents with half maximal inhibitions at concentrations of 10 nM for HeLa and half maximal responses between 10 nM and 1 μM for the NADH

Table III. Response of Plasma Membrane of Rat Liver and Hepatoma and HeLa Plasma Membranes to Sulfhydryl Reagents

Reagent	Concentration (μM)	NADH oxidase (nmol/min/mg protein)		
		Liver	Hepatoma	HeLa
None	—	1.7 ± 0.15	2.7 ± 0.2	0.43 ± 0.05
PCMB	0.1	1.6 ± 0.2	2.0 ± 0.6[a]	0.21 ± 0.07
NEM	0.1	2.0 ± 0.15	5.7 ± 0.09	0.18 ± 0.07
DTNB	10	2.7 ± 0.15	4.3 ± 0.3	0.42 ± 0.10
DTT	0.1	2.1 ± 0.2	3.5 ± 0.2	0.2 ± 0.04
GSH	0.1	2.1 ± 0.2	3.1 ± 0.2	0.13 ± 0.03

[a] In 3 of 6 plasma preparations, PCMB was inhibitory and in 3 of 6 plasma membrane preparations, PCMB stimulated (4.5 ± 0.3 nmol/min/mg protein).

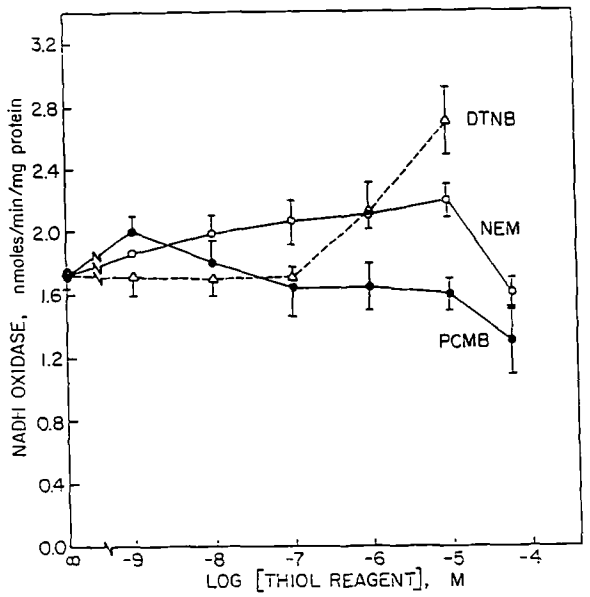

Fig. 1. NADH oxidase activity of rat liver in response to the logarithm of the concentration of the thiol reagents, 5,5′-dithiobis(2-nitrophenylbenzoate) (DTNB), *N*-ethylmaleimide (NEM), and *p*-chloromercuribenzoate (PCMB). PCMB was without effect over a wide range of concentrations. NEM and DTNB were without effect or slightly stimulatory. Duplicate determinations in each experiment were averages. Results are means from three such experiments ($n = 6$) ± standard deviations among experiments. Differences were significant from control ($P < 0.01$) only at 10^{-6} and 10^{-5} M DTNB and NEM.

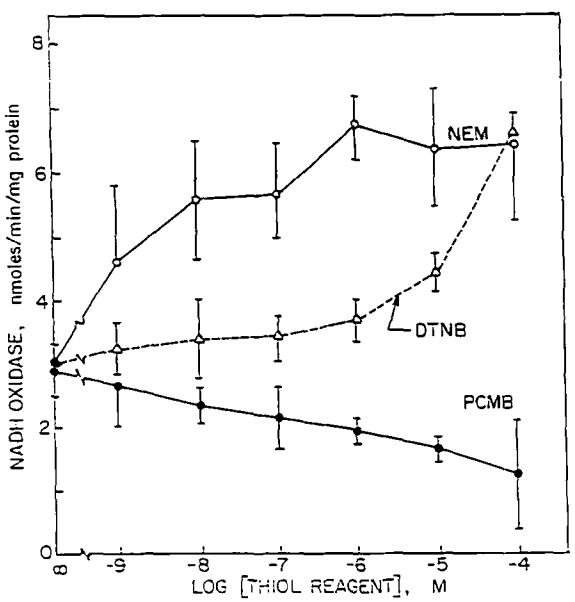

Fig. 2. NADH oxidase activity of plasma membranes of rat hepatoma in response to the logarithm of the concentration of thiol reagents. 5,5′-Dithiobis(2-nitrophenylbenzoate) (DTNB) and *N*-ethylmaleimide (NEM) were stimulatory. With some membrane preparations, *p*-chloromercuribenzoate was inhibitory. With other preparations, the PCMB stimulated (not shown). Duplicate determinations in each experiment were averaged. Results are means from three or four such experiments ($n = 6$ or 8) ± standard deviations among experiments. Differences were significantly different from control ($P < 0.01$) at all concentrations $>10^{-8}$ for NEM, at 10^{-5} and 10^{-4} M DTNB, and for concentrations of PCMB of 10^{-6} M or greater.

oxidase of hepatoma plasma membranes. For example, PCMB at a concentration of 0.1 μM was without effect or slightly stimulatory with rat liver plasma membranes (Fig. 1). With plasma membranes of hepatomas (Fig. 2) and of HeLa cells (Fig. 3), PCMB inhibited. With HeLa cell plasma membranes, 0.1 μM PCMB was sufficient to inhibit the NADH oxidase activity by about 50% (Fig. 3). Similar results were obtained with NEM and DTNB (Table III). However, with hepatoma plasma membranes, NEM and DTNB stimulated, and with HeLa cell plasma membranes, they inhibited (Table III).

As illustrated in Fig. 1, the NADH oxidase activity of rat liver plasma membranes was unaffected or stimulated slightly by two additional thiol reagents, NEM and DTNB over a wide range of concentrations. NEM appeared to stimulate the activity only very slightly at low concentrations (1 nM to 0.1 μM), whereas DTNB stimulated at 1 μM and greater, and PCMB was without effect over the entire range of concentrations tested.

The NADH oxidase activity of hepatoma plasma membranes responded as well to both the thiol reagents DTNB and NEM in proportion to the logarithm of concentration between 1 nM and 0.1 mM (Fig. 2). PCMB was either inhibitory or stimulatory (Table III). However, both DTNB and NEM only stimulated with hepatoma plasma membranes.

NADH oxidase activities of plasma membranes of rat liver, rat hepatoma, and HeLa cells also responded differently to glutathione (GSH) and dithiothreitol (DTT) (Fig. 4). The NADH oxidase activities of rat liver and of hepatoma plasma membranes were substantially stimulated by both GSH and DTT in proportion to the logarithm of concentration (Figs. 4A and 4B) but not to the same extent. Overall, the NADH oxidase activity of hepatoma plasma membranes was stimulated 2-fold by GSH and DTT (Fig. 4A), whereas with plasma membranes of rat liver the stimulations were less than 50% (Fig. 4B). With plasma membranes of HeLa cells, DTT and GSH inhibited (Fig. 4C). As with PCMB, NEM, and DTNB, the activity was inhibited by 50% by DTT and GSH at about 10 nM. The responses to

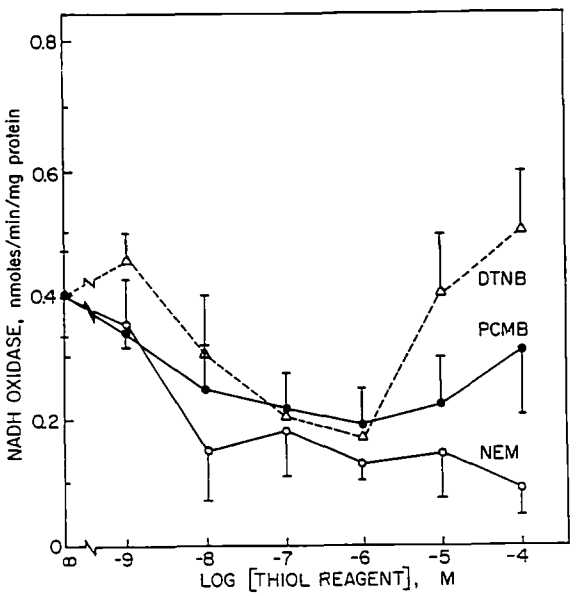

Fig. 3. NADH oxidase activity of plasma membranes of HeLa cells in response to the logarithm of the concentration of thiol reagents. In contrast to results with liver and hepatoma plasma membranes, all three thiol reagents, 5,5′-dithiobis(2-nitrophenylbenzoate) (DTNB), *N*-ethylmaleimide (NEM), and *p*-chloromercuribenzoate (PCMB), inhibited the NADH oxidase activity. Duplicate determinations in each experiment were averaged. Results are means from three such experiments ($n = 6$) ± standard deviations among experiments. Inhibitions were significantly different from controls ($P < 0.05$) for all concentrations of PCMB and DTNB > 10^{-8} and ($P < 0.01$) for all concentrations of NEM > 10^{-8} M. DTNB at 10^{-5} and 10^{-4} M was not inhibitory and not different from control rates.

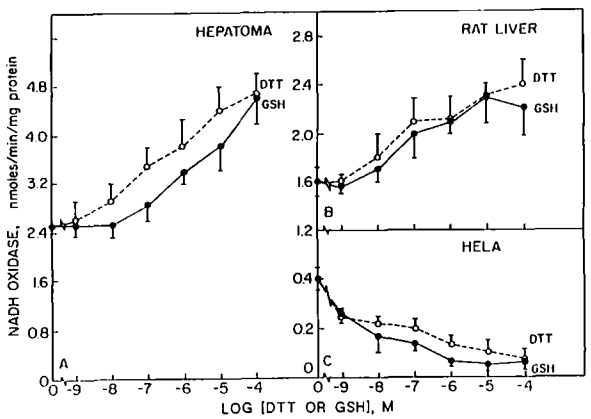

Fig. 4. NADH oxidase of plasma membranes in response to the logarithm of concentration of dithiothreitol (DTT) (dashed lines and open symbols) and glutathione (GSH) (solid line and symbols). (A) Rat hepatoma. (B) Rat liver. (C) HeLa cells. Duplicate determinations in each experiment were averaged. Results are means from three such experiments ($n = 6$) ± standard deviations among experiments. Stimulations of NADH oxidase activity were significantly different ($P < 0.01$) for concentrations of DTT > 10^{-7} M and of GSH > 10^{-6} M for hepatoma plasma membranes and for concentrations of GSH and DTT of 10^{-6} M or greater for both DTT and GSH for rat liver. NADH oxidase activity of HeLa plasma membranes was inhibited significantly ($P < 0.01$) at all concentrations of GSH and DTT tested.

all thiol reagents were rapid and occurred within the first few minutes of incubation.

Thiol contents of membranes estimated by reaction with DTNB (Ellman's reagent) (Ellman, 1958) were increased slightly (<10%) by incubation with NADH. Based on a cysteine standard, thiol contents varied ± 15% among different plasma membrane preparations on a protein basis but did not correlate with responsiveness to inhibition or stimulation by thiol reagent (liver plasma membrane ≃ hepatoma plasma membrane ≃ HeLa plasma membrane).

DISCUSSION

Studies of the growth factor- and hormone-responsive NADH oxidase activities of rat liver plasma membranes (Brightman *et al.*, 1992; Morré and Brightman, 1991; Morré *et al.*, 1992) in comparison to the growth factor- and hormone-unresponsive NADH oxidase activities of hepatoma plasma membranes (Bruno *et al.*, 1992) has revealed responses to thiol reagents that further distinguish the two activities. An especially clear difference was seen with NEM where the NADH oxidase activity of rat liver plasma membranes was unaffected, that of hepatoma plasma membranes was stimulated, and that of HeLa plasma membranes was inhibited. The impermeant DTNB stimulated at high concentrations with plasma membranes of liver and hepatoma but inhibited with plasma membranes of HeLa. In contrast, the NADH oxidase of plasma membranes from rat liver was unaffected by PCMB, whereas PCMB inhibited (or stimulated) the activity with hepatoma plasma membrane preparations but inhibited with plasma membranes from HeLa cells.

The NADH oxidase of HeLa cells, a human transformed cell line of cervical carcinoma derivation, was especially sensitive to inhibition by thiol reagents. The inhibitions by PCMB, NEM, and DTNB were substantial (50% inhibition at 10 nM to 100 nM thiol reagent) but not complete. That the NADH oxidase activity was not completely inhibited suggested the presence in HeLa plasma membranes of thiol reagent-susceptible and -resistant forms of the

activity. Moreover, the susceptible activity was responsive to concentrations of thiol reagent as low as 10 nM, with significant inhibitions being observed within 5 min of incubation.

Thiol protectants glutathione (GSH) and dithiothreitol (DTT) also affected the NADH oxidase activity. With hepatoma plasma membranes, NADH oxidase activity was stimulated strongly by these compounds. The NADH oxidase activity of rat liver plasma membranes also was stimulated although to a lesser extent than with hepatoma plasma membranes. With HeLa plasma membranes, both GSH and DTT inhibited. The inhibitions were not complete but were observed at relatively low concentration of GSH and DTT (50% inhibition at about 10 nM). As with NEM, PCMB, and DTNB, the results with DTT and GSH suggest fundamental differences between the NADH oxidase activity of plasma membranes from rat liver, rat hepatoma, or HeLa cells.

The responses to mild oxidizing agents, 0.0003% hydrogen peroxide or 1 to 100 μM *N*-chlorosuccinamide, also were determined (results not shown). Neither treatment inhibited the NADH oxidase activity nor resulted in the same levels of stimulation as were observed with DTT or GSH. However, with both HeLa and liver plasma membranes, the oxidizing agents appeared to retard losses of enzymatic activity with prolonged times of incubation either at 37°C or at 4°C.

The results show that at least a portion of the NADH oxidase activity of the mammalian plasma membrane in hepatoma and HeLa cells was distinct in its response to thiol reagents from that of rat liver. The latter is relatively resistant to these agents, whereas that of HeLa plasma membranes is very susceptible. Thus, it appears that alkylation of free thiol groups of the plasma membrane occurs differently or affects the response of NADH oxidase activity of plasma membranes of transformed cells differently from that of nontransformed cells. This interaction appears as well to take place at the cell surface.

DTNB, especially, is considered to be an impermeant thiol reagent. With plasma membrane vesicles of HeLa cells, they appear to be sealed and right-side out. That sealed, right-side-out vesicles are responsive to DTNB suggests an involvement of thiol groups at the vesicle (cell) surface. These thiol groups could be located either on one or more NADH oxidases or on a surface protein altered in transformation that is somehow functionally linked to the oxidase activity. A drug target at the cell surface specific to cancer would be especially advantageous in that antineoplastic agents directed to such a target need not enter the cells to be effective.

A role for thiols in transmembrane signalling in mammalian cells is well established. Previously demonstrated have been involvements of thiols in the action of several mammalian hormones at the plasma membranes including those mediated by insulin (Maturo *et al.*, 1983), muscarinic (Aronstam *et al.*, 1978), dopamine (Sidhu *et al.*, 1986; Suen *et al.*, 1980), opiate (Larsen *et al.*, 1981), β-adrenergic (Bottari *et al.*, 1979), vasopressin (Pavo and Fehrenholz, 1990), leukotriene B4 (Falcone and Aharony, 1990), and peptide (El Battari *et al.*, 1988) receptors.

Thioredoxin and thioredoxin reductase activities also are associated with mammalian plasma membranes (Holmgren, 1985), and a phospholipase C active on inositol phosphatides when cloned was shown to contain a thioredoxin-like domain in its structure (Bennett *et al.*, 1988). However, the specificity of the thioredoxin reductase for NADPH makes it unlikely as the link between NADH oxidation and the oxidase activity responsive to thiol reagents. Also thioredoxin is without effect on the activity of the NADH oxidase activity of rat liver plasma membranes. Selenite, which reacts strongly with thioredoxin and thioredoxin reductase, is without effect on the NADH oxidase activity as well.

Our results with HeLa plasma membranes demonstrate an involvement of thiols in the alterations of an NADH oxidase activity associated with transformation. It appears that once the critical thiol groups are reacted with NEM, activity is lost. Membranes pretreated with reduced glutathione or dithiothreitol also are inhibited in their steady-state NADH oxidase activities. Thus not only do thiol groups of NAOH oxidase appear altered in response to transformation, but thiol modifications also may influence the response of the NADH oxidase of the plasma membrane to growth factors and/or hormones. Reagents reacting covalently with thiols, including the impermeant DTNB, as well as reagents reducing thiols inhibited the activity. The findings, taken together with the lack of stimulation of the oxidase by growth factors and hormones, suggest that the response of the NADH oxidase activity of plasma membrane to transformation may involve alterations in critical thiols at or near the external cell surface. We are unable to distinguish between a mechanism where

the proteins responsible for the NADH oxidase activity of the plasma membrane are modified in transformation or where differences in membrane structure associated with transformation alter the oxidase activity. However, with either mechanism, the NADH oxidase activity of the plasma membrane of cancer cells might provide a drug target unique for the development of antineoplastic agents.

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