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RESEARCH COMMUNICATION

Stimulation of NADH oxidase activity from rat liver plasma membranes by growth factors and hormones is decreased or absent with hepatoma plasma membranes

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Plasma membranes of rat liver isolated by aqueous two-phase partition exhibited basal levels of NADH oxidase activity that were increased approx. 2-fold by addition of hormones and growth factors to which liver cells were known to respond. In contrast, hepatoma plasma membranes demonstrated an intrinsically increased level of NADH oxidase, which was not stimulated further by addition of growth factors. The results suggest that the NADH oxidase of the hepatoma plasma membrane is no longer correctly coupled to hormone and growth-factor receptors. This biochemical defect may parallel the loss of growth control that is characteristic of neoplastic transformation in hepatocarcinogenesis and other transformation systems.

INTRODUCTION

An NADH oxidase activity (transfer of electrons from NADH to oxygen) of plasma membranes purified from rat liver was described that was stimulated by the growth factor transferrin [1]. The stimulated activity was not inhibited by cyanide and was not seen in plasma membranes prepared from hyperplastic nodules from livers of animals given the hepatocarcinogen 2-acetylaminofluorene, nor was it due to reduction of iron associated with diferric transferrin [1]. In the present study, these findings were extended to a comparison of the effects of several growth factors and hormones to which liver cells are known to respond for both liver plasma membranes and plasma membranes prepared from rat hepatomas carried in syngeneic recipients. The findings show that the cyanide-resistant NADH oxidase was stimulated by several other growth factors in addition to diferric transferrin. Additionally, the ability of the growth factors and hormones to stimulate the NADH oxidase activity was lost in the plasma membranes of hepatomas. The latter exhibited intrinsically higher levels of activity, comparable with the hormone-stimulated activity levels for liver plasma membranes. The findings point to a fundamental alteration of the hepatoma plasma membrane involving regulation of the activity of the NADH oxidase, which may be important to the unregulated growth that is characteristic of hepatomas *in situ*.

MATERIALS AND METHODS

Animals and diets

Male Fischer 344 rats weighing 100–125 g were purchased from Harlan Animal Supply (Indianapolis, IN, U.S.A.). Hepatocellular carcinomas induced initially with diethylnitrosamine and designated RLT-N were propagated *in vitro* in syngeneic recipients as described by Kloppel & Morré [2]. In brief, the tumours 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, NY, U.S.A.) on the left mid-lateral surface. The animals were fed *ad libitum* and killed after about 3–4 weeks.

Preparation of homogenates

Rats were killed by decapitation, and livers or tumours were removed quickly. Necrotic and capsular material and blood clots were trimmed from the tumours. Thereafter, the same procedure was followed for both livers and hepatomas, and all subsequent operations were at 0–4 °C. Tissues were minced into approx. 2 mm^2 fragments. The minced tissue was then placed in 2 vol. (v/w) of cold homogenization medium (37.5 mM-Tris/maleate, pH 6.4, 0.5 M-sucrose, 1 % dextran and 5 mM-$MgCl_2$) and homogenized with a Polytron 20 ST (Kinematica, Lucern, Switzerland) for 45 s at 6000 rev./min. Homogenates were centrifuged at 5000 *g* for 15 min (5500 rev./min, Sorvall RC-2B centrifuge, with a swinging-bucket rotor). Supernatant and the upper one-third of the yellow–brown portion of the first differential layer were removed, and the lower two-thirds was resuspended in 1–2 ml of 1 mM-$NaHCO_3$ by using a pipette. The resuspension was homogenized in a stainless-steel Dounce homogenizer (Duragrind) with 10–15 up–down strokes. Another 5 ml of 1 mM-$NaHCO_3$ was added, and the mixture was centrifuged for 10 min at 5000 *g*. The supernatant was removed, and the friable and light-brown top portion of the pellet was collected by resuspension in 1 mM-$NaHCO_3$.

Aqueous two-phase partition

The homogenates were fractionated with 16 g two-phase systems [3]. A 2 g portion of concentrated homogenate was added to a mixture of 6.4 % (w/w) Dextran T-500 (Pharmacia), 6.4 % poly(ethylene glycol) 3350 (Fisher), 0.25 M-sucrose and 5 mM-potassium phosphate (pH 7.2). The two-phase system was then mixed by 40 inversions in the cold (4 °C) and separated by centrifugation in a swinging-bucket rotor at 750 *g* for 5 min. The upper phase, which contained the plasma membranes, was diluted with 1 mM-$NaHCO_3$ and the membranes were collected by

Abbreviation used: EGF, epidermal growth factor.

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Table 1. NADH oxidase of plasma membranes of rat liver and hepatomas

Plasma-membrane fractions were prepared by aqueous two-phase partition and assayed spectroscopically for NADH oxidase activity by the disappearance of the NADH in the absence of added external acceptor (electron transfer to oxygen) in the presence or absence of hormones and growth factors. Results are means from four experiments ± S.D. among experiments. Within each experiment determinations were in duplicate and were averaged. Numbers not followed by the same letter were significantly different ($P < 0.01$).

		NADH oxidase activity (nmol/min per mg of protein)	
Addition	Concn.	Liver	Hepatoma
None		1.05 ± 0.07[a]	2.46 ± 0.12[c]
Insulin	0.1 nM	2.35 ± 0.29[b,c]	2.54 ± 0.16[c]
EGF	10 nM	2.23 ± 0.21[b,c]	2.52 ± 0.13[c]
Transferrin	12.5 μM	1.96 ± 0.27[b,c]	2.06 ± 0.20[c]
Lactoferrin	25 μM	1.62 ± 0.32[b,c]	2.27 ± 0.10[c]
Vasopressin	3 μM	1.65 ± 0.20[d]	2.67 ± 0.14[c]
Glucagon	3 nM	1.37 ± 0.30[a,d]	2.71 ± 0.13[c]

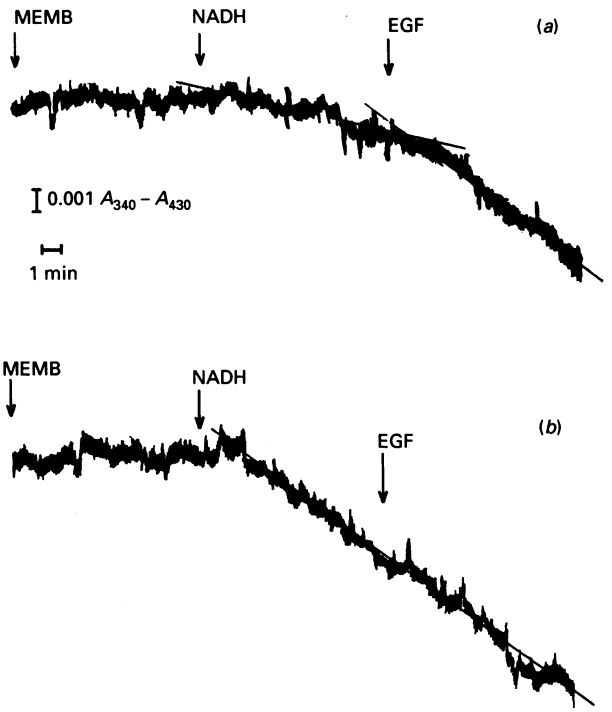

Fig. 1. Spectrophotometric traces of NADH oxidation by plasma membranes where the reaction is initiated by the addition of NADH

After 5 min, EGF was added. Absorbance was measured at 340 nm with a reference at 430 nm. (*a*) Liver plasma membranes. Upon EGF addition, an accelerated rate of NADH oxidation was observed within the time of temperature equilibration and mixing of the system (less than 1 min). (*b*) Hepatoma (RLT-N) plasma membranes. A response to added EGF was not readily discernible. Each reaction contained 0.1 mg of membrane protein. Abbreviation: MEMB, membranes.

centrifugation for 25 min at 80000 *g*. The pellets were resuspended in 300–500 μl of 20 mM-Tris/Mes (pH 7.2).

NADH oxidase activity

The assay medium contained 20 mM-Tris/Mes (pH 7.2), 1 mM-KCN, 150 μM-NADH and 0.1–0.2 mg of protein in a final volume of 2.6 ml. Absorbance was monitored at 340 nm with reference at 430 nm in a SLM-2000 (Aminco) dual-beam spectrophotometer in the dual-wavelength mode of operation. The cuvette was stirred with a magnetic stirrer and the temperature was constant at 37 °C. A blank rate was determined for an additional 10 min. Growth factor was then added, and the rate was determined in the presence of concentrations of growth factor for a further 10 min. Alternatively, NADH was added and the rate determined for two consecutive 10 min periods, followed by addition of hormone or growth factor, after which the stimulated rate was determined also for two consecutive 10 min scans. The absorption coefficient used for NADH was 6.21×10^{-3} mM^{-1}·cm^{-1}. Protein was determined by the bicinchoninic acid procedure [4], with BSA as standard.

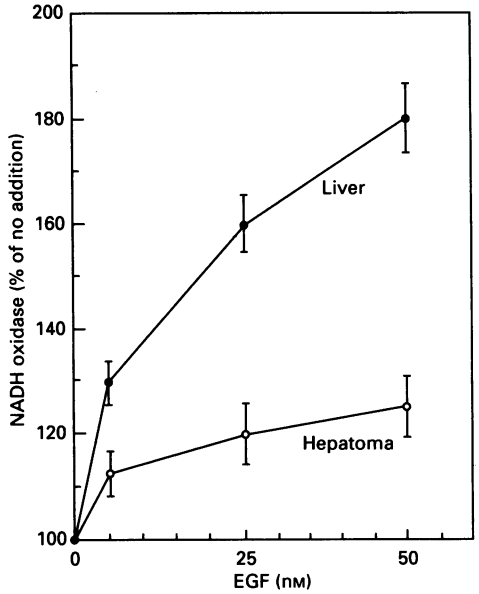

Fig. 2. Oxidation of NADH by rat liver (●) and rat hepatoma RLT-N (○) plasma membranes in response to increasing concentrations of EGF

With hepatoma plasma membranes, the initial specific activity was about 2.5 nmol/min per mg of protein, whereas that of the liver plasma membrane was about 1 nmol/min per mg of protein. Relative activities are reported to contrast better the magnitude of response to EGF.

Electron microscopy

The plasma-membrane pellets were fixed in 2% (v/v) glutaraldehyde in 0.1 M-phosphate buffer, pH 7.2. The glutaraldehyde was removed by rinsing three times with 0.1 M-phosphate buffer, pH 7.2, and the pellet was post-fixed overnight in cold 1% OsO_4 in the same buffer and then dehydrated in an acetone series. Samples were embedded in Epon [5], sectioned, stained with lead, and examined and photographed with a Philips EM/200 electron microscope. For morphometry, a transparent overlay containing lines 1 cm apart was placed over a micrograph of ×35000 enlargement [6]. Intercepts of membranes with lines on the overlay were counted both for total membrane and for each cell component to be analysed. Results are reported as intercepts with the plasma membrane per 100 total intercepts with all membranes present.

RESULTS

The NADH oxidase activities of the plasma membrane preparations purified from rat liver and the RLT-N rat hepatoma

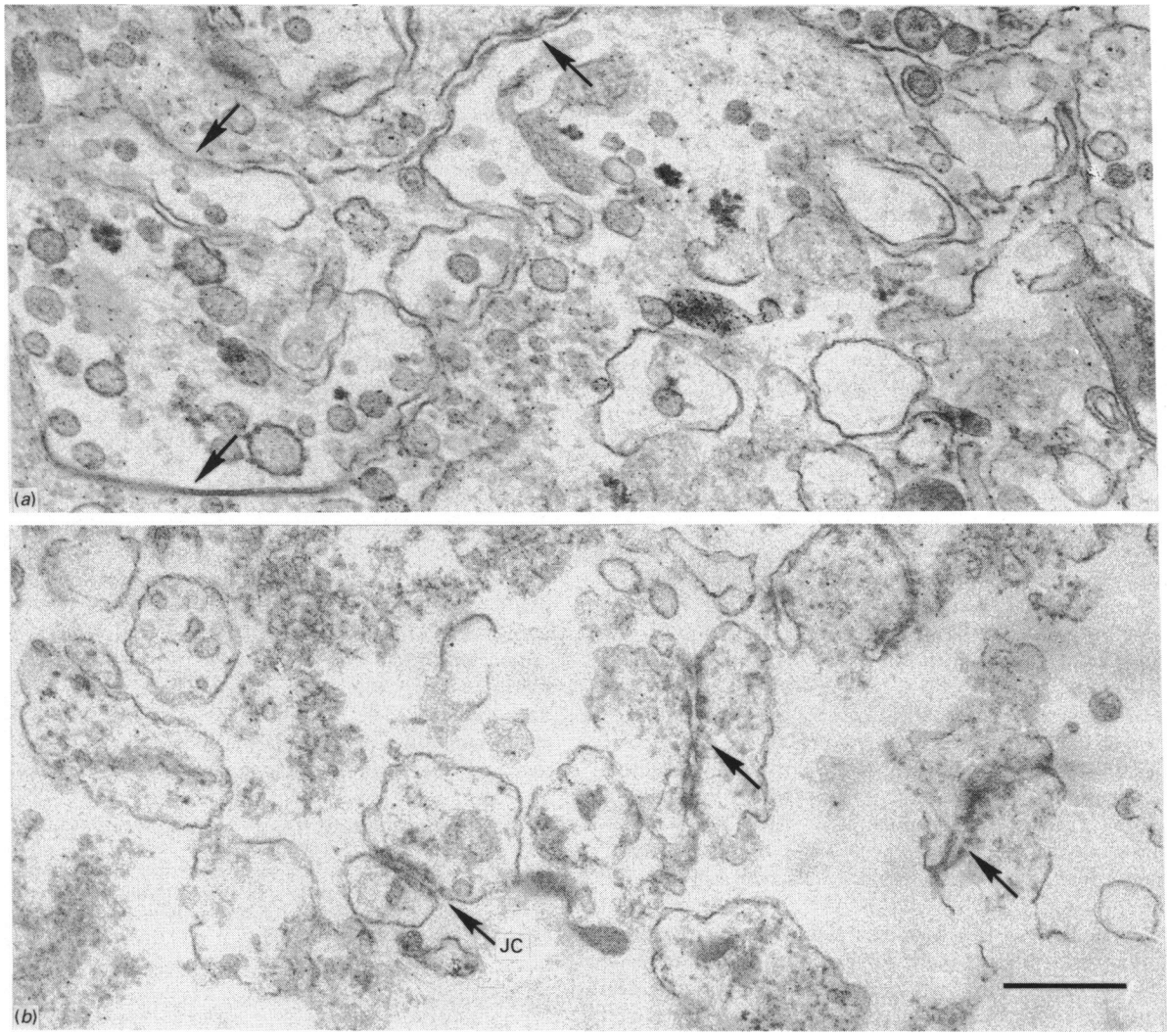

Fig. 3. Electron micrographs of plasma-membrane fractions prepared by aqueous two-phase partition: (*a*) rat liver; (*b*) rat hepatoma

Arrows mark junctional complexes (JC). Scale bar = 0.5 μm.

showed altered specific activities and responses to growth factors and hormones (Table 1). The specific activity of the NADH oxidase of the hepatoma plasma membranes was approximately twice that of the liver plasma membranes and, in contrast with liver plasma membranes, the plasma membranes of hepatomas were relatively unresponsive to growth factors and hormones.

When the response of the NADH oxidase to a series of growth factors and hormones was compared, the activity of the plasma membranes from liver was increased to a level approximately equal to that of the hepatoma membranes (Table 1). The response was greatest for insulin and epidermal growth factor (EGF) and least for glucagon. The responses to diferric transferrin, lactoferrin and vasopressin were intermediate. However, with hepatoma plasma membranes, in no instance was the activity of the NADH oxidase in the presence of hormone or growth factor significantly greater than the rate with no additions.

The NADH oxidase measurements were done in the presence of 1 mM-cyanide, so that a cyanide-resistant non-mitochondrial activity was measured. The activities were linear with time for 30 min or longer under the conditions of assay. With liver plasma membranes, the response to EGF was very rapid and occurred within the time required for the incubation mixture to equilibrate, usually within 1 min or less (Fig. 1*a*). With hepatoma plasma membranes, the activity was also cyanide-resistant and linear with time, but was much less responsive to growth factors (Fig. 1*b*). Neither activity was inhibited by rotenone [7], nor was NADH a substrate for the growth-factor-stimulated activity.

The NADH oxidase activity was stimulated optimally between 50 nM- and 100 nM-EGF for plasma membranes of rat liver (Fig. 2). With hepatoma plasma membranes, the EGF induced a much smaller response than with liver plasma membranes, which was optimal at about 50 nM.

The morphological appearance of the plasma-membrane fractions prepared by aqueous two-phase partition from liver (Fig. 3*a*) and from hepatomas (Fig. 3*b*) demonstrated the predominant plasma-membrane composition of the fractions analysed for NADH oxidase activities. From liver, the plasma membranes were isolated as large sheets and vesicles clearly identified as plasma membranes by the presence of junctional complexes. With hepatomas the membranes were more fragmented, but many junctional complexes were still evident. Analysis of the fractions by morphometry showed that the preparations were 92 ± 2 and 90 ± 3% plasma membranes respectively for rat liver and hepatoma. Contaminants were chiefly mitochondria (3–7%), nuclear envelope (1–2%) and endoplasmic reticulum (1–2%). Lysosomes, Golgi apparatus and unidentified membranes combined were 1–2% of the total.

Both the hepatoma and the liver plasma membranes prepared

as described above have been characterized extensively both morphologically and from detailed analyses of marker enzymes [8]. Based on analyses of enzyme markers, the hepatoma preparations are decreased below homogenate values for specific activities of glucose-6-phosphatase and NADPH–cytochrome *c* reductase for endoplasmic reticulum, succinate reductase for mitochondria and galactosyltransferase for Golgi apparatus. At the same time, the preparations are 21-fold enriched for the specific plasma-membrane marker K^+-stimulated ouabain-inhibited *p*-nitrophenylphosphatase. With liver, where purified reference fractions are available for comparison, total contamination does not exceed 10%, on the basis of analysis of marker enzymes, and the enrichment for the K^+-stimulated ouabain-inhibited *p*-nitrophenylphosphatase is 30-fold and that for a second liver plasma-membrane marker, 5′-nucleotidase, is 22.5-fold [8].

DISCUSSION

The previous report of a growth-factor-stimulated NADH oxidase activity associated with the plasma membrane of rat liver [1] provided an important new perspective to the concept of a redox system of the plasma membrane that may be important to the regulation of growth [9]. A growth-hormone-responsive NADH oxidase activity had been previously described for plasma membranes of plants [10] and was detected on the assumption that the natural acceptor for the plasma-membrane redox system might be oxygen [11]. At least a portion of the transferrin-stimulated oxidation of NADH previously ascribed to NADH–diferric transferrin reductase [12] may now be ascribed also to the stimulation of the plasma-membrane NADH oxidase by diferric transferrin.

The NADH oxidase has been characterized extensively, is cyanide-insensitive, and is unaffected by common inhibitors of oxidoreductases of endoplasmic reticulum or of mitochondria [13]. Addition of coenzyme Q_{10} stimulated the activity and partially reversed the inhibition of NADH oxidase activity by quinone analogues [7,14]. The pH optimum for the activity was about 7.0 in both the absence and the presence of growth factors. The stoichiometry of the electron transfer reaction from NADH to $\frac{1}{2}O_2$ was 2:1, indicative of a two-electron transfer. Ion-exchange chromatography separated the NADH oxidase activity from that of the major plasma-membrane dehydrogenases which transfer electrons from NADH to external electron acceptors such as ferricyanide [14]. Taken together, the evidence suggests that the NADH oxidase of the plasma membrane was a unique oxidoreductase that may be also important to the regulation of cell growth.

The present study was predicated on the observation [1] that the NADH oxidase of plasma membranes purified from hyperplastic nodules of livers of rats given the hepatocarcinogen 2-acetylaminofluorene was no longer responsive to stimulation by diferric transferrin. With liver nodule plasma membranes the intrinsic activity was greater, as would have been predicted if NADH oxidase activities were proportional to growth rate but the control by diferric transferrin was lost. The liver nodule plasma membranes were unaffected by the addition of diferric transferrin, even though the number of transferrin receptors on the plasma-membrane preparations analysed was increased [1].

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These properties observed previously with the nodule plasma membranes for diferric transferrin are retained by plasma membranes of transplanted hepatomas. In addition to showing a decreased response to diferric transferrin, the NADH oxidase of the hepatoma plasma membranes exhibited a decreased responsiveness to EGF, insulin and other growth factors and hormones that stimulate the oxidase of liver plasma membranes to greater or lesser extents than does diferric transferrin [13]. Additionally, the NADH oxidase activity of the hepatoma plasma membrane was elevated in the absence of added growth factor to approximately the same level as was the NADH oxidase of liver plasma membranes in the presence of growth factor.

The present findings may have relevance to the loss of growth control in liver nodules and hepatomas. The interesting possibility is that the coupling of NADH oxidase to hormone and growth-factor receptors that is observed with plasma membranes of liver is lost or decreased in parallel to the loss of growth control that is characteristic of hepatomas and other neoplasms.

In liver, it appears that growth-factor and hormone receptors are somehow linked to the NADH oxidase in a manner where presence of growth factor is required for maximal NADH oxidase activity. However, with the hepatoma, the NADH oxidase activity may now be coupled to constitutively active growth-factor receptors, with a subsequently higher level of NADH oxidase activity that is no longer dependent on the presence of growth factor. The function of this NADH oxidase activity in either hepatoma or liver plasma membranes, or how it may be involved in growth control, remains to be elucidated.

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