

# Multiple proteins with single activities or a single protein with multiple activities: The conundrum of cell surface NADH oxidoreductases

Debbie-Jane G. Scarlett<sup>a</sup>, Patries M. Herst<sup>a,b</sup>, Michael V. Berridge<sup>a,\*</sup>

<sup>a</sup>Malaghan Institute of Medical Research, PO Box 7060, Wellington, New Zealand

<sup>b</sup>Department of Radiation Therapy, Wellington School of Medicine and Health Sciences, University of Otago, PO Box 7343, Wellington South, New Zealand

Received 3 October 2004; received in revised form 13 March 2005; accepted 14 March 2005

Available online 2 April 2005

## Abstract

Reduction of the cell-impermeable tetrazolium salt WST-1 has been used to characterise two plasma membrane NADH oxidoreductase activities in human cells. The *trans* activity, measured with WST-1 and the intermediate electron acceptor *m*PMS, utilises reducing equivalents from intracellular sources, while the *surface* activity, measured with WST-1 and extracellular NADH, is independent of intracellular metabolism. Whether these two activities involve distinct proteins or are inherent to a single protein is unclear. In this work, we have attempted to address this question by examining the relationship between the *trans* and *surface* WST-1-reducing activities and a third well-characterised family of cell surface oxidases, the ECTO-NOX proteins. Using blue native-polyacrylamide gel electrophoresis, we have identified a complex in the plasma membranes of human 143B osteosarcoma cells responsible for the NADH-dependent reduction of WST-1. The dye-reducing activity of the 300 kDa complex was attributed to a 70 kDa NADH oxidoreductase activity that cross-reacted with antisera against the ECTO-NOX protein CNOX. Differences in enzyme activities and inhibitor profiles between the WST-1-reducing NADH oxidoreductase enzyme in the presence of NADH or *m*PMS and the ECTO-NOX family are reconciled in terms of the different purification methods and assay systems used to study these proteins.

© 2005 Elsevier B.V. All rights reserved.

**Keywords:** CNOX; gp96; NADH oxidase; NADH oxidoreductase; *Trans*-plasma membrane electron transport; WST-1

## 1. Introduction

The existence of electron transport pathways in cellular compartments other than those of the well-characterised inner

mitochondrial and thylakoid membranes has been known for some time. Only recently, however, has the biological significance of these non-mitochondrial pathways been recognised. Of particular interest are the electron transport systems associated with the plasma membrane (reviewed in Ref. [1]). *Trans*-plasma membrane electron transport (tPMET) pathways have been identified in all living cells investigated, and function in biological processes as diverse as cellular defense [2], intracellular redox homeostasis [3], and control of cell growth and survival [4]. Although our understanding of the functional roles of these processes has progressed significantly over the last decade, the molecular identities of the majority of the proteins involved are still unclear. One protein that has received considerable attention, however, is the cell surface NADH oxidase tNOX [5], the gene for which was cloned recently [6].

tNOX is a tumour-associated member of the external NADH oxidase (ECTO-NOX) family of proteins [7].

**Abbreviations:** BN-PAGE, blue native polyacrylamide gel electrophoresis; capsaicin, 8-methyl-*N*-vanillyl-*trans*-6-nonenamide; CNOX, constitutive NADH oxidase; ECTO-NOX, external NADH oxidase; EP, pH 5 eluted cell surface proteins; FBS, foetal bovine serum; HBSS, Hanks' Balanced Saline Solution; KCN, potassium cyanide; *m*PMS, 1-methoxy-5-methylphenazinium methylsulfate; NBT, nitro blue tetrazolium; PBS, phosphate buffered saline; pCMBS, *p*-chloromercuriphenylsulfonic acid; PI, preimmune; PM, plasma membrane; PMOX, plasma membrane oxidoreductase; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; RT, room temperature; SDS-PAGE, SDS polyacrylamide gel electrophoresis; tNOX, tumour NADH oxidase; tPMET, *trans*-plasma membrane electron transport; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt

\* Corresponding author. Tel.: +64 4 499 6914x825; fax: +64 4 499 6915.

E-mail address: [mberridge@malaghan.org.nz](mailto:mberridge@malaghan.org.nz) (M.V. Berridge).

Other members of this family include the constitutive NADH oxidase, CNOX [8], and an aging-related protein, arNOX [9]. These proteins exhibit a hydroquinone (NADH) oxidase activity, and a protein disulfide–thiol interchange activity, that cycles every 22 to 26 min [10]. The full-length tNOX gene encodes a protein with a predicted molecular mass of 70.1 kDa [6]. However, the majority of published work has focused on a 34 kDa form of this protein that is shed from the cell surface into culture media [11] or sera [12]. Although the oxidase activity of the ECTO-NOX proteins is usually measured directly by NAD(P)H oxidation, the physiological substrate for these proteins is thought to be plasma membrane hydroquinones [7,13].

To measure a major tPMET pathway in proliferating cells, we have used the cell-impermeable tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium), in conjunction with the intermediate electron acceptor *m*PMS (1-methoxy-5-methylphenazinium methylsulfate) [14]. Whole cell reduction of WST-1 under these conditions is sensitive to inhibitors of glycolysis, stimulated by mitochondrial poisons such as potassium cyanide (KCN), and upregulated in mitochondrial gene knockout ( $\rho^0$ ) cells that are dependent on glycolytic ATP for survival [15,16]. The substitution of *m*PMS for extracellular NADH provides a second (albeit non-physiological) measure of plasma membrane electron transport, with somewhat different characteristics to that described above [17]. In this latter pathway, the reduction of WST-1 is dependent on added cofactor (NADH or NADPH), but not on intracellular metabolism [17]. The inhibitor profiles of the NADH- and *m*PMS-dependent WST-1-reducing activities differ both from each other and from those of the ECTO-NOX proteins [15]. This is particularly true for the quinone analogue capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide), which stimulates WST-1 reduction in the presence of NADH [17], inhibits *m*PMS/WST-1 reduction [15], and inhibits tNOX [5], but has no effect on CNOX [8]. Although the simplest interpretation of these results is that the different assay systems measure different oxidase proteins, other explanations are possible, and the absence of molecular data for the WST-1-reducing protein(s) makes it difficult to draw definitive conclusions.

To address this issue directly, we set out to identify the cell surface WST-1-reducing NADH oxidoreductase protein from human 143B osteosarcoma cells. This particular cell line was chosen for study because of its interesting cofactor specificity (a preference for NADH over NADPH) with respect to WST-1 reduction [17]. Using blue native polyacrylamide gel electrophoresis, we identified an approximately 300 kDa complex from purified 143B plasma membranes that reduced WST-1 in the presence of NADH. The WST-1-reducing NADH oxidoreductase activity in this complex was attributed to a 70 kDa protein that cross-reacted with antisera against CNOX. In support of the involvement of ECTO-NOX

proteins in cell surface WST-1 reduction, we show that previously reported differences in enzyme activities and inhibitor profiles between CNOX and the WST-1-reducing enzyme can be explained in terms of the different purification methods and assay systems used to study these proteins. Taken together, these results suggest that the CNOX and WST-1-reducing NADH oxidoreductase activities are intrinsic to a single multifunctional cell surface protein.

## 2. Materials and methods

### 2.1. Materials

The anti-CNOX antiserum [8] was kindly provided by Dr. James Morré (Purdue University, West Lafayette, IN, USA), and anti-gp96 antiserum [18] by Dr. Christopher Nicchitta (Duke University Medical Center, Durham, NC, USA). Human 143B osteosarcoma  $\rho^0$  and parental cells [19] were obtained from Dr. Michael Murphy (Medical Research Council Dunn Human Nutrition Unit, Cambridge, UK). WST-1 and *m*PMS were purchased from Dojindo Laboratories (Kumamoto, Japan). Foetal bovine serum (FBS), GlutaMAX-1, penicillin-streptomycin, and RPMI-1640 medium were from GibcoBRL Life Technologies (Rockville, MD, USA). TEMED, 30% (w/v) acrylamide/bis solution (37.5:1), ammonium persulfate, nitrocellulose and 1 mL Econo-Pac<sup>®</sup> High Q columns were from Bio-Rad Laboratories (Hercules, CA, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Pall Corporation (East Hills, NY, USA), and Coomassie G250 (SERVA Blue G) from SERVA Electrophoresis GmbH (Heidelberg, Germany). Peroxidase-conjugated goat anti-rabbit immunoglobulins were purchased from DAKO (Glostrup, Denmark), SuperSignal<sup>®</sup> chemiluminescent substrate from Pierce (Rockford, IL, USA), and 0.5 mL Vivaspins concentrators (5000 MWCO) from Vivascience (Hanover, Germany). All other reagents were purchased from Sigma Chemical (St. Louis, MO, USA).

### 2.2. Cell culture conditions

Cells were cultured in RPMI-1640 medium supplemented with 5% (v/v) heat-inactivated FBS, 2 mM GlutaMAX-1, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, 50 µg/mL uridine and 1 mM pyruvate, at 37 °C, in a humidified atmosphere with 5% CO<sub>2</sub>.

### 2.3. Preparation of whole cell protein lysates

Cells were washed twice in phosphate buffered saline pH 7.4 (PBS), and resuspended in a lysis buffer containing 50 mM Tris–HCl pH 8, 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Nonidet-P40, 0.9% (v/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF), at a final concen-

tration of  $10^8$  cells/mL. Following incubation on ice for 15 min, the cell lysates were centrifuged at  $12,000 \times g$  for 10 min at 4 °C, and the cleared supernatants containing solubilised proteins transferred to fresh tubes.

#### 2.4. Elution of an NADH oxidoreductase activity from the surface of 143B cells

143B cells ( $10^7$ ) were washed twice in Hanks' Balanced Saline Solution pH 7.4 (HBSS) or PBS, and the cell pellets resuspended in 50  $\mu$ L 0.1 M sodium acetate pH 5. The cell suspensions were then incubated at 37 °C for 60 min, and the eluted protein supernatants (EP) collected for both enzyme activity and Western blot analyses.

#### 2.5. Plasma membrane purification

Plasma membranes were purified from PBS-washed 143B cells essentially as described in [20], the only exception being that 1 mM PMSF was added to the cell pellet suspensions prior to homogenisation. The isolated membranes were stored in 50 mM Tris–HCl pH 7.5, at –70 °C. The purity of the membranes was assessed to be 90% using enzyme marker assays as described in [21].

#### 2.6. Determination of protein concentrations

Protein concentrations were determined using the Lowry protein assay [22].

#### 2.7. Solubilisation of proteins from plasma membranes

Prior to detergent solubilisation, plasma membranes were concentrated to 7.5–10 mg/mL using 0.5 mL Vivaspins concentrators (5000 MWCO), according to the manufacturers' instructions. Of the different detergents examined, optimal solubilisation was obtained in the presence of 0.9% (v/v) Triton X-100. Under these conditions, the plasma membrane proteins retained full enzymatic activity as assessed by the NADH-dependent reduction of WST-1. For blue native polyacrylamide gel electrophoresis, plasma membranes (at a final concentration of 5 mg/mL) were solubilised in a mixture containing 0.9% (v/v) Triton X-100, 0.75 M aminocaproic acid and 50 mM Bis-Tris–HCl pH 7, on ice for 1 h. Solubilised proteins were then harvested by centrifugation at  $16,000 \times g$  for 5 min at 4 °C. For anion exchange chromatography, plasma membranes were solubilised in a buffer containing 0.9% (v/v) Triton X-100 and 25 mM Tris–HCl pH 8.

#### 2.8. Enzyme assays

The *trans* and *surface* WST-1-reducing activities of 143B cells were measured as described in [17]. Briefly, cells ( $5 \times 10^5$ /mL) were washed in HBSS buffer, and transferred (100  $\mu$ L) to a flat-bottomed 96-well microtitre plate. For the

*trans* activity, the cells were incubated with 500  $\mu$ M WST-1 and 20  $\mu$ M *m*PMS at 37 °C in a shaking FLUOstar OPTIMA microplate reader (BMG Labtechnologies Pty. Germany), in the presence or absence of added inhibitors. Optimal inhibitor concentrations were determined by titration as described previously [16]. WST-1 reduction was monitored in real time at 450 nm over a 40–60 min time interval. For *surface* WST-1 reduction, 20  $\mu$ M *m*PMS was replaced with 200  $\mu$ M NADH. WST-1 reduction assays with isolated proteins (2.5  $\mu$ g) were also performed in HBSS buffer with 500  $\mu$ M WST-1 and 200  $\mu$ M NADH. Rates of WST-1 reduction are expressed as mA450/min (change in absorbance at 450 nm/min, multiplied by 1000).

NADH oxidation assays were performed in 200  $\mu$ L reaction volumes in UV compatible 96-well microtitre plates (UV-star, Greiner Labortechnik, Germany). Cells ( $2 \times 10^6$ /mL) or proteins (25  $\mu$ g) were washed in HBSS buffer, then incubated with 200  $\mu$ M NADH in the presence or absence of added inhibitors at 37 °C in a shaking FLUOstar OPTIMA microplate reader. Rates of NADH oxidation were calculated in real time from a decrease in absorbance at 340 nm over a 40–60 min time interval.

#### 2.9. Blue native polyacrylamide gel electrophoresis

Blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed on 5–13% (w/v) linear acrylamide gradient gels using the Mini-PROTEAN II™ system (Bio-Rad) according to [23]. Typically, 50–250  $\mu$ g Triton X-100-solubilised plasma membrane proteins mixed with 500 mM aminocaproic acid, 5% (w/v) SERVA Blue G (1 part dye suspension to 16 parts protein), were loaded per lane. Following electrophoresis, individual lanes were cut out of the first-dimension gel with a razor blade and incubated overnight at room temperature (RT) in 2 mM Tris–HCl pH 7.4, 100  $\mu$ g/mL NAD(P)H, and 2.5 mg/mL nitro blue tetrazolium (NBT) in the presence or absence of 100  $\mu$ M *p*-chloromercuriphenylsulfonic acid (pCMBs).

For second dimension SDS polyacrylamide gel electrophoresis (SDS-PAGE), and activity analysis of the dye-reducing NADH oxidoreductase protein(s), the complex was excised from an unstained gel (its position determined by comparison with a stained lane) and transferred to a 1.5 mL microtube. The gel slice was homogenised in 100  $\mu$ L of 50 mM Tris–HCl pH 7.5, or 100  $\mu$ L HBSS and left on ice for 30 min. The acrylamide was then pelleted by centrifugation ( $16,000 \times g$  for 5 min at 4 °C), and the supernatant assayed for NADH oxidoreductase activity, or mixed with loading buffer and fractionated by SDS-PAGE as described below.

#### 2.10. Anion exchange chromatography

Detergent solubilised plasma membrane proteins (~1 mg) were fractionated on a 1 mL Econo-Pac® High Q column using the BioLogic LP™ chromatography system

(Bio-Rad). Bound proteins were eluted with a linear gradient of 0 to 750 mM NaCl in 25 mM Tris–HCl pH 8, and 0.9% (v/v) Triton X-100 at a flow rate of 1 mL/min, and 0.5 mL fractions collected.

### 2.11. SDS-PAGE and immunoblotting

Proteins (typically 20 µg/lane) were fractionated by SDS-PAGE using the Mini-PROTEAN II™ system (Bio-Rad) according to Laemmli [24] and stained with colloidal Coomassie Blue G-250 as described in [25]. For Western blot analyses, SDS-PAGE-resolved proteins were transferred to nitrocellulose membranes at 100 V for 1 h using a Mini Trans-Blot system (Bio-Rad), according to the manufacturers' instructions. The membranes were blocked in Tris-buffered saline (40 mM Tris–HCl pH 7.6, 150 mM NaCl and 0.05% (v/v) Tween 20) with 5% (w/v) skim milk powder for 1 h at RT, then incubated overnight at 4 °C with rabbit anti-gp96 (1:5000 dilution), anti-CNOX (1:500 dilution) or anti-β-actin (1:1000 dilution) antisera. After washing off the primary antibodies with Tris-buffered saline/1% (w/v) skim milk powder, the blots were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulins (1:2000 dilution) for 1 h, and the protein bands visualised by chemiluminescence (Pierce), according to the manufacturers' instructions. Densitometric analyses were performed using the Kodak Digital Science 1 D Image Analysis Software.

### 2.12. N-terminal protein sequencing

For N-terminal sequence analysis, proteins were transferred to PVDF membrane as described above. The membrane was stained with 0.025% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) methanol for 30 s, and the position of the protein band to be sequenced marked with a needle. The membranes were then destained in 50% (v/v) methanol for 2 h, and washed overnight in multiple changes of milli-Q water (Millipore). Automated Edman degradation was carried out using an Applied Biosystems 494 Procise Protein Sequencing System by the Australian Proteome Analysis Facility (APAF). The sequence obtained was compared to known protein sequences using the Basic Local Alignment Search Tool (BLAST) of NCBI.

## 3. Results

### 3.1. Identification of a 300 kDa redox-active complex in the plasma membranes of 143B cells

Plasma membranes were isolated from human 143B osteosarcoma cells and solubilised in 0.9% (v/v) Triton X-100. The solubilised plasma membranes reduced WST-1 in the presence of NADH and, with the exception of

capsaicin (discussed below), showed a similar inhibitor and substrate profile to that of the surface WST-1-reducing activity of intact 143B cells (Table 1).

To identify plasma membrane protein(s) involved in the NADH-dependent reduction of WST-1, we used blue native polyacrylamide gel electrophoresis (BN-PAGE). This procedure, developed by Schagger and von Jagow to characterise the inner mitochondrial membrane complexes involved in oxidative phosphorylation [26], facilitates the fractionation of heteromultimeric complexes without dissociating them into their constituent polypeptides. Since complexes separated by BN-PAGE retain enzymatic activity, the position of the plasma membrane NADH oxidoreductase (PMOX) was determined by the NADH-dependent reduction of NBT (Fig. 1A). WST-1 could not be used in the in-gel activity assays because it produces a soluble formazan upon reduction [14]. Of the two protein bands visualised by the in-gel activity assay (indicated with arrowheads), only the lower molecular weight protein complex was inhibited by *p*-chloromercuriphenylsulfonic acid (pCMBS), a thiol-binding non-competitive inhibitor of the cell surface NADH oxidoreductase [15,17]. The reduction of NBT by the smaller protein complex (labeled PMOX) was specific for NADH and did not occur when the electron donor was NADPH (Fig. 1A), a substrate profile similar to that of the 143B cell surface NADH oxidoreductase (Table 1). To determine if the protein(s) could also reduce WST-1, the complex was eluted from an equivalent region of unstained gel and assayed for WST-1 reduction. The gel-eluted PMOX complex showed titratable NADH-dependent reduction of WST-1 (Fig. 1B, circles), and this reduction was shown to be sensitive to pCMBS (Fig. 1B, triangles).

The molecular mass of the PMOX protein complex isolated by BN-PAGE was estimated to be approximately 300 kDa by comparison with protein complexes of

Table 1  
WST-1-reducing properties of 143B cells and detergent-solubilised 143B plasma membrane proteins

	WST-1 reduction [mM450/min] (activity % control)		
	Trans ( <i>m</i> PMS)	Surface (NADH)	PM (NADH)
Control	13.2 ± 0.6 (100)	2.2 ± 0.2 (100)	6.3 ± 0.7 (100)
KCN	47.3 ± 1.8 (358)	2.6 ± 0.3 (118)	6.5 ± 1.3 (103)
pCMBS	10.1 ± 0.8 (77)	0.3 ± 0.1 (14)	2.7 ± 0.3 (42)
Capsaicin	1.2 ± 0.3 (9)	12.6 ± 1.1 (573)	6.1 ± 0.5 (97)
		Surface (NADPH)	PM (NADPH)
Control		0.6 ± 0.2 (26)	0.9 ± 0.1 (14)

WST-1 reduction activities were assayed as described in Materials and methods. Control values for whole cell *surface* and isolated PM protein WST-1-reducing activities were determined in the presence of WST-1 (500 µM) and NADH or NADPH (200 µM), while whole cell *trans* WST-1-reducing activities were measured with WST-1 (500 µM) and *m*PMS (20 µM). Inhibitor concentrations were as follows: 1 mM KCN, 25 µM pCMBS, and 200 µM capsaicin. Results are the average ± S.E.M. of three separate experiments.

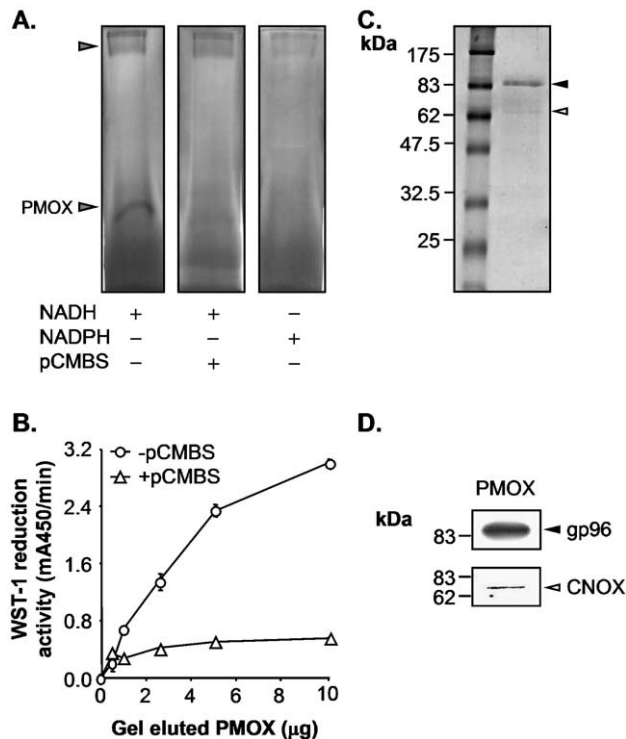


Fig. 1. Identification of plasma membrane proteins involved in the NADH-dependent reduction of WST-1. (A) 100 μg Triton X-100 (0.9% v/v) solubilised plasma membrane proteins from 143B osteosarcoma cells were fractionated on a 5–13% native polyacrylamide gel, and stained with 2 mM Tris–HCl pH 7.5, 0.1 mg/mL NAD(P)H, and 2.5 mg/mL NBT at RT for 4–16 h, in the presence or absence of 100 μM pCMBS. Two protein bands were visualised by the in-gel activity assay (indicated with arrowheads), only one of which, labeled PMOX, was sensitive to pCMBS. (B) WST-1 reduction activity of the gel-eluted PMOX complex in the presence (triangles) or absence (circles) of 25 μM pCMBS. Results are the average  $\pm$  S.E.M. of at least two separate experiments. (C) Resolution of the proteins in the gel-eluted PMOX complex into two predominant polypeptides with estimated molecular masses of 96 kDa (closed arrowhead) and 70 kDa (open arrowhead) following fractionation on a 12% SDS polyacrylamide gel and colloidal Coomassie Blue G-250 staining. (D) Western blots of the SDS-PAGE resolved gel-eluted PMOX complex (25 μg) immunoblotted with rabbit antisera against gp96 (top panel) or CNOX (bottom panel).

known molecular mass (data not shown). Following second dimension SDS-PAGE, the gel-eluted 300 kDa complex resolved into two predominant polypeptides (Fig. 1C) with estimated molecular masses of 96 kDa (closed arrowhead) and 70 kDa (open arrowhead) respectively. The combined molecular weight of the two proteins did not account for the size of the native complex, suggesting either that other proteins were also present in the complex, or that the 96 and 70 kDa proteins existed in higher order structures. N-terminal sequence analysis of the larger molecular weight protein identified it as the glucose-regulated heat shock protein gp96/GRP94 that functions in the regulation of protein folding and assembly (reviewed in Ref. [27]). This assignment was confirmed by immunoblotting of the gel-eluted PMOX complex with anti-gp96 antisera, which reacted with a protein of the predicted molecular mass for

gp96 (96 kDa; Fig. 1D, top panel). Although we had difficulties purifying enough of the  $\sim$ 70 kDa protein using BN-PAGE for direct sequence analysis, the screening of the PMOX complex with antisera against known redox-active plasma membrane proteins suggested that this second protein was related to CNOX (Fig. 1D, bottom panel), a constitutively expressed member of the ECTO-NOX family of cell surface oxidases [8].

### 3.2. The plasma membrane WST-1-reducing NADH oxidoreductase is related to CNOX

To determine which of the identified proteins (CNOX or gp96) were responsible for dye reduction, Triton X-100 (0.9% v/v) solubilised plasma membrane proteins were fractionated by anion exchange chromatography, and bound proteins eluted with a gradient of 0 to 750 mM sodium chloride (Fig. 2A). Gp96 has a *pI* of 4.58 and has been reported in the literature to elute at  $\sim$ 500 mM sodium chloride [18]. Eluted fractions were assayed for both WST-1 (open circles) and NBT (closed circles) NADH oxidoreductase activities (Fig. 2A). Several enzyme activities have been attributed to gp96, including protein phosphorylation [28], aminopeptidase [29] and ATP hydrolysis [30]. However, it appears that most of these activities are the result of copurifying proteins rather than gp96 itself [18]. This was also the situation here because peak activities for the reduction of either tetrazolium salt were observed in fractions 36 to 42 (peak 38), well separated from fractions containing gp96 (Fig. 2B, upper panels). In contrast, relative levels of the anti-CNOX-reactive protein in the column eluted fractions correlated closely with both NBT and WST-1-reducing activities (Figs. 2A and B), suggesting that CNOX, or a closely related protein, was the plasma membrane NADH oxidoreductase responsible for cell surface dye reduction. In support of this proposal, preincubation of whole cells with anti-CNOX antiserum inhibited both the *trans* and *surface* WST-1-reducing activities (Fig. 2C).

### 3.3. The cell surface NADH oxidoreductase is a multifunctional protein that interacts with quinone molecules in the plasma membrane

The identification of CNOX as the likely WST-1-reducing plasma membrane NADH oxidoreductase raised a number of questions. Previously, the *trans* and *surface* WST-1-reducing activities had been considered as separate proteins, which were thought to both differ from the ECTO-NOX proteins [17]. These views were based on apparent differences in activity and on responses to compounds such as capsaicin, which inhibits *trans* WST-1 reduction ([15] and Table 1), stimulates *surface* WST-1 reduction ([15] and Table 1), and inhibits NADH oxidation by tNOX [31] but has no effect on CNOX [8]. It is important to note, however, that the above enzyme activities are measured using different assay systems.

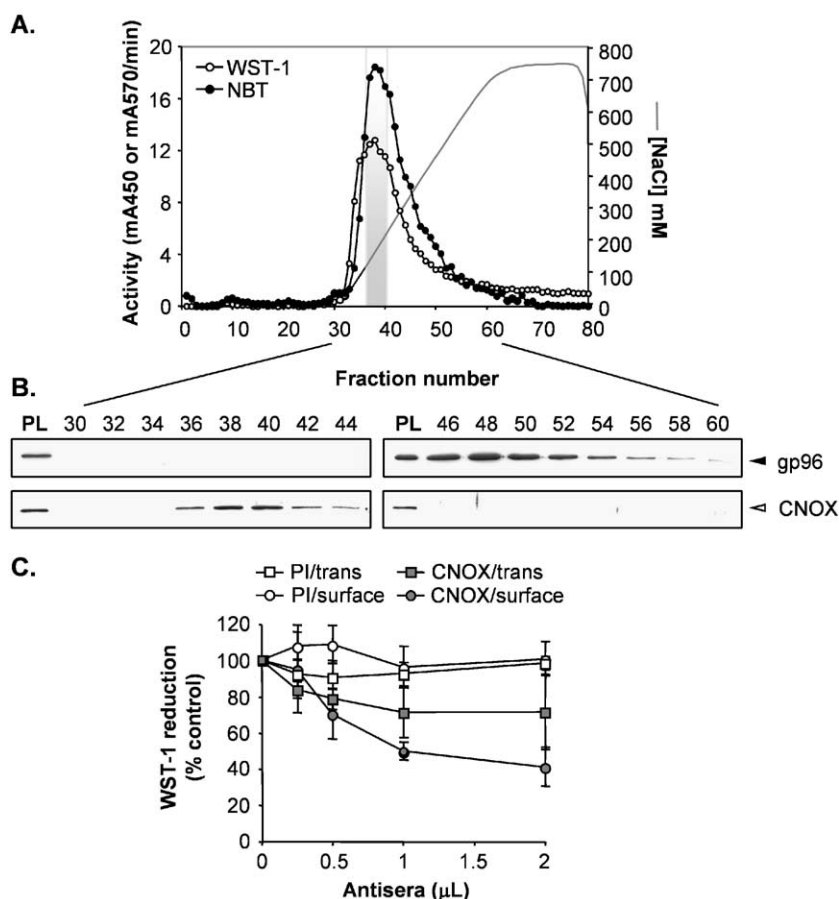


Fig. 2. The plasma membrane dye-reducing NADH oxidoreductase activity is related to CNOX. (A) Triton X-100-solubilised plasma membrane proteins were fractionated by anion exchange chromatography on a High Q (BioRad) column. Bound proteins were eluted with a gradient of 0 to 750 mM sodium chloride (grey line), and individual fractions (0.5 mL) assayed for both NADH-dependent WST-1 (mA450/min, open circles) and NBT (mA570/min, closed circles) reduction. (B) Western blots of eluted fractions (30 µL) immunoblotted with anti-gp96 (top panels) and anti-CNOX (bottom panels) antisera. PL, pre-column load sample (25 µg) of Triton X-100-solubilised plasma membranes. (C) Effect of preincubation of 143B cells with the indicated volume of preimmune (open symbols) or anti-CNOX (closed symbols) antisera for 30 min at RT on *trans* (squares) and *surface* (circles) WST-1 reduction. Results are the average  $\pm$  S.E.M. of at least two separate experiments.

The *trans* activity is measured with WST-1 and the obligate intermediate electron acceptor *m*PMS. Compounds that interfere with glycolysis inhibit this pathway of dye reduction, suggesting that the electron donor for *m*PMS-mediated WST-1 reduction is intracellular NADH. The electron donor for *surface* WST-1 reduction is extracellular NADH. Extracellular NADH is also the electron donor in NADH oxidation measurements (the main assay used to analyse ECTO-NOX activity). However, in this latter system, it is the loss of the substrate that is measured (oxidation of NADH to  $\text{NAD}^+$ ), rather than the appearance of a product (the NADH-dependent reduction of WST-1). In support of a link between at least three of these activities, antisera against CNOX inhibited cellular *trans* and *surface* WST-1 reduction (Fig. 2C). If the different NADH oxidoreductase activities were indeed related, the different effects of capsaicin on these enzymatic activities might then be explained in terms of the different assay systems used to measure them.

We therefore compared the effects of capsaicin, pCMBS, and KCN on the rates of NADH oxidation, the main assay

system used to measure ECTO-NOX activities [10], and on *surface* WST-1 reduction by 143B cells. Both assay systems showed very similar results with respect to substrate profile (a preference for NADH over NADPH) and inhibition by pCMBS, and neither activity was inhibited by potassium cyanide (KCN; Fig. 3A). Capsaicin on the other hand stimulated *surface* WST-1 reduction, as shown previously, while inhibiting NADH oxidation. The observed stimulation of *surface* WST-1 reduction by capsaicin was significant ( $\sim 4$ – $5$  fold) and was shown to be dependent on both capsaicin (Fig. 3B) and NADH (Fig. 3C) concentrations. Interestingly, this stimulation was not seen with capsaicin-treated solubilised plasma membrane proteins (Table 1). Since vanilloids inhibit electron flow through plasma membrane ubiquinone, one interpretation of these results is that in addition to passing electrons from extracellular NADH directly to WST-1, the plasma membrane oxidoreductase is also able to transfer electrons to membrane-associated ubiquinone. The stimulation of *surface* WST-1-reducing activity observed in the presence

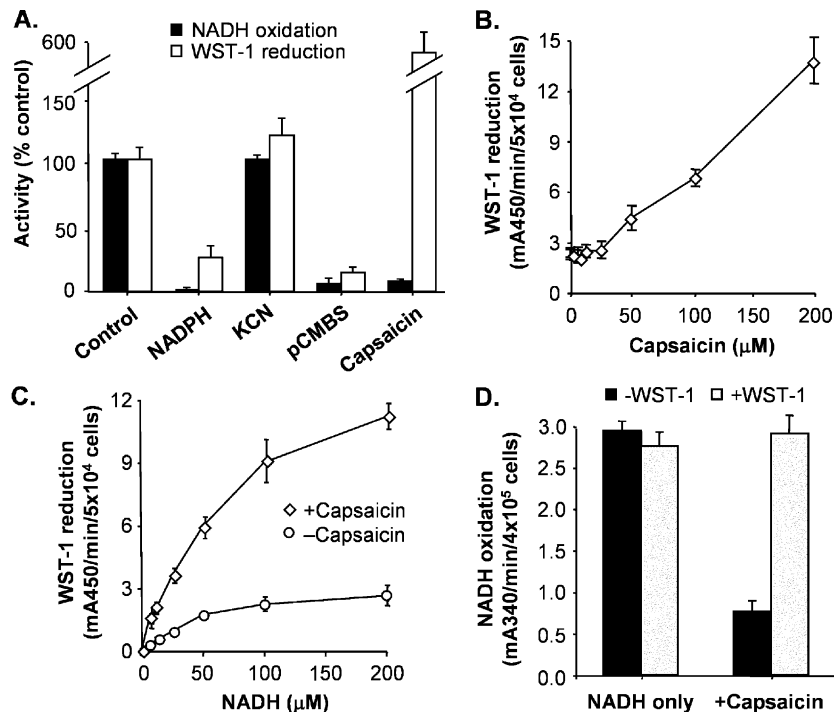


Fig. 3. The cell surface NADH oxidoreductase is a multifunctional protein that interacts with quinone molecules in the plasma membrane. (A) Comparison of the substrate and inhibitor profiles of the surface (WST-1 reduction, open bars) and NADH oxidase (NADH oxidation, closed bars) activities of 143B cells. Cells were incubated with 200  $\mu\text{M}$  NADH in the presence or absence of 1 mM KCN, 25  $\mu\text{M}$  pCMBS or 200  $\mu\text{M}$  capsaicin, and rates of WST-1 reduction or NADH oxidation determined over 40–60 min. To examine the substrate specificity of the oxidoreductase activities, in one set of experiments, NADH was substituted with 200  $\mu\text{M}$  NADPH. (B) Concentration dependence of the capsaicin ‘stimulatory’ effect on surface WST-1 reduction by  $5 \times 10^4$  143B cells. (C) The effect of NADH concentration on surface WST-1 reduction in the presence (diamonds) or absence (circles) of 200  $\mu\text{M}$  capsaicin. (D) NADH oxidation rates of  $4 \times 10^5$  143B cells  $\pm$  200  $\mu\text{M}$  capsaicin in the presence (grey bars) or absence (black bars) of 55  $\mu\text{M}$  WST-1. Results are the average  $\pm$  S.E.M. of three separate experiments.

of capsaicin could then be interpreted as a result of a blockade in the latter competing pathway. In support of this proposal, the capsaicin-induced block in NADH oxidation was overridden by the addition of WST-1 (Fig. 3D), presumably by providing an alternative pathway for electron flow.

### 3.4. Exposure of CNOX to low pH cleaves the protein into lower molecular weight products that exhibit different enzyme activities

The majority of work characterising the ECTO-NOX proteins has been performed on the ‘eluted’ form of the protein, obtained following incubation of cells at 37  $^{\circ}\text{C}$  in 0.1 M sodium acetate pH 5 [20]. Under these conditions, we were also able to detect a time-dependent increase in the appearance of a WST-1-reducing NADH oxidoreductase activity in the supernatants of low pH-treated 143B cells (Fig. 4A). However, the eluted protein (EP) exhibited only very weak WST-1-reducing activity compared with an equivalent amount of solubilised 143B plasma membrane (PM) protein (Fig. 4B, open bars). Conversely, the NADH oxidation activity of the eluted protein was considerably higher than that of solubilised plasma membranes (Fig. 4B, closed bars).

A further property of the ECTO-NOX proteins is their remarkable resistance to both heat and protease treatments [8,32]. In support of this observation, following incubation of both the eluted and plasma membrane proteins at 50  $^{\circ}\text{C}$  for 10 min, the NADH oxidation activities of the proteins were unchanged (Fig. 4C, closed bars). Under the same conditions, however, the WST-1-reducing activities of the proteins were significantly reduced (Fig. 4C, open bars). Similar results were obtained following treatment of the proteins with proteinase K (data not shown). Immunoblotting of the low pH-eluted activity and the Triton X-100 solubilised PM proteins with antisera against CNOX (Fig. 4D, top panel) and gp96 (Fig. 4D, bottom panel) revealed that following the pH 5 treatment, both CNOX and gp96 were cleaved into smaller molecular weight species. The distinct molecular compositions of the eluted and plasma membrane proteins might account for the observed differences in WST-1 reduction and NADH oxidation specific activities.

### 3.5. Modulation of cellular WST-1 reduction and NADH oxidation activities by inhibitors of the hsp90 family of heat shock proteins

A second protein identified in the WST-1-reducing plasma membrane complex was the heat-shock protein

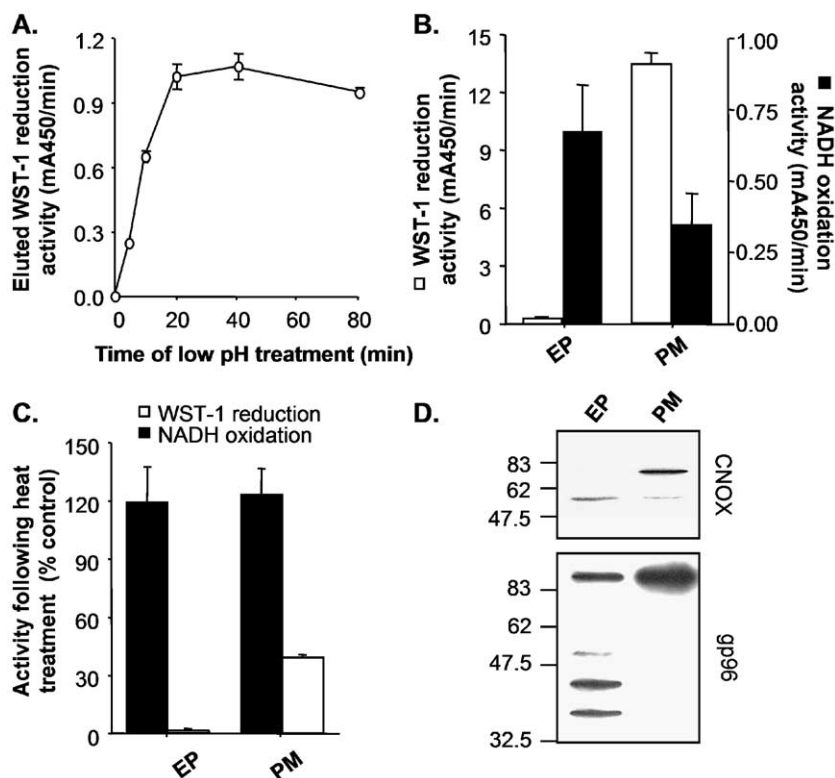


Fig. 4. Comparison of the activities of the eluted and plasma membrane NADH oxidoreductase proteins. (A) Release of a WST-1-reducing activity into the supernatants of 0.1 M sodium acetate pH 5-treated 143B cells. Individual aliquots of 143B cells ( $1 \times 10^7$  cells/aliquot) were washed in HBSS and resuspended in 50  $\mu$ L 0.1 M sodium acetate pH 5. Following incubation at 37  $^{\circ}$ C for the indicated times, 20  $\mu$ L of the culture supernatants were assayed for WST-1 reduction in the presence of 200  $\mu$ M NADH. (B) Rates of WST-1 reduction (open bars, 2.5  $\mu$ g protein) and NADH oxidation (closed bars, 25  $\mu$ g protein) of low pH eluted proteins (EP) and Triton X-100 solubilised plasma membranes (PM). (C) Heat sensitivity of NADH oxidation (closed bars) and NADH-dependent WST-1 reduction (open bars) by 143B EP and PM proteins treated at 50  $^{\circ}$ C for 10 min. Rates have been normalised to control samples incubated at 37  $^{\circ}$ C for 10 min. All assay results are the average  $\pm$  S.E.M. of three separate experiments. (D) CNOX and gp96 compositions of the eluted and plasma membrane protein fractions. Samples (25  $\mu$ g) were fractionated on a 12% (w/v) SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with rabbit antisera against CNOX (top panel) or gp96 (bottom panel).

gp96 (Fig. 1). To determine if this association was important for the activity and/or stability of CNOX, or simply an artifact of preparation, we next examined the effects of compounds known to modulate gp96 activity on our model assay systems. The macrocyclic antifungal antibiotic radicicol interacts with the nucleotide-binding domain of gp96, thereby disrupting its association with various client proteins [33]. With respect to plasma membrane electron transport, radicicol was shown to stimulate the *surface* WST-1-reducing activity of 143B cells (Fig. 5A, circles) but inhibit *trans* WST-1 reduction (Fig. 5A, squares) and whole cell oxidation of NADH (Fig. 5B) at concentrations greater than 50  $\mu$ M. It was also interesting to note that both CNOX and, to a lesser extent, gp96 expressions were elevated in 143B mitochondrial gene-knockout ( $\rho^0$ ) cells in comparison to parental cells (Fig. 5C). The  $\rho^0$  cell model is an example of cells that have adapted to long-term impaired energy metabolism resulting from defective mitochondrial respiration. Under these conditions, elevated CNOX expression was associated with increased *trans* WST-1-reducing activity (Fig. 5D).

#### 4. Discussion

The reduction of the cell-impermeable tetrazolium salt WST-1 is a convenient measure of a tPMET pathway used by proliferating cells [14–16]. In this work, we have identified two proteins in a 300 kDa plasma membrane complex from human 143B osteosarcoma cells that appear to function in this pathway. The first is an NADH oxidoreductase activity related to CNOX, while the second is gp96/GRP94, a glucose-regulated member of the Hsp90 family of heat shock proteins. This is the first demonstration of a potential role for gp96 in tPMET and of its association with an NADH oxidoreductase activity in the plasma membrane.

To identify the NADH oxidoreductase protein(s) involved in WST-1 reduction, we used a combination of BN-PAGE and standard chromatographic techniques. In both procedures, an approximately 70 kDa plasma membrane protein was identified that reacted with antisera against CNOX (Figs. 1D and 2B respectively). The relative levels of the immunoreactive protein in fractions eluting from an anion exchange column were shown to correlate

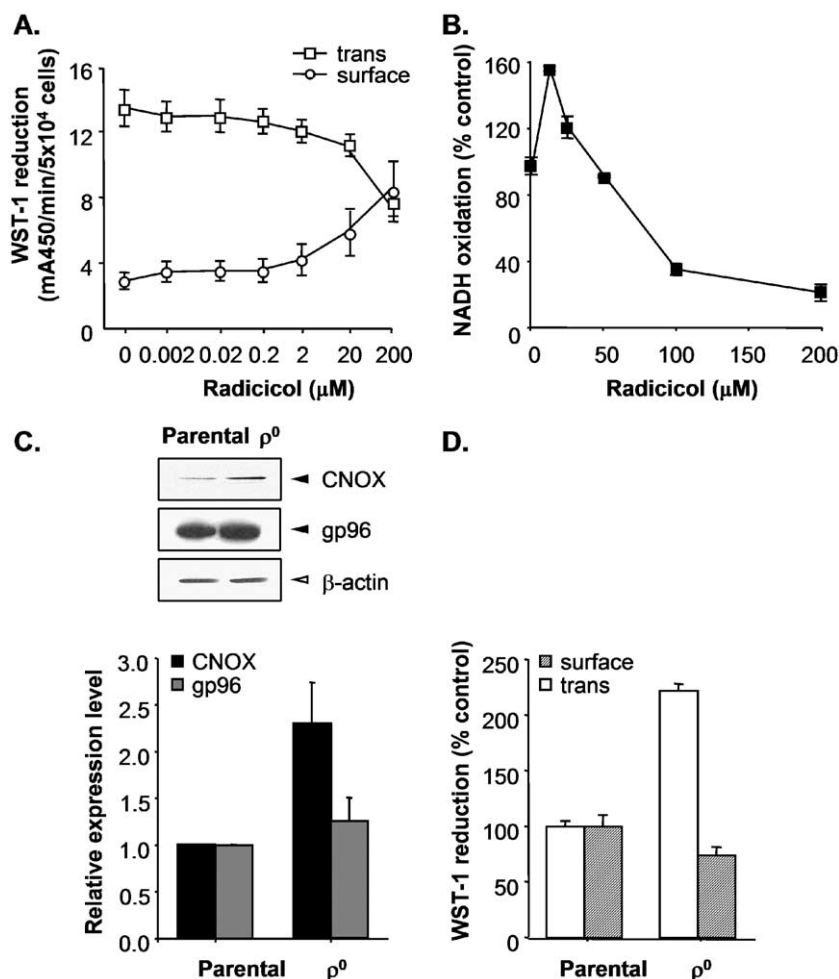


Fig. 5. Modulation of cellular WST-1 reduction and NADH oxidation activities by inhibitors of the hsp90 family of heat shock proteins. (A) *Trans* (squares) and *surface* (circles) WST-1 reduction activities and (B) NADH oxidation activities of  $5 \times 10^4$  143B cells in the presence of increasing concentrations of the gp96 inhibitor radicicol. (C) Representative Western blots of whole cell lysates showing CNOX and gp96 expression levels in 143B parental and  $\rho^0$  cells. Band intensities from three separate experiments were quantified using densitometry, normalised to actin, and the relative expression levels of CNOX and gp96 in parental 143B cells assigned a value of one. (D) *Trans* (open bars) and *surface* (hatched bars) WST-1-reducing activities of 143B parental and  $\rho^0$  cells. Results have been normalised to the activity of parental cells, and are the average  $\pm$  S.E.M. of three separate experiments.

directly with the NADH-dependent reduction of both WST-1 and NBT tetrazolium salts (Figs. 2A, B). In support of the involvement of CNOX in WST-1 reduction, the preincubation of 143B cells with anti-CNOX antisera resulted in decreased *surface* and *trans* activity (Fig. 2C). These results were quite unexpected because several of the properties of the ECTO-NOX proteins such as inhibitor profile, size, and specific activity (reviewed in Refs. [7,34]) differed from that reported for the WST-1-reducing activities [15,17]. In order to determine whether the WST-1-reducing protein was indeed related to CNOX, it was therefore important to reconcile these differences.

Two classes of inhibitors that have been used extensively to characterise CNOX are quinone redox cycle antagonists, such as capsaicin, and thiol-binding compounds, such as pCMBS or its non-sulfonated derivative pCMB. Capsaicin did not affect the NADH oxidase activity of the isolated CNOX protein [8,12,31]. This was also true for the isolated

plasma membrane WST-1-reducing activity (Table 1). In whole cell assays, however, NADH oxidase activity and *trans* WST-1 reduction were inhibited by capsaicin, while *surface* activity was stimulated (Table 1 and Fig. 3A). These responses can be explained in terms of a single multifunctional NADH oxidase protein complex that participates in competing pathways of electron transport. Our results suggest that when associated with the cell surface, the NADH oxidoreductase protein interacts with and is able to transfer electrons from extracellular NADH to membrane-bound ubiquinone. We propose that the same enzyme also accepts two single electrons from semiquinone. In the presence of capsaicin, redox cycling of ubiquinone is blocked so electron transport via this pathway decreases. However, the enzyme is still able to pass electrons from extracellular NADH directly to WST-1, and in the absence of the competing pathway of electron flow, WST-1 reduction increases (Fig. 3). The capsaicin resistance of

the isolated NADH oxidoreductase proteins can then be explained simply as a consequence of no longer being in direct contact with membrane-bound ubiquinone. Although this model accounts for the observed effects of capsaicin on the different whole cell, plasma membrane and eluted protein assay systems, it does not explain the relative sensitivity of the isolated tNOX protein to capsaicin [5,6]. It is possible, however, that tNOX differs structurally from CNOX, accounting for the different responses to capsaicin.

Although the NADH oxidase activity of CNOX is reported to be resistant to thiol-binding reagents [8], the results for this class of compounds are less clear. We routinely work with pCMBS in the 25–100  $\mu$ M range, and have shown that all WST-1-reducing activities show some sensitivity to this compound under these conditions (Table 1 and Refs. [15,17]). pCMB also inhibits the serum CNOX activity isolated from healthy volunteers [12], albeit at higher concentrations than those required to inhibit tNOX from cancer patient serum. However, the antitumour sulfonylurea LY181984 appears to have little effect on CNOX activity (reviewed in Ref. [34]). These results suggest that both the CNOX and WST-1-reducing NADH oxidoreductase activities possess an active site sulfhydryl group important for activity, but that it is not accessible to all thiol-binding drugs.

The molecular mass of CNOX purified from human sera was 24 kDa [8], while the WST-1-reducing activity identified in 143B plasma membranes was estimated to be 70 kDa (Fig. 1). Both 68 and 72 kDa CNOX-like activities have been identified in rat liver plasma membranes [35], and it is possible that the 24 kDa form of CNOX is in fact a proteolytic fragment of a larger molecular weight protein. This is suggested because the smaller forms of CNOX were isolated following digestion with proteinase K and heating at 70 °C for 20 min [8]. Although the NADH oxidase domain of CNOX can withstand these treatments, overall tertiary and secondary structures could be compromised, as was seen with the 143B enzyme following low pH treatment (Fig. 4D). Interestingly, the lower molecular weight fragments of the 143B plasma membrane protein exhibited higher NADH oxidase activity and lower WST-1 reducing activity than the full-length 70 kDa protein did (Fig. 4B), suggesting that certain changes in protein conformation might change enzyme activity. Although the defining characteristic of CNOX is its ability to cycle between NADH oxidase and protein disulfide interchange activities every 24 min [10], we have yet to successfully demonstrate this functionality in our laboratory.

Global profiling of the plasma membrane proteome of cancer cells revealed, somewhat surprisingly, that the most abundantly expressed family of proteins at the cell surface are heat shock proteins [36]. An intriguing finding of the present work was the identification of the heat shock protein gp96 as a component of the 300 kDa redox-active plasma membrane complex. In its native form, gp96 exists as a dimer [37] and is located

predominantly in the endoplasmic reticulum of cells. In some cell types, however, gp96 is also expressed at the cell surface [38–40], at relative levels correlating with tumour immunogenicity [39]. Gp96 belongs to a family of molecular chaperone proteins that prevent the aggregation of misfolded proteins by facilitating correct protein folding [41,42]. Interestingly, the protein disulfide–thiol interchange activity of CNOX has been suggested to function in a similar manner to maintain the structure of proteins that make up the cytoskeleton of the cell [43]. In support of an association between gp96 and CNOX, inhibitors of the heat shock protein such as radicicol were shown to disrupt tPMET (Fig. 5). In contrast to the interaction between Hsp70 and the redox-active plasma membrane associated NAD(P)H:quinone oxidoreductase 1 (NQO1) [44], this interaction is not an obligate requirement for the functionality and stability of the CNOX protein, but does become important during cellular adaptation to stress (Scarlett, D.J., Jerram, P. and Berridge, M.V., unpublished data).

Although the specific involvement of gp96 in modulating CNOX function is unknown, it is possible that an interaction between these two proteins might arise during normal cellular trafficking. Cell surface expressed gp96 has been shown to retain its endoplasmic reticulum KDEL retention signal [38,45] and is thought to travel to the cell surface as part of a larger molecular complex [38]. It is therefore possible that CNOX is one of the proteins involved in this process. Although CNOX is predominantly recognised as a cell surface-associated protein [5,46,47], a CNOX-like activity has been observed in the endoplasmic reticulum [48,49] and the Golgi apparatus [50]. The protein disulfide interchange activity of both the Golgi- and plasma membrane-associated forms of CNOX are inhibited by brefeldin A [51], a fungal macrolide antibiotic that blocks membrane trafficking. This has led to the suggestion that CNOX might play a role in the physical membrane displacements encountered during cell enlargement or vesicle budding along the endoplasmic reticulum vesicle–Golgi apparatus–plasma membrane pathway [51]. Interestingly, both the chaperone activity of gp96 [33] and the NADH oxidase activity [52,53] and stability of CNOX [53] appear to be regulated by ATP. Since CNOX may also function in a plasma membrane electron transport pathway that sustains glycolytic ATP production [43], it is possible that CNOX expression and activity are regulated by a feedback mechanism that responds to changes in intracellular energy metabolism.

In summary, the experiments detailed in this work provide compelling evidence that the *trans* and *surface* WST-1-reducing activities of human 143B osteosarcoma cells are intrinsic to the multifunctional ECTO-NOX protein CNOX. These studies highlight the difficulties of defining enzyme activities using single assay systems in the absence of molecular data.

## Acknowledgements

We would like to acknowledge Dr. James Morré and Dr. Christopher Nicchitta for the generous provision of anti-CNOX and anti-gp96 antisera, respectively, Dr. Michael Murphy for the 143B parental and  $\rho^0$  cell lines, and Martijn Jasperse for his technical assistance with generating graphics. This work was supported by the Royal Society of New Zealand Marsden Fund, a Royal Society James Cook Research Fellowship awarded to M.V.B., and the Cancer Society of New Zealand, and was facilitated by access to the Australian Proteome Analysis Facility established under the Australian Government's Major National Research Facilities program.

## References

- [1] J.D. Ly, A. Lawen, Transplasma membrane electron transport: enzymes involved and biological function, *Redox Rep.* 8 (2003) 1–19.
- [2] A. del Castillo-Olivares, I. Núñez de Castro, M.A. Medina, Dual role of plasma membrane electron transport systems in defense, *Crit. Rev. Biochem. Mol. Biol.* 35 (2000) 197–220.
- [3] M.A. Baker, D.J. Lane, J.D. Ly, V. De Pinto, A. Lawen, VDAC1 is a transplasma membrane NADH:ferricyanide reductase, *J. Biol. Chem.* 279 (2004) 4811–4819.
- [4] M.A. Medina, A. del Castillo-Olivares, I. Núñez de Castro, Multi-functional plasma membrane redox systems, *Bioessays* 19 (1997) 977–984.
- [5] F. Yantiri, D.J. Morré, Isolation and characterization of a tumor-associated NADH oxidase (tNOX) from the HeLa cell surface, *Arch. Biochem. Biophys.* 391 (2001) 149–159.
- [6] P.-J. Chueh, C.P. Kim, N.M. Cho, D.M. Morré, D.J. Morré, Molecular cloning and characterization of a tumor-associated, growth-related, and time-keeping hydroquinone (NADH) oxidase (tNOX) of the HeLa cell surface, *Biochemistry* 41 (2002) 3732–3741.
- [7] D.J. Morré, Quinone oxidoreductases of the plasma membrane, *Methods Enzymol.* 378 (2004) 179–199.
- [8] D. Sedlak, D.M. Morré, D.J. Morré, A drug-unresponsive and protease-resistant CNOX protein from human sera, *Arch. Biochem. Biophys.* 386 (2001) 106–116.
- [9] D.M. Morré, F. Guo, D.J. Morré, An aging-related cell surface NADH oxidase (arNOX) generates superoxide and is inhibited by coenzyme Q, *Mol. Cell. Biochem.* 254 (2003) 101–109.
- [10] D.J. Morré, P.J. Chueh, J. Pletcher, X. Tang, L.Y. Wu, D.M. Morré, Biochemical basis for the biological clock, *Biochemistry* 41 (2002) 11941–11945.
- [11] F. Wilkinson, C. Kim, N. Cho, P.J. Chueh, S. Leslie, S. Moya-Camarena, L.Y. Wu, D.M. Morré, D.J. Morré, Isolation and identification of a protein with capsaicin-inhibited NADH oxidase activity from culture media conditioned by growth of HeLa cells, *Arch. Biochem. Biophys.* 336 (1996) 275–282.
- [12] D.J. Morré, S. Caldwell, A. Mayorga, L.Y. Wu, D.M. Morré, NADH oxidase activity from sera altered by capsaicin is widely distributed among cancer patients, *Arch. Biochem. Biophys.* 342 (1997) 224–230.
- [13] T. Kishi, D.M. Morré, D.J. Morré, The plasma membrane NADH oxidase of HeLa cells has hydroquinone oxidase activity, *Biochim. Biophys. Acta* 1412 (1999) 66–77.
- [14] M.V. Berridge, A.S. Tan, *Trans*-plasma membrane electron transport: a cellular assay for NADH- and NADPH-oxidase based on extracellular, superoxide-mediated reduction of the sulfonated tetrazolium salt WST-1, *Protoplasma* 205 (1998) 74–82.
- [15] M.V. Berridge, A.S. Tan, High-capacity redox control at the plasma membrane of mammalian cells: *trans*-membrane, cell surface, and serum NADH-oxidases, *Antioxid. Redox. Signal.* 2 (2000) 231–242.
- [16] P.M. Herst, A.S. Tan, D.-J.G. Scarlett, M.V. Berridge, Cell surface oxygen consumption by mitochondrial gene knockout cells, *Biochim. Biophys. Acta* 1656 (2004) 79–87.
- [17] M.V. Berridge, A.S. Tan, Cell-surface NAD(P)H-oxidase: relationship to *trans*-plasma membrane NADH-oxidoreductase and a potential source of circulating NADH-oxidase, *Antioxid. Redox Signal.* 2 (2000) 277–288.
- [18] R.C. Reed, T. Zheng, C.V. Nicchitta, GRP94-associated enzymatic activities. Resolution by chromatographic fractionation, *J. Biol. Chem.* 277 (2002) 25082–25089.
- [19] R.D. Appleby, W.K. Porteous, G. Hughes, A.M. James, D. Shannon, Y.H. Wei, M.P. Murphy, Quantitation and origin of the mitochondrial membrane potential in human cells lacking mitochondrial DNA, *Eur. J. Biochem.* 262 (1999) 108–116.
- [20] D.J. Morré, D. Sedlak, X.Y. Tang, P.J. Chueh, T. Geng, D.M. Morré, Surface NADH oxidase of HeLa cells lacks intrinsic membrane binding motifs, *Arch. Biochem. Biophys.* 392 (2001) 251–256.
- [21] P. Navas, D.D. Nowack, D.J. Morré, Isolation of purified plasma membranes from cultured cells and hepatomas by two-phase partition and preparative free-flow electrophoresis, *Cancer Res.* 49 (1989) 2147–2156.
- [22] C.R. Harrington, Lowry protein assay containing sodium dodecyl sulfate in microtiter plates for protein determinations on fractions from brain tissue, *Anal. Biochem.* 186 (1990) 285–287.
- [23] L.G.J. Nijtmans, N.S. Henderson, I.J. Holt, Blue native electrophoresis to study mitochondrial and other protein complexes, *Methods* 26 (2002) 327–334.
- [24] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [25] N.L. Anderson, R. Esquer-Blasco, J.P. Hofmann, N.G. Anderson, A two-dimensional gel database of rat liver proteins useful in gene regulation and drug effects studies, *Electrophoresis* 12 (1991) 907–930.
- [26] H. Schägger, G. von Jagow, Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form, *Anal. Biochem.* 199 (1991) 223–231.
- [27] C.V. Nicchitta, Re-evaluating the role of heat-shock protein-peptide interactions in tumour immunity, *Nat. Rev., Immunol.* 3 (2003) 427–431.
- [28] P. Csermely, Y. Miyata, T. Schnaider, I. Yahara, Autophosphorylation of grp94 (endoplasmic), *J. Biol. Chem.* 270 (1995) 6381–6388.
- [29] A. Menorét, Z. Li, M.L. Niswonger, A. Altmeyer, P.K. Srivastava, An endoplasmic reticulum protein implicated in chaperoning peptides to major histocompatibility of class I is an aminopeptidase, *J. Biol. Chem.* 276 (2001) 33313–33318.
- [30] Z. Li, P.K. Srivastava, Tumor rejection antigen gp96/grp94 is an ATPase: implications for protein folding and antigen presentation, *EMBO J.* 12 (1993) 3143–3151.
- [31] D.J. Morré, P.J. Chueh, D.M. Morré, Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture, *Proc. Natl. Acad. Sci. U. S. A.* 92 (1995) 1831–1835.
- [32] M. Kelker, C. Kim, P.J. Chueh, R. Guimont, D.M. Morré, D.J. Morré, Cancer isoform of a tumor-associated cell surface NADH oxidase (tNOX) has properties of a prion, *Biochemistry* 40 (2001) 7351–7354.
- [33] J.J. Wassenberg, R.C. Reed, C.V. Nicchitta, Ligand interactions in the adenosine nucleotide-binding domain of the Hsp90 chaperone, GRP94: II. Ligand-mediated activation of GRP94 molecular chaperone and peptide binding activity, *J. Biol. Chem.* 275 (2000) 22806–22814.
- [34] D.J. Morré, NADH oxidase: a multifunctional ectoprotein of the eukaryotic cell surface, in: H. Asard, A. Berczi, R.J. Caubergs (Eds.), *Plasma Membrane Redox Systems and Their Role In Biological Stress and Disease*, Kluwer Academic Publishers, Dordrecht, 1998, pp. 121–156.

- [35] D.J. Morré, A.O. Brightman, NADH oxidase of plasma membranes, *J. Bioenerg. Biomembr.* 23 (1991) 469–489.
- [36] B.K. Shin, H. Wang, A.M. Yim, F. Le Naour, F. Brichory, J.H. Jang, R. Zhao, E. Puravs, J. Tra, C.W. Michael, D.E. Misek, S.M. Hanash, Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function, *J. Biol. Chem.* 278 (2003) 7607–7616.
- [37] P.A. Wearsch, C.V. Nicchitta, Endoplasmic reticulum chaperone GRP94 subunit assembly is regulated through a defined oligomerization domain, *Biochemistry* 35 (1996) 16760–16769.
- [38] A. Altmeyer, R.G. Maki, A.M. Feldweg, M. Heike, V.P. Protopov, S.K. Masur, P.K. Srivastava, Tumor-specific cell surface expression of KDEL-containing, endoplasmic reticular heat shock protein gp96, *Int. J. Cancer* 69 (1996) 340–349.
- [39] J. Robert, A. Menorét, N. Cohen, Cell surface expression of the endoplasmic reticular heat shock protein gp96 is phylogenetically conserved, *J. Immunol.* 163 (1999) 4133–4139.
- [40] M.E. Calvert, L.C. Digilio, J.C. Herr, S.A. Coonrod, Oolemmal proteomics—identification of highly abundant heat shock proteins and molecular chaperones in the mature mouse egg and their localization on the plasma membrane, *Reprod. Biol. Endocrinol.* 1 (2003) 27–34.
- [41] C.V. Nicchitta, Biochemical, cell biological and immunological issues surrounding the endoplasmic reticulum chaperone GRP94/gp96, *Curr. Opin. Immunol.* 10 (1998) 103–109.
- [42] P. Csermely, T. Schnaider, C. Soti, Z. Prohaszka, G. Nardai, The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review, *Pharmacol. Ther.* 79 (1998) 129–168.
- [43] D.J. Morré, D.M. Morré, Cell surface NADH oxidases (ECTO-NOX proteins) with roles in cancer, cellular time-keeping, growth, aging and neurodegenerative diseases, *Free Radic. Res.* 37 (2003) 795–808.
- [44] A. Anwar, D. Siegel, J.K. Kepa, D. Ross, Interaction of the molecular chaperone Hsp70 with human NAD(P)H:quinone oxidoreductase 1, *J. Biol. Chem.* 277 (2002) 14060–14067.
- [45] D.L. Wiest, A. Bhandoola, J. Punt, G. Kreibich, D. McKean, A. Singer, Incomplete endoplasmic reticulum (ER) retention in immature thymocytes as revealed by surface expression of “ER-resident” molecular chaperones, *Proc. Natl. Acad. Sci. U. S. A.* 94 (1997) 1884–1889.
- [46] D.J. Morré, NADH oxidase activity of HeLa plasma membranes inhibited by the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl) urea (LY181984) at an external site, *Biochim. Biophys. Acta* 1240 (1995) 201–208.
- [47] C. Kim, W.C. MacKellar, N.M. Cho, S.R. Byrn, D.J. Morré, Impermeant antitumor sulfonylurea conjugates that inhibit plasma membrane NADH oxidase and growth of HeLa cells in culture. Identification of binding proteins from sera of cancer patients, *Biochim. Biophys. Acta* 1324 (1997) 171–181.
- [48] E. Jacobs, D.J. Morré, R. de Cabo, M. Sweeting, D.M. Morré, Response of a protein disulfide isomerase-like activity of transitional endoplasmic reticulum to all-*trans* retinol, *Life Sci.* 59 (1996) 273–284.
- [49] P. Sun, D.J. Morré, D.M. Morré, Periodic NADH oxidase activity associated with an endoplasmic reticulum fraction from pig liver. Response to micromolar concentrations of retinol, *Biochim. Biophys. Acta* 1498 (2000) 52–63.
- [50] D.J. Morré, M. Paulik, J.L. Lawrence, D.M. Morré, Inhibition by brefeldin A of NADH oxidation activity of rat liver Golgi apparatus accelerated by GDP, *FEBS Lett.* 346 (1994) 199–202.
- [51] D.J. Morré, Hormone- and growth factor-stimulated NADH oxidase, *J. Bioenerg. Biomembr.* 26 (1994) 421–433.
- [52] D.J. Morré, J.C. Rodríguez-Aguilera, P. Navas, D.M. Morré, Redox modulation of the response of NADH oxidase activity of rat liver plasma membranes to cyclic AMP plus ATP, *Mol. Cell. Biochem.* 173 (1997) 71–77.
- [53] D.J. Morré, P. Navas, J.C. Rodríguez-Aguilera, D.M. Morré, J.M. Villalba, R. de Cabo, J. Lawrence, Cyclic AMP-plus ATP-dependent modulation of the NADH oxidase activity of porcine liver plasma membranes, *Biochim. Biophys. Acta* 1224 (1994) 566–574.