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Original Paper

Capsaicin Inhibits Plasma Membrane NADH Oxidase and Growth of Human and Mouse Melanoma Lines

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Hormone- and growth factor-stimulated NADH oxidase of the mammalian plasma membrane is thought to be involved in the control of normal cell proliferation. The aim of this study was to determine the effect of the naturally occurring quinone analogue capsaicin (8-methyl-*N*-vanillyl-6-noneamide) on the NADH oxidase activity of plasma membranes and cell growth of human primary melanocytes, the A-375 and SK-MEL-28 human melanoma cell cultures. NADH oxidase activity was inhibited preferentially in the A-375 melanoma cells but not in the primary melanocytes, by capsaicin. Inhibition of growth and the NADH oxidase by capsaicin could be induced in resistant SK-MEL-28 melanoma cells by co-administration of capsaicin with *t*-butyl hydroperoxide, a mild oxidising agent. Death of the inhibited cells was accompanied by nuclear changes suggestive of apoptosis. With B16 mouse melanoma, capsaicin inhibited both the NADH oxidase activity and growth in culture. Growth of B16 melanoma, transplanted in C57BL/6 mice, was significantly inhibited by capsaicin injected directly into the tumour site when co-administered with *t*-butyl hydroperoxide. The findings correlate the inhibition of cell surface NADH oxidase activity with inhibition of growth and capsaicin-induced apoptosis, and also suggest that the extent of inhibition may relate to the oxidation state of the plasma membrane.

Key words: melanoma, cancer, capsaicin, growth, NADH oxidase, redox, plasma membrane, mouse, human

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INTRODUCTION

GROWTH FACTOR- and hormone-stimulated NADH oxidase activity of rat liver plasma membranes has been reported previously by our laboratory [1, 2]. Evidence for the involvement of this growth factor-responsive NADH oxidase in the control of cell proliferation has come from correlative studies with inhibitors [3]. Its activity in transformed cells and tissues is distinct from that in the liver as the growth factor- and hormone-responsiveness appears to be lost. These studies were with hyperplastic nodules of liver induced by the liver carcinogen, 2-acetylaminofluorene [4], and transplanted rat hepatomas [5].

The NADH oxidase activity of liver plasma membranes differs from that of the NADH oxidase of hepatomas and from other oxidoreductase activities not only in its response to growth factors and hormones, but, also in its response to other inhibitors and activators [3, 6, 7]. One such response has been found with the quinone analogues [8, 9]. The plasma membrane NADH oxidase activity has been shown to require quinones [8]. That of transformed cells is inhibited by low concentrations of the quinone analogue, capsaicin (8-methyl-*N*-vanillyl-6-noneamide) [9] whereas that of normal cells is not. Other quinone analogues such as chloroquine may result in inhibition, but millimolar rather than

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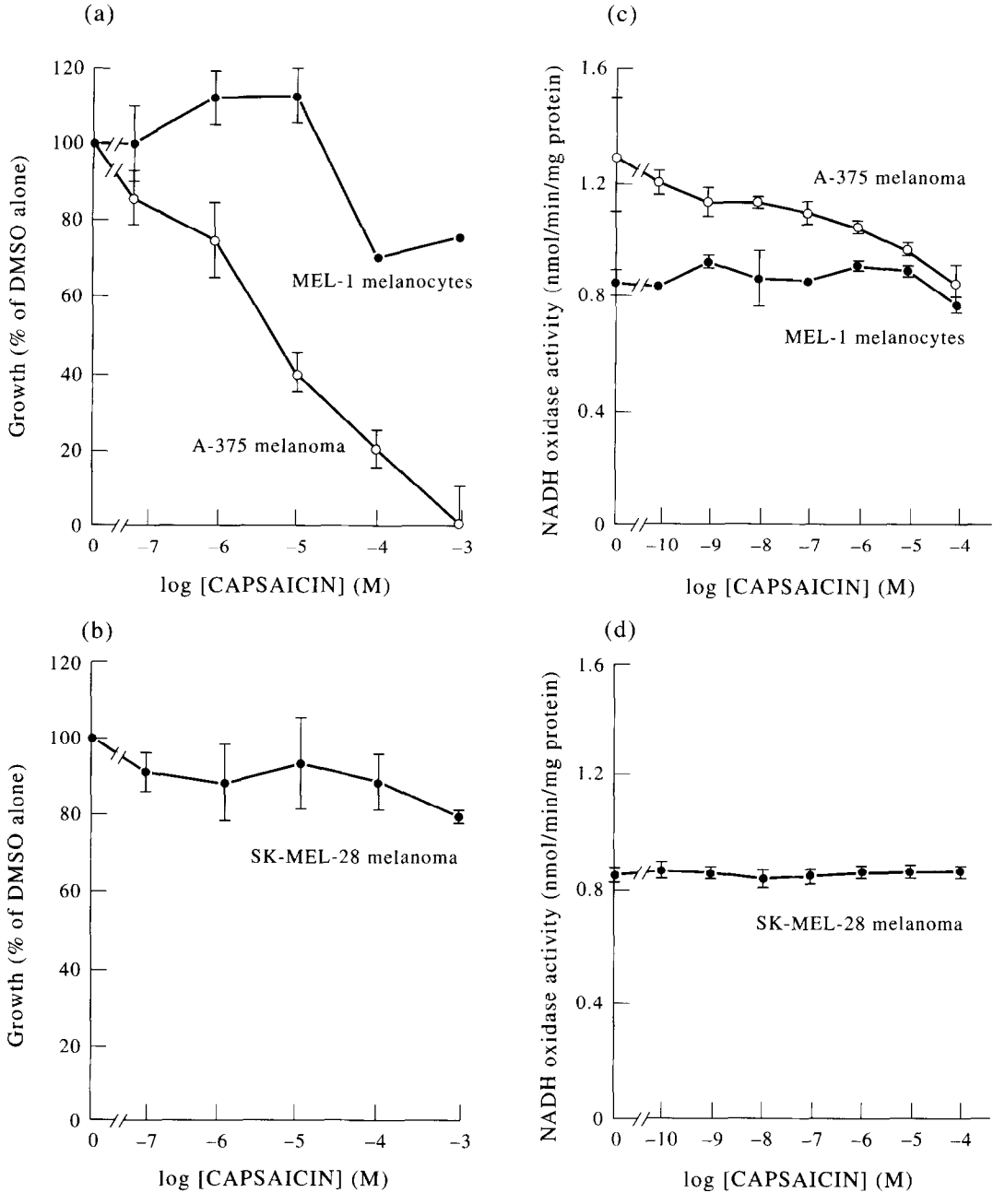

Figure 1. Inhibition by capsaicin of cell growth and NADH oxidase activity in isolated plasma membrane vesicles.

micromolar concentrations are usually required and the inhibition is not specific to transformed cell lines [8].

The aim of this study was to determine the effect of capsaicin on the NADH oxidase activity of plasma membranes and the growth of primary melanocytes and A-375 melanoma cell cultures. The plasma membrane NADH oxidase activity of a melanoma cell line resistant to capsaicin, SK-MEL-28, was also investigated.

MATERIALS AND METHODS

Melanocyte culture

Epidermal cell suspensions were obtained from the foreskins of children by overnight digestion in trypsin (0.25% in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}) at 4°C, and were transferred to complete melanocyte medium (CMM) as previously described [10, 11]. The CMM consisted of MCDB 153 (Biochrom, Berlin, Germany) supplemented with amino acids [12], 2 mM Ca^{2+}, 5 μg/ml insulin (Sigma, St Louis, Missouri, U.S.A.), 10 μg/ml human transferrin (Sigma), 0.4% v/v whole bovine pituitary extract (Clonetics, Inc., San Diego, California, U.S.A.), 2 ng/ml bovine basic fibroblast growth factor (Boehringer-Mannheim, Germany) in 1 nM cholera toxin (Calbiochem, San Diego, California, U.S.A.), 50 μM hydrocortisone (Serva, Heidelberg, Germany) and antibiotics. Foetal calf serum (2%, Biochrom) plus 100 μg/ml geneticin (Gibco BRL, Gaithersburg, Maryland, U.S.A.) was added for the first 3 days of primary culture and just foetal calf serum for the first 24 h of subculture. The keratinocytes were unable to proliferate in this medium and died after 15–20 days.

Pure primary melanocyte cultures were obtained as verified by staining with S-100 antibodies [11].

Growth of melanoma cells

A-375 cells were grown in 175 cm^2 flasks in Dulbecco's modified Eagle's medium with 4.5 g/l glucose, pH 7.4, at 37°C with 10% foetal bovine serum (heat inactivated). Cells were harvested by scraping and suspending in PBS and 25 mM Tris, pH 7.4, to a final cell concentration of 0.1 g wet weight (gww) per ml. The medium was renewed every 2–3 days.

SK-MEL-28 cells were grown as above, except that Eagle's minimum essential medium was used with non-essential amino acids and sodium pyruvate pH 7.4, at 37°C with 10% foetal bovine serum (heat inactivated). For growth experiments, cells were cultured in 96-well flat bottomed microtitre plates, as previously described [13], to which different concentrations of capsaicin in DMSO (final concentrations 0.1%) were added. DMSO at 0.1% was without effect on growth.

Cell lines were from the American Type Culture Collection (Rockville, Maryland, U.S.A.).

Cell proliferation was estimated using a rapid fluorometric assay [13] based on a hydrolysis of the fluorogenic substrate, 4-methylumbelliferyl heptanoate, by cell esterases (Figure 1) or by counting cells over defined areas consisting of a grid of 1 mm squares.

Purification of plasma membranes

In the purification of plasma membranes, cells were collected by centrifugation for 6–15 min at 1000–3000 rpm [14]. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM sodium bicarbonate at an approximate concentration of 10^8 cells/ml and incubated on ice for 10–30 min to enlarge the cells. Homogenisation was with a Polytron Homogeniser for 30–40 s at 10,500 rpm using a PT-PA 3012/23 or ST-10 probe in 7–8 ml aliquots. To estimate breakage, the cells were monitored by light microscopy before and after homogenisation. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at 1000 rpm (175*g*) to remove unbroken cells and nuclei, and the supernatant was centrifuged a second time at $1.5 \times 10^6 g$ min (25,000*g* for 30 min) to prepare a plasma membrane-enriched microsome fraction [14]. 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 on to the two-phase system constituted on a weight basis, as previously described [15].

The two-phase system contained 6.6% (w/w) Dextran T-500 (Pharmacia), 6.6% (w/w) polyethylene glycol 3350 (Fisher), and 5 mM potassium phosphate buffer (pH 7.2) [15]. The plasma membrane-enriched microsome fraction (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 40 times at 4°C. The phases were separated by centrifugation at 750 rpm (150*g*) in a Sorval HB 4 rotor for 5 min. The upper phases were withdrawn carefully with a Pasteur pipette, divided in half, and transferred to 40 ml plastic centrifuge tubes. The tube contents were diluted with cold 1 mM sodium bicarbonate and collected by centrifugation at 33 000*g* in an HB rotor for 30 min. Plasma membrane pellets were resuspended in 50 mM Tris–Mes buffer (pH 7.0) and stored at −70°C. Proteins were determined using the bicinchoninic acid (BCA) assay [16] with bovine serum albumin as standard. The upper phase, containing the plasma membranes, was diluted 5-fold with 1 mM sodium bicarbonate and the membranes were collected by centrifugation. The purity of the plasma membranes was determined to be more than 90% by electron microscope morphometry. The yield was 20 mg plasma membrane protein from 10^{10} cells.

Spectrophotometric assay of NADH oxidase

NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mixture containing 50 mM Tris–Mes buffer (pH 7.2), 1 mM KCN (to inhibit low levels of mitochondrial oxidase activity), and 150 μM NADH at 37°C with stirring. Activity was measured using a Hitachi U3210 or SLM Aminco model DW2000 spectrophotometer with continuous recording over two intervals of 5 min each. A millimolar extinction coefficient of 6.22 was used to determine specific activity. Capsaicin was added as a DMSO solution at the final concentrations indicated at the beginning of the assay and was present during the assay period. Controls received an equivalent amount of DMSO (0.1%).

Fluorescence microscopy

Cells were grown for 72 h on glass coverslips placed in small culture dishes with media containing 100 μM capsaicin in DMSO or an equivalent amount of DMSO alone. The coverslips were rinsed and the cells fixed in methanol. Nuclear DNA was stained using a fluorescent dye DAPI (4′,6-diamidino-2-phenylindole), as previously described [17]. Cells were observed and photographed at a primary magnification of 400×.

Animal studies

B16 F_0 mouse melanomas were carried in female C57BL/6 mice (16 g, Harlan Industries, Indianapolis, Indiana, U.S.A.). Tumours were initiated by subdermal injection of approximately 10^6 cells. When the tumour size was approximately 0.5 g, capsaicin or *t*-butyl hydroperoxide alone or in combination were administered daily at the tumour site in 100 μl DMSO for 7 days. Eight days after the initiation of treatment, the animals were sacrificed and the tumours were excised and weighed.

All chemicals were from Sigma (St. Louis, Missouri, U.S.A.) unless otherwise specified.

Statistical analyses

Values are duplicate determinations in two separate experiments ± mean average deviations. The data shown in Figure 4 represent single experiments where a lack of a significant growth response to micromolar or submicromolar *t*-butyl hydroperoxide concentrations was confirmed in subsequent studies summarised in Figures 3 and 5–7.

RESULTS

Low concentrations of capsaicin inhibited the growth of A-375 melanoma cells in culture (Figure 1a). Capsaicin was very effective with an EC_{50}, after 48 h of growth, of

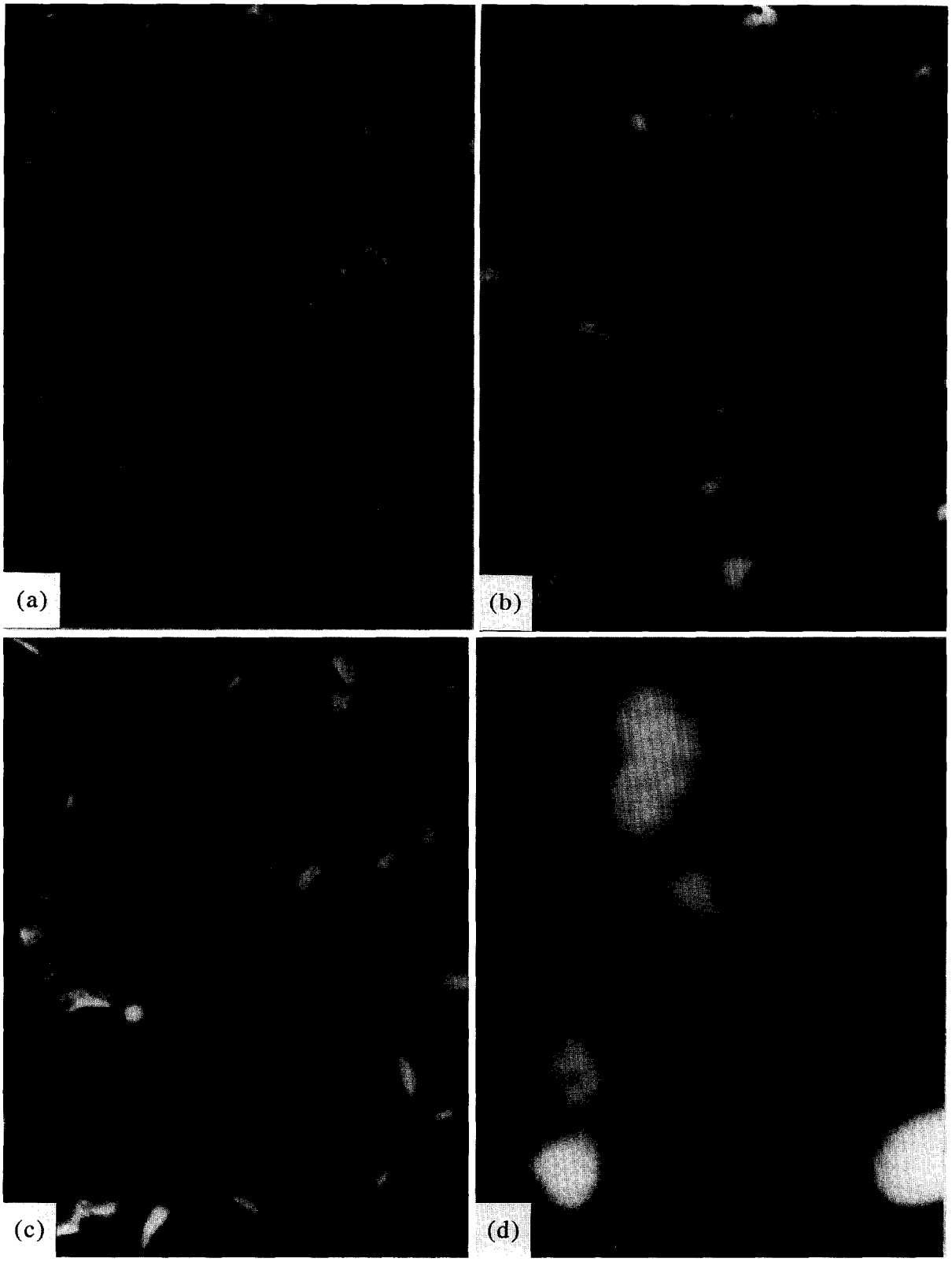

Figure 2. Photomicrograph of A-375 melanoma with DAPI (4′,6-diamidino-2-phenylindole) to show condensed chromatin characteristic of growth after 72 h with 100 μM capsaicin. (a) 0.1% DMSO, (b)–(d) 100 μM capsaicin plus 0.1% DMSO. (a)–(c) ×750. (d) ×3000.

approximately 6 μM. Effects were also observed at 24, 72 and 96 h of growth (not shown). In contrast, growth of primary melanocytes was not inhibited at 10 μM capsaicin and was only inhibited by approximately 20% at 1 mM capsaicin.

Capsaicin was also without effect on the NADH oxidase of plasma membrane vesicles isolated from the primary melanocytes (Figure 1c). With plasma membranes from the A-375 melanoma cells, the specific activity of the plasma membrane NADH oxidase was about 60% greater than that of the primary melanocytes. In addition, the activity was inhibited by capsaicin to near the basal level found in the plasma membranes of primary melanocytes (Figure 2).

The diameters of treated A-375 melanoma cells were, on average, less for cells treated with 100 μM capsaicin in DMSO, and approximately 50% of the volume of control cells. When the cells treated with capsaicin were stained to reveal DAPI fluorescence, a very large percentage of the treated cells showed the characteristic condensed and fragmented appearance characteristic of apoptotic cells (Figure 2).

In contrast to the results with A-375 melanoma cells, cells of the melanoma line SK-MEL-28 were resistant to capsaicin. These cells were not killed by capsaicin and appeared to grow normally even at the highest concentration tested (Figure 1b). Similarly, the NADH oxidase activity of plasma membrane vesicles isolated from the SK-MEL-28 melanoma did not respond to capsaicin (Figure 1d).

In an effort to understand the basis for the lack of response of the resistant SK-MEL-28 melanoma cell line to capsaicin, a series of experiments was initiated to attempt to modify the response of the plasma membrane NADH oxidase to capsaicin. One treatment that enhanced the response of the NADH oxidase of plasma membranes of HeLa cells to an antitumour sulphonylurea was to incubate the vesicles with reduced glutathione or dithiothreitol (results unpublished). However, treatment with reduced glutathione or dithiothreitol was ineffective in enhancing the capsaicin responsiveness of the SK-MEL-28 membrane vesicles (not shown).

In contrast, mild oxidising conditions, such as with 0.003% hydrogen peroxide, did enhance the capsaicin response of the NADH oxidase (Figure 3a). Other mild oxidising agents were also effective. For example, co-administration of capsaicin with *t*-butyl hydroperoxide resulted in capsaicin inhibition of the NADH oxidase activity of the isolated plasma membrane vesicles of the SK-MEL-28 line (Figure 3a). The inhibition was nearly the same as that observed with capsaicin and the A-375 melanoma line in the absence of *t*-butyl hydroperoxide (Figure 1). Similarly, when growth of the SK-MEL-28 cells was determined in the presence of both *t*-butyl hydroperoxide and capsaicin, growth was inhibited (Figure 3b). *t*-Butyl hydroperoxide at 50 μM alone was without effect on the growth of the SK-MEL-28 cells and was utilised in preference to hydrogen peroxide, which was more toxic.

The enhancement of capsaicin inhibition of the plasma membrane NADH oxidase was observed over the range of 1–100 μM *t*-butyl hydroperoxide and 0.0003–0.03% hydrogen peroxide. Initial experiments were with 100 μM *t*-butyl hydroperoxide and 0.003% hydrogen peroxide, and use of these concentrations was continued. For growth experiments with the cells, the highest concentrations of *t*-butyl hydroperoxide without a significant effect on cell growth by itself was selected for testing in combination with capsaicin.

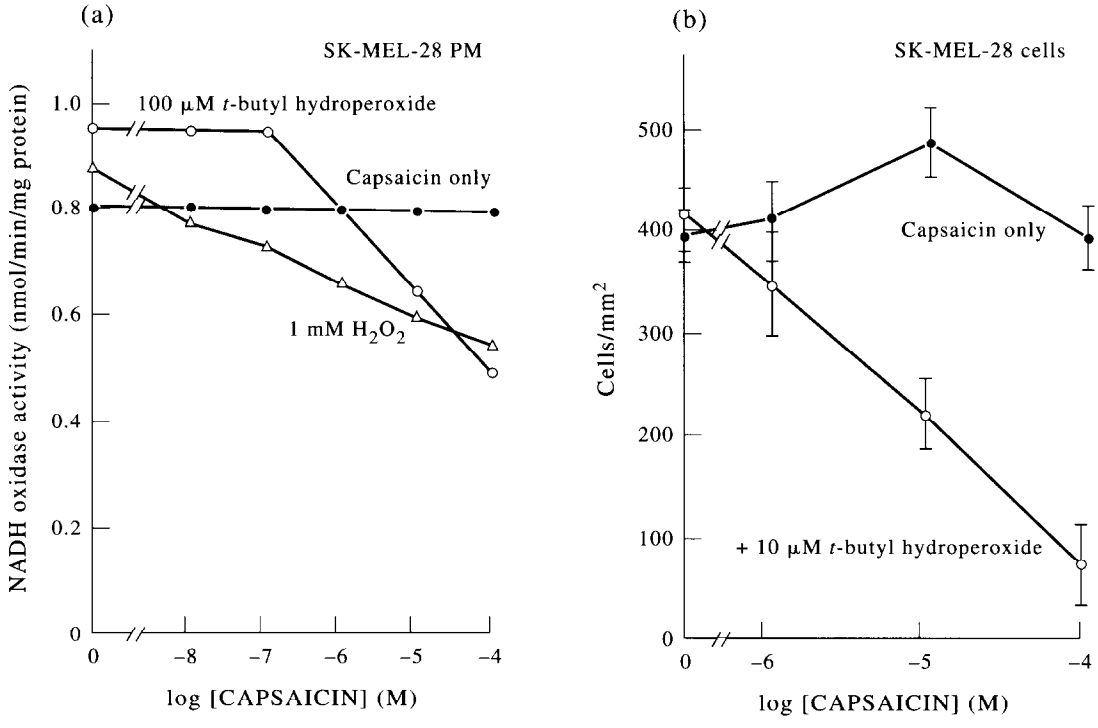

Figure 3. NADH oxidase activity of isolated plasma membrane (PM) vesicles (a) and growth in culture (b) of SK-MEL-28 cells in response to capsaicin alone or co-administered with mild oxidising agents.

In contrast to its lack of effect on the NADH oxidation by plasma membrane vesicles, *t*-butyl hydroperoxide alone inhibited the growth of cells at high concentrations. In addition, the growth responses of the different cell lines varied in their sensitivity to *t*-butyl hydroperoxide (Figure 4). To avoid complications arising from additive effects, growth experiments were conducted with concentrations of *t*-butyl hydroperoxide that would have little or no effect on growth when administered alone, as determined both from the dose–response curves of Figure 4 and in each of the experiments with capsaicin. The interval between the addition of capsaicin and the mild oxidising agent was always short (<1 min) and appeared not to affect the results.

The NADH oxidase activity of the plasma membrane vesicles (Figure 5a, b) and the growth of cells (Figure 5) of the normally capsaicin-responsive A-375 human melanoma line were inhibited to approximately the same extent by capsaicin, either in the presence or absence of *t*-butyl hydroperoxide. Similar results were obtained for the NADH oxidase activity of the isolated plasma membrane vesicles and 1 mM hydrogen peroxide (Figure 5).

Both *t*-butyl hydroperoxide and hydrogen peroxide stimulated NADH oxidation in membrane preparations from SK-MEL-28 and A-375 cells at zero concentrations of capsaicin (Figures 3 and 5). Oxidation of NADH by isolated plasma membrane vesicles was normally stimulated by the addition of mild oxidising agents. This stimulation was not concentration-dependent and may have related to the redox status of the oxidase itself, but the mechanisms of the stimulation have not yet been investigated.

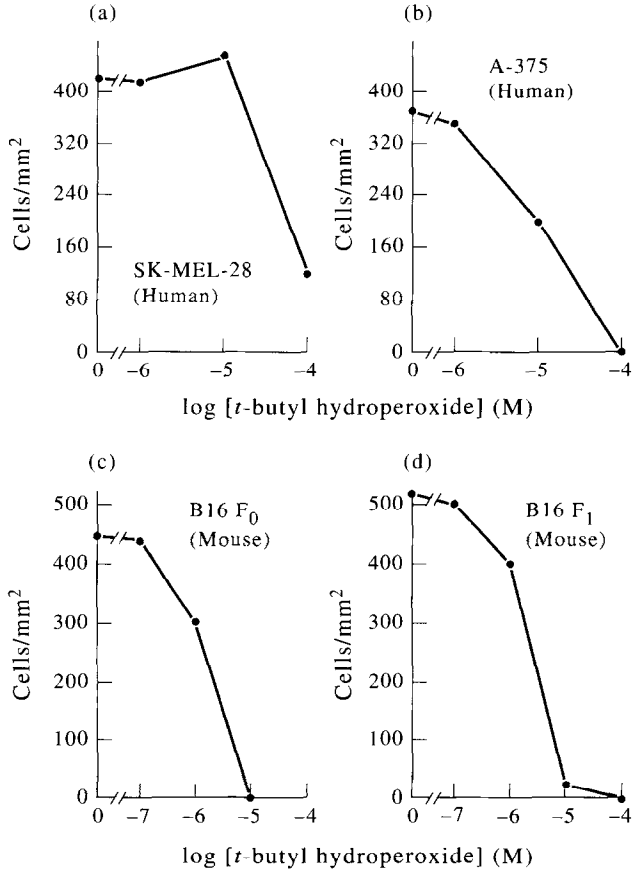

Figure 4. Dose–response of growth of melanoma cell lines to *t*-butyl hydroperoxide.

In order to examine the response of melanoma cells to capsaicin *in vivo*, experiments were extended to B16 mouse melanoma cells. Both the B16 F_1 and the B16 F_0 mouse melanoma lines responded to capsaicin in terms of the inhibition of NADH oxidase activity of isolated plasma membrane vesicles (Figures 6 and 7) and the growth of the cells in culture (Figures 6 and 7).

When carried in the C57BL/6 mice, both the B16 F_0 (Table 1) and the B16 F_1 (data not shown) melanomas gave small but statistically non-significant responses to the daily administration of 5 or 50 μg capsaicin per animal (the maximum single dose tolerated in 50% of the mice was approximately 150 μg). These experiments were subsequently continued with the B16 F_0 cells, and, when administered together with *t*-butyl hydroperoxide, an enhanced reduction in tumour growth by 5 μg capsaicin per animal per day was observed. *t*-Butyl hydroperoxide alone was without effect on tumour growth (Table 1).

DISCUSSION

NADH oxidase activity of the plasma membrane has been identified from rat liver [1–3], keratinocytes [6] and plant stems [18] stimulated by hormones and growth factors. In cancer, the activity appears to be activated constitutively and no longer responds to hormones and growth factors [4, 5]. Quinones are required for the stimulation of the NADH oxidase activity of rat liver plasma membranes [8]. Stimulation or inhibition of the activity correlates closely with the inhibition of stimulation of growth in plants [19, 20] and in cultured mammalian cells [9, 21].

In this study, low concentrations of the natural quinone analogue, capsaicin, were found to inhibit the NADH oxidase activities of the plasma membrane vesicles from the human melanoma A-375 and the B-16 mouse melanoma cell lines. We have reported similar results in HeLa, mammary adenocarcinoma, Caov-3 ovarian adenocarcinoma and HL-60 [9] cells (normal cells are not affected). Capsaicin exerts parallel responses on growth, nearly completely inhibiting the growth of A-375 melanoma, HeLa and HL-60 cells, and affecting to a much lesser extent normal melanocytes, rat kidney [9] and HL-60 cells induced to differentiate with DMSO [9]. This preferential inhibition of plasma membrane NADH oxidase activity and cell growth by capsaicin in transformed but not in normal cells suggests that the capsaicin inhibition site might represent a novel anticancer drug target deserving of additional study.

In the present report, we demonstrate further that, in contrast to the NADH oxidase activity of the A-375 human melanoma and the B16 mouse melanoma cell lines, the NADH oxidase activity of plasma membranes from primary melanocytes and a capsaicin-resistant melanoma, SK-MEL-28, was not inhibited. With the capsaicin-resistant SK-MEL-28 melanoma cell line, it appeared that resistance of both the NADH oxidase activity of plasma membrane vesicles and cell growth in culture was overcome if capsaicin was co-administered with a mild oxidising agent, such as *t*-butyl hydroperoxide or low concentrations of hydrogen peroxide. The drug responsiveness of the plasma membrane NADH oxidase and growth of HeLa cells was also observed to be altered by an antitumour sulphonylurea, *N*-(4-methyl-

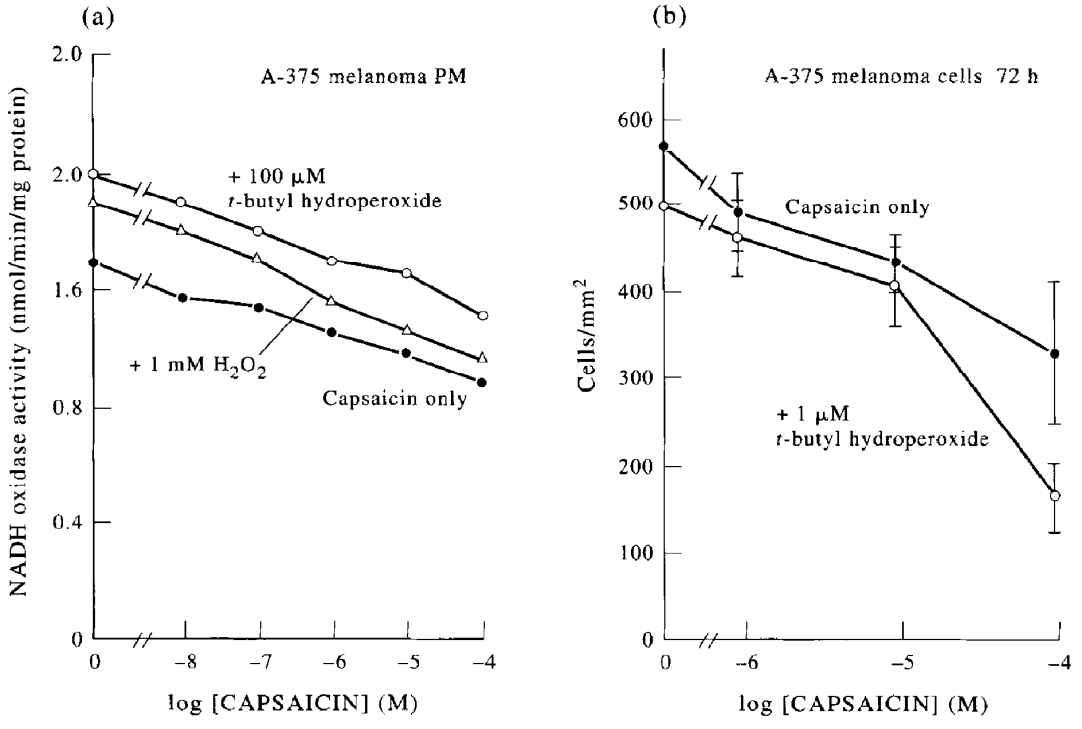

Figure 5. NADH oxidase activity of isolated plasma membrane vesicles (a) and growth in culture (b) of A-375 melanoma cells in response to capsaicin alone or co-administered with mild oxidising agents.

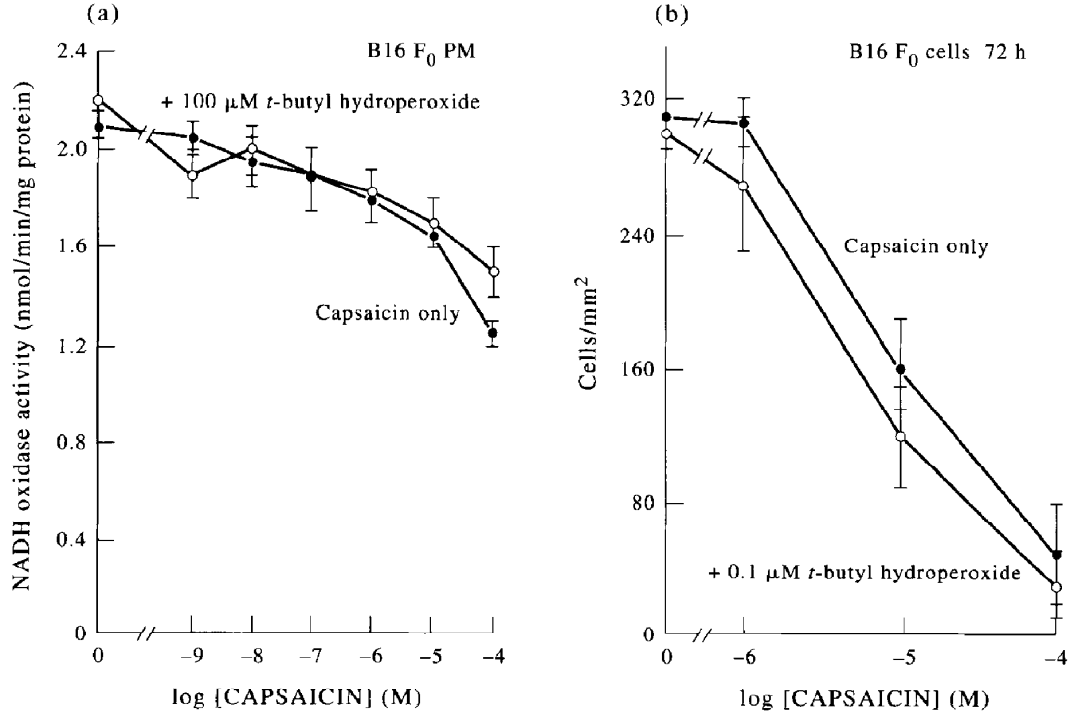

Figure 6. NADH oxidase activity of isolated plasma membrane vesicles (a) and growth in culture (b) of B16 F_0 mouse melanoma cells in the presence or absence of *t*-butyl hydroperoxide.

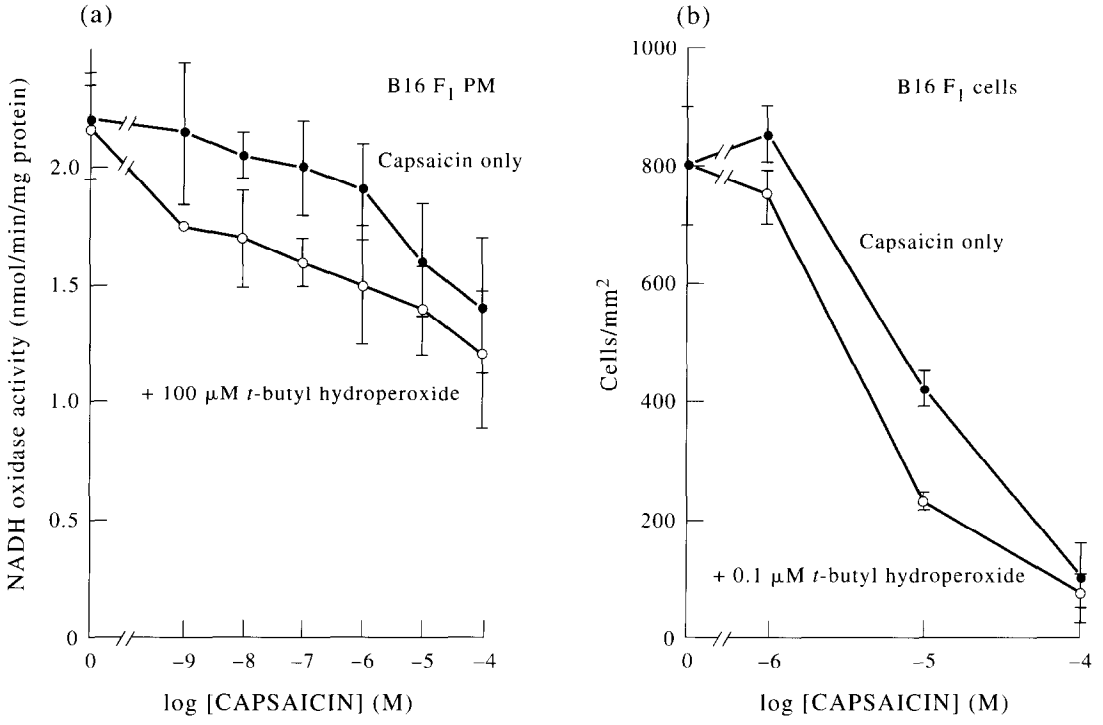

Figure 7. NADH oxidase activity of isolated plasma membrane vesicles (a) and growth in culture (b) of B16 F_1 mouse melanoma cells in the presence or absence of *t*-butyl hydroperoxide.

phenyl-sulfonyl)-*N'*-(4-chlorophenyl)urea. Here, binding of radiolabelled drug to high affinity sites of the plasma membrane [22] was greatly enhanced by reducing conditions, for example, with 1 mM DTT. With capsaicin and the SK-MEL-28 cells, just the opposite was observed where the mild oxidising agents induced capsaicin responsiveness in both NADH oxidase activity and growth in the otherwise capsaicin-resistant SK-MEL-28 cell line.

Apparently, the redox state of the oxidase or its immediate environment affects its drug responsiveness, with oxidation resulting in the inhibition of SK-MEL-28 cells by capsaicin and reduction resulting in inhibition of HeLa cells by the antitumour sulphonylurea. Reactive oxygen intermediates as potentially widely utilised intermediates in transmembrane signalling and the cardioprotective effects of antioxidants are well known [23, 24]. However, it is important to note that addition of the mild oxidising agent, in our studies, exerted no further marked effects on either the capsaicin inhibition of the NADH oxidase activity in the isolated plasma membrane vesicles or on cell growth for both the capsaicin-responsive A-375 and B16 melanoma cell lines in culture. Only with the normally capsaicin-resistant SK-MEL-28 line did the mild oxidising agent elicit a response, and then only to approximately the same level as observed in the capsaicin-susceptible lines.

Table 1. *B16 mouse melanoma response in mice*

Treatment	Tumour weight (g) ± S.D.
Controls (DMSO alone)	5,2 ± 0.9*
Capsaicin (5 μg) in DMSO	4.4 ± 0.3*
t-Butyl hydroperoxide (5 μl) in DMSO	5.2 ± 1.6*
Capsaicin + *t*-butyl hydroperoxide in DMSO	1.8 ± 1.0†

* Non-significant compared to controls; † statistically significant ($P < 0.001$). Drug plus *t*-butyl hydroperoxide in 5 μl DMSO were administered daily at the tumour site for 7 days. Results are from four experiments, three mice per treatment per experiment ($n = 12$). Initial tumour weight was approximately 0.5 g; S.D., standard deviation.

The results with the B16 melanoma in mice were unexpected, as the B16 melanoma cells in culture were remarkably capsaicin susceptible. However, when carried in mice, the combination of capsaicin plus the mild oxidising agent *t*-butyl hydroperoxide was required to exert a substantial slowing response on tumour growth. One interpretation suggested by the findings was that the oxidation state of the target protein or proteins with the B16 melanoma was at approximately the correct oxidation state, with cells in culture to respond to capsaicin without further modification. However, when carried in mice, the target protein or proteins became sufficiently reduced to exhibit capsaicin resistance so that re-oxidation was necessary to generate capsaicin susceptibility. With the SK-MEL-28 human melanoma, oxidation was required even in cell cultures to induce a response to capsaicin. Thus, what began to emerge from these studies was a pattern of response to capsaicin where inhibition of NADH oxidase activity and inhibition of growth appear subject to modulation by modification of the redox conditions of a putative drug target. While modification of the redox condition may not be the only approach to modulation of the capsaicin response, the findings do afford an opportunity to probe the antitumour action of capsaicin and at least one possible mechanism of resistance, with the expectation that alternative strategies, not dependent on co-administration of drugs and oxidising agents, might ensue. More important, however, is the observation

that the inhibition of plasma membrane NADH oxidase activity of transformed cell lines correlates with inhibition of growth of cancer cells. As such, it may represent a novel drug site for potential antitumour agents of which capsaicin may be only an early example.

Note added in proof—The F_1 line is derived from the F_1 line as originally described by Fidler (Fidler IJ. Biological behavior of malignant melanoma cells correlated to their survival *in vivo. Cancer Res* 1975, **35**, 218–224). The F_0 designation refers to the parental cell liine of low lung-metastatic capacity (El-Sabban ME, Pauli BU. Cytoplasmic dye transfer between metastatic tumor cells and vascular endothelium. *J Cell Biol* 1991, **115**, 1375–1382).

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