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NADH oxidase of liver plasma membrane stimulated by diferric transferrin and neoplastic transformation induced by the carcinogen 2-acetylaminofluorene

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NADH oxidase of purified plasma membranes (electron transfer from NADH to oxygen) was stimulated by the growth factor diferric transferrin. This stimulation was of an activity not inhibited by cyanide and was not seen in plasma membranes prepared from hyperplastic nodules from liver of animals fed the hepatocarcinogen, 2-acetylaminofluorene, nor was it due to reduction of iron associated with diferric transferrin. With plasma membranes from nodules, the activity was already elevated and the added transferrin was without effect. The stimulation by diferric transferrin did not correlate with the absence of transferrin receptors which were increased at the nodule plasma membranes. With liver plasma membranes, the stimulation by diferric transferrin raised the plasma membrane NADH oxidase specific activity to approximately that of the nodule plasma membranes. In contrast to NADH oxidase, which was markedly stimulated by the diferric transferrin, NADH ferricyanide oxidoreductase or reduction of ferric ammonium citrate by liver plasma membranes was approximately equal to or slightly greater than that of the nodule plasma membrane and unaffected by diferric transferrin. The results suggest the possibility of coupling of NADH oxidase activity to a growth factor response in mammalian cells as observed previously for this enzyme in another system.

Introduction

Evidence for the presence of constituents of a redox system associated with the plasma membranes of eukaryotic cells and tissues has been reviewed extensively [1–4]. Most attention has been directed to the transmembrane dehydrogenases because of their possible involvements in transport and growth. These dehydrogenases transfer electrons to oxygen and various extracellular iron compounds.

Both oxygen and iron-containing compounds are available as acceptors for electron transport across the plasma membrane. However, with the plasma membrane the relationship of the transmembrane transfer of electrons to oxygen is less clear than to iron compounds [5]. An NADH-acceptor oxidoreductase activity using oxygen as electron acceptor is widely distributed among isolated plasma membranes from a variety of tissue and cell types [6–12] and has been implicated as an important step in the control of cell enlargement in plants [13–16]. Our interest in the plasma membrane redox system of the mammalian plasma membrane has been stimulated by these observations with plants that suggest that agents that stimulate the oxidase stimulate growth and that agents that inhibit the oxidase inhibit growth [16]. Additionally, the action of plant growth hormones at the cell surface and activity of the NADH oxidase are somehow linked [5]. The plant oxidase has been isolated from and partially purified from plasma

Abbreviations: BSA, bovine serum albumin; Fe_2Tf, diferric transferrin; Tf, apotransferrin; SHAM, salicylhydroxamic acid.

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membranes. The partially purified NADH oxidase is still hormone responsive [17].

In this report, we have examined the response of the NADH oxidase of plasma membranes highly purified from rat liver by aqueous two-phase partition [18,19] to the liver growth factor diferric transferrin and compared the responses of plasma membranes isolated from normal liver to those of plasma membranes from liver nodules induced by intermittent feeding of the carcinogen 2-acetylaminofluorene. The findings suggest that the NADH oxidase of liver is responsive to the growth factor diferric transferrin for which it has receptors on the plasma membrane. In contrast, the nodule plasma membranes, which contain elevated numbers of transferrin receptors, exhibit an NADH oxidase activity that, while near the stimulated rate in liver, is completely unresponsive to further stimulation by diferric transferrin. These findings suggest a fundamental alteration of the nodule plasma membrane involving regulation of NADH oxidase activity which may be important to unregulated growth, characteristic of the liver nodules in situ [20].

Materials and Methods

Animals and diets. Male Wistar rats weighing 140 to 160 g were purchased from Møllegaards Ayls-laboratorium, Ejby, Denmark. The basal diet was brood stock food for rats and mice (R3; Ewos, Södertälje, Sweden). Hepatocellular liver nodules were produced by feeding ad libitum the basal diet containing 0.05% 2-acetylaminofluorene according to the regimen introduced by Epstein et al. as described [21] and modified for Wistar rats by Eriksson et al. [22]. The 2-acetylaminofluorene was from Fluka, Buchs, Switzerland and mixed with the diet by Ewos. Rats with nodules were fed the basal diet for at least 6 weeks after termination of the feeding protocol. Control animals of the same strain and age were fed basal diet through the whole experiment. Animals were anesthetized with an ip injection of sodium hexabarbital, 20 mg/100 g body weight and livers were perfused with cold 0.25 M sucrose. Tissue surrounding nodules was collected as well and processed as for normal liver.

Preparation of homogenates. Liver, liver nodules and liver tissue surrounding nodules were homogenized using an Ultra-Turrax tissue homogenizer (Janke and Kunkel) for 45 s at about 6000–8000 rpm in a medium containing 37 mM Tris-maleate (pH 6.4), 0.5 M sucrose, 1% dextran (Sigma; average molecular weight 225000) and 5 mM $MgCl_2$ [19]. The homogenate was then centrifuged at low speed ($5000 \times g_{av}$) for 15 min to concentrate the plasma membrane vesicles. The supernatant and the top one-fourth of the pellet (the cream-colored upper layer enriched in Golgi apparatus) was removed and the middle one-third to one-half of the pellet (avoiding the underlying nuclei and unbroken cells) was resuspended in 4–6 ml 1 mM sodium bicarbonate using 20 up-and-down strokes with a Duragrind tissue homogenizer (Wheaton, Millville, NJ). Another 15–20 ml of sodium bicarbonate was added after the pellet had been resuspended and the mixture was centrifuged for 15 min at $5000 \times g$. The supernatant was discarded and the light brown, top portion of the pellet was used for preparation of plasma membranes.

Aqueous two-phase partition. Membranes resuspended in 1 mM sodium bicarbonate were fractionated using 16 g two-phase systems [23]. The concentrated membranes were added to a mixture of 6.4% (w/w) Dextran T500 (Pharmacia), 6.4% poly(ethylene glycol) 3350 (Fisher), 0.25 sucrose, and 5 mM potassium phosphate (pH 7.2) [18,19]. The contents of the two-phase system were then mixed by 40 inversions of the tubes in the cold (4° C). The two phases were separated by centrifugation in a swinging bucket rotor at $750 \times g$ for 5 min. The upper phase, enriched in plasma membranes, was diluted with 1 mM bicarbonate and the plasma membranes were collected by centrifugation for 20 min at $80000 \times g_{av}$.

Electron microscopy. Fractions were fixed by immersion of small wedges cut from pellets in 2% glutaraldehyde in 0.05 M sodium phosphate (pH 7.2) for 1 h to overnight followed by transfer to 1% osmium tetroxide in the same buffer for 1 to several hours. Samples were dehydrated in an acetone series and embedded in Epon [24]. Lead-stained thin sections were examined and photographed with a Philips EM/200 electron microscope.

NADH oxidase activity. The assay medium contained 25 mM Tris-HCl, 25 μM NADH, 1 mM KCN, 150 mM NaCl and 5 mM KCl (pH 7.4) and 0.2 to 0.5 mg protein in a final volume of 1.5 ml. Absorbance was monitored at 340 nm with reference at 430 nm using a Shimadzu dual-beam spectrophotometer in the dual wavelength mode of operation. The cuvette was stirred with a magnetic stirrer. The extinction coefficient used for NADH was $6.22 \cdot 10^3\ M^{-1}\ cm^{-1}$.

NADH-ferricyanide oxidoreductase (EC 1.6.99.3). The assay medium contained 25 mM Tris-HCl, 25 μM NADH, 0.2 mM $K_3Fe(CN)_6$, 150 mM NaCl and 5 mM KCl (pH 7.4) and 0.05 to 0.2 mg protein in a final volume of 1.5 ml. Absorbance was monitored at 420 nm with reference at 500 nm using a Shimadzu dual beam spectrophotometer in the dual wavelength mode of operation. The cuvette was stirred with a magnetic stirrer. The extinction coefficient used for ferricyanide was $1 \cdot 10^3\ M^{-1}\ cm^{-1}$.

Ferric ammonium citrate reductase. Ferric ammonium citrate reductase was assayed as for NADH oxidase except that the difference in absorbance between 535 and 600 nm was determined in the presence of 7.5 μM ferric ammonium citrate, 15 μM bathophenanthrolene-

phosphosulfonate and 25 μM NADH with 0.05 to 0.2 mg membrane protein in 1.5 ml final volume.

Transferrin receptor assays. Membrane fractions prepared, as described above were frozen at −20°C and assayed for transferrin receptors as described [22,25]. Storage of membrane fractions at this temperature did not affect receptor binding. Aliquots of the membrane fractions containing 25 μg protein were diluted with receptor assay buffer (50 mM Hepes, 50 mM NaCl (pH 7.4), containing 0.1 g/100 ml BSA). The membranes were incubated with 200 fmol of [^{125}I]transferrin (spec. act. 15–20 μCi/μg) per incubation. Human transferrin was saturated with iron and iodinated according to Fisher and Speck [26]. Non-specific binding was determined using a 100-fold excess of unlabeled transferrin. The total incubation volume was 0.5 ml. Binding was performed in triplicate incubations. Following incubation at 4°C for 16 h, $MgCl_2$ was added to a final concentration of 20 mM and membranes were collected as a pellet by centrifugation at 15000 × g_{av} for 20 min. The surfaces of the pellets were carefully rinsed with receptor assay buffer and the pellets were counted in a gamma counter. Specific binding was calculated by subtracting nonspecific from total binding.

Results

The morphological appearance of the plasma membrane fractions prepared by aqueous two-phase partition from liver, liver nodules and tissue surrounding nodules demonstrates the predominant plasma membrane composition of the fractions analyzed for NADH oxidase activity (Fig. 1). The plasma membranes were isolated as large sheets and vesicles clearly identified as plasma membranes by the presence of junctional complexes. An analysis of the fractions by morphometry showed that the preparations were 92 ± 2%, 87 ± 3% and 90 ± 1% plasma membrane for liver, hyperplastic nodules and liver surrounding nodules, respectively. Contaminants were chiefly mitochondria (3 to 5%), nuclear envelope (1%) and endoplasmic reticulum (2%). Lysosomes, Golgi apparatus and unidentified membranes combined were 2 to 5% of the total.

TABLE I

NADH oxidase of plasma membranes from liver and liver nodules of rats

Plasma membrane fractions were prepared by aqueous two-phase partition and assayed spectroscopically for NADH oxidase activity from the disappearance of NADH in the absence of added external acceptor (electron transfer to oxygen) in the presence or absence of 3 μM diferric transferrin. Values are averages of three or four determinations ± S.D. from three experiments (I–III) comparing animals from two different feeding trials. Livers and (nodules) were combined from two different animals for each experiment. n.d., not determined.

Tissue source	Addition	nmol/min per mg protein		
		I	II	III
Liver	none	0.8 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
	3 μM diferric transferrin	1.3 ± 0.02	1.1 ± 0.1	1.9 ± 0.4
Liver nodules	none	1.5 ± 0.1	1.1 ± 0.2	1.4 ± 0.1
	3 μM diferric transferrin	1.6 ± 0.1	1.4 ± 0.1	2.2 ± 0.2
Liver surrounding nodules	none	n.d.	0.7 ± 0.1	1.2 ± 0.3
	3 μM diferric transferrin	n.d.	1.0 ± 0.1	1.2 ± 0.1

The NADH oxidase activity of the different plasma membrane preparations (Table I) revealed a marked difference comparing those of liver and those of the liver nodules. Specific activities of nodule preparations were 2- (first feeding experiment trial I and second feeding experiment, trial II) to 3-fold (second feeding experiment, trial III) those of normal liver. Results from tissue surrounding the nodules were similar to those for

TABLE II

Ferricyanide and ferric ammonium citrate reduction by plasma membranes from liver and liver nodules of rat

The preparations analyzed are the same as those of Table I except that NADH oxidation was measured in the presence of added external electron acceptors ferricyanide and ferric ammonium citrate. In Expt. III, external coenzyme Q was added to determine whether this electron transport chain constituent might be rate-limiting to activity. Values are from duplicate determinations from two or three experiments comparing animals from the second feeding trial. Livers (and nodules) were combined from two different animals from each experiment. n.d., not determined.

Tissue source	Addition	nmol/min per mg protein					
		ferricyanide			ferric ammonium citrate		
		I	II	III	I	II	III
Liver	none	898	656	1140	37	59	45
	+10 μM coenzyme Q			1330			
Liver nodules	none	775	721	880	37	40	31
	+10 μM coenzyme Q			1080			
Liver surrounding nodules	none	n.d.	750	750	38	39	30

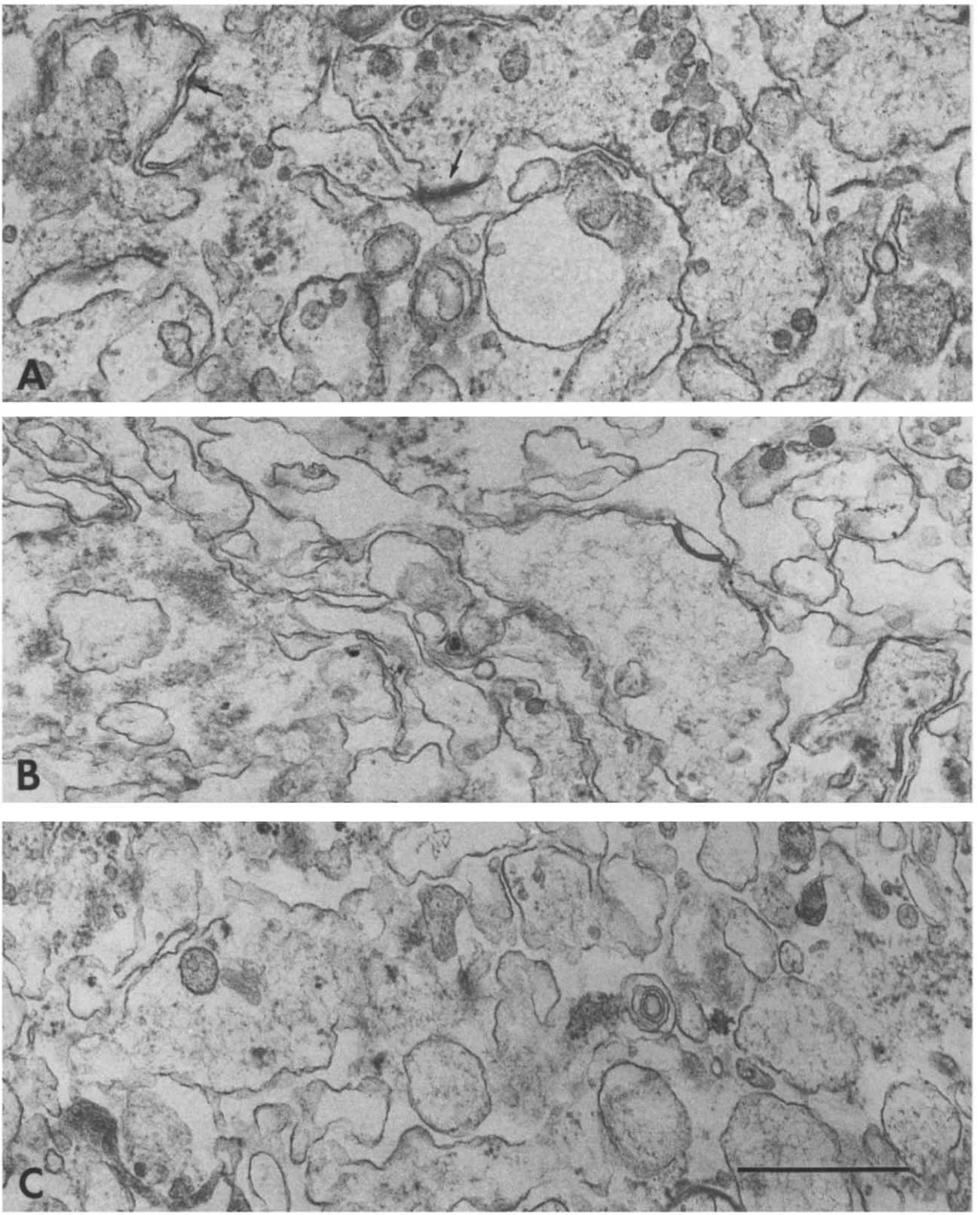

Fig. 1. Electron micrographs of plasma membrane fractions prepared by aqueous two phase partition. (A) Rat liver. Arrows mark junctional complexes. (B) Liver nodules. (C) Liver surrounding nodules. Scale bar = 0.5 μm.

nodules. Histological examination of these tissues revealed small nodules as the predominant component of this tissue.

Not only were the specific activities of the NADH oxidase of the plasma membranes from nodules and tissue surrounding nodules higher than those from normal liver, the activity was unresponsive to stimulation by diferric transferrin in contrast to that of normal liver. As shown in Table I, NADH oxidase of liver plasma membrane was stimulated by 3 μM diferric transferrin more than 50% (first feeding experiment, trial I) to more than 3-fold (second feeding experiment, trial III). As with liver nodules, tissue surrounding nodules yielded plasma membranes whose NADH oxidase was unresponsive to diferric transferrin (Table I).

The stimulation of diferric transferrin of NADH oxidase was presumably through some interaction with the transferrin receptor and not through the reduction of iron bound to the transferrin. Estimation of iron reduced by diferric transferrin (10 μM) using bathophenanthrolene disulfonate as a capture reagent revealed rates of reduction of transferrin-bound iron of less than 0.4 nmoles/min/mg protein, far too low to account for the diferric transferrin stimulation of NADH oxidation.

In contrast to NADH oxidation, reduction of ferricyanide and reduction of ferric ammonium citrate by the isolated plasma membrane fractions was little affected by the carcinogen treatment. Specific activities of plasma membranes from liver, liver nodules and tissue surrounding the nodules were very similar (Table II). If anything, specific activities of the oxidoreductases of NADH with added external electron acceptors were somewhat higher for liver than for nodules and tissue surrounding nodules. Also, there was no effect of diferric transferrin in ferric ammonium citrate reduction with either liver or liver nodule plasma membranes. Coenzyme Q added to the isolated membranes stimulated by about 20% ferricyanide reduction for both liver and liver nodule plasma membranes.

Arguing against a direct reduction of diferric transferrin iron as the basis for the diferric transferrin stimulation of the NADH oxidase was the lack of concentration dependency for diferric transferrin (Fig. 2). The NADH oxidase was stimulated optimally between 1 and 5 μM diferric transferrin, whereas if transferrin-bound iron were being reduced, the stimulation should have been proportional to diferric transferrin concentration over these concentration ranges [27]. With hyperplastic nodules, diferric transferrin was without effect at any of the concentrations tested (Fig. 2).

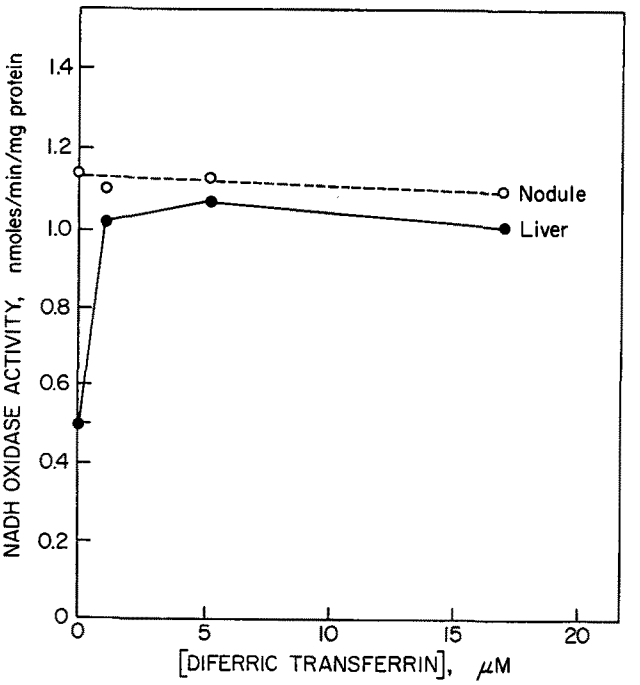

Fig. 2. Dose dependency of NADH oxidase activity on concentration of diferric transferrin in the assay comparing plasma membrane from rat liver and from liver nodules. The nodule plasma membranes were unresponsive to diferric transferrin over the range of 1 to 20 μM, whereas the NADH oxidase of liver plasma membranes was optimally stimulated between 1 and 5 μM. These analyses were from membranes prepared from Expt. III of the second feeding trial.

Transferrin binding of nodule plasma membranes was increased 3–4-fold compared to plasma membranes from liver (Table III).

TABLE III

Distribution of transferrin (Tf) receptors comparing plasma membranes from liver and liver nodules of rat

Values are from duplicate determinations comparing animals from Expts. II and III of the second feeding trial.

Tissue source	fmol Tf bound/mg protein
Liver	4520 ± 80
Liver nodules	16020 ± 1760
Liver surrounding nodules	9620 ± 840

Discussion

The discovery of a growth hormone-responsive NADH oxidase associated with the plasma membrane of plants [13–17] gives new perspective to the concept of plasma membrane redox regulation of growth. On the assumption that the natural acceptor for plasma membrane redox activity might be oxygen, a search was initiated for a mechanism whereby flow of electrons from cytoplasmic NADH could be coupled to molecular oxygen. Results with cells were ambiguous such that we turned to plasma membranes, highly purified by aqueous two-phase partition as described [18,19]. These membranes from a variety of sources (both animal and

plant) were found to catalyze the transfer of electrons from NADH to oxygen with a stoichiometry of about 2. The activity is a NADH oxidase having very unique properties [28] that, based on studies with plants [5,16], may be rate-limiting to growth under certain conditions.

NADH oxidase activity of plasma membrane vesicles and sheets prepared from rat liver by aqueous two-phase partition showed a cyanide-insensitive rate of NADH oxidation (measured as decrease in 340 nm absorbance of NADH) of about 5 nmol/min per mg protein. The K_m of the activity (NADH) was about 200 μM and the pH optimum was broad (pH 6.5 to 8.5) (Juan Wang, MS Thesis, Purdue University, 1989). Of several compounds known to be inhibitory to redox enzyme activities of mitochondria or endoplasmic reticulum, none was inhibitory to the plasma membrane NADH oxidase (e.g., 1 mM cyanide, 1 mM azide, 1 μM antimycin A, 10 μM rotenone, 1 mM SHAM).

The activity measured with isolated plasma membranes from rat liver as NADH oxidase can be distinguished clearly from that of, for example, a residual activity of a plasma membrane-associated dehydrogenase. The oxidase and dehydrogenase activities are separated on a DE-52 column after detergent solubilization. The purified dehydrogenase is incapable of transfer of electrons to oxygen, whereas the partially purified oxidase shows much reduced or no electron transfer to an artificial electron acceptor such as ferricyanide. Erythrocytes, which represent a classic example of a non-growing cell type, yield membranes with an active dehydrogenase but lacking an NADH oxidase [29,30]. The oxidase and dehydrogenase activities exhibit different sensitivities to inhibitors. Simple gangliosides, such as the monosialoganglioside G_{M3}, which are inhibitory to the growth of cultured mammalian cells [31], inhibit the NADH oxidase of rat liver plasma membranes but not the dehydrogenase [32]. An inhibitory effect of gangliosides on platelet-derived and epidermal growth factor receptor responses has been reported from the work of Bremer et al. [33].

This report, which compares the NADH oxidase activity of plasma membranes of rat liver and liver nodules, reveals two additional interesting properties of the NADH oxidase of the mammalian plasma membrane. The liver growth factor, diferric transferrin, stimulates markedly (0.5- to 3-fold) the NADH oxidase of the plasma membrane of liver. With liver nodule plasma membranes, the intrinsic activity is greater, as would be expected if NADH oxidase were proportional to growth rate, but the control by diferric transferrin is lost. These properties of increased NADH oxidase activity compared to normal liver and lack of response to growth factors is retained in plasma membranes of transplanted hepatomas of the rat originally induced by feeding of the carcinogen 2-acetylaminofluorene (unpublished results). In addition to showing a reduced response to diferric transferrin, the NADH oxidase of hepatoma plasma membrane exhibits a reduced responsiveness to epidermal growth factor and insulin, both of which stimulate the oxidase of liver plasma membrane to the same extent as diferric transferrin.

A characteristic feature of liver nodules is their inability to accumulate iron even under conditions where siderosis is produced in the liver by heavy dietary iron overloads [34–36]. This deficiency in iron storage has been used as a marker for altered foci and preneoplastic liver nodules [34]. Eriksson et al. [22] previously have demonstrated that cells forming liver nodules contain a 60-fold higher number of high-affinity binding sites for transferrin than normal liver cells. These 'induced' binding sites were very similar to those of normal liver cells in molecular weight and binding affinity. However, cellular uptake of ^{59}Fe-transferrin in vivo was lower in nodules than in normal liver. Similarly, in the present study the transferrin receptors, even though increased, do not appear to be coupled to NADH oxidase as with plasma membranes from normal liver. The growth rate of cells in liver nodules in vivo is significantly higher than in normal liver and in liver tissue surrounding liver nodules [37]. With time, the growth fraction, expressed as mitotic index or labeling index, increases in liver nodules reaching levels around 7–8% at the age of 8–10 months. Also in vitro, in primary liver cell cultures, where liver cells are seeded on growth permitting collagen type I biomatrix, nodular cells show a higher spontaneous background cell proliferation than normal cells. Wollenberg et al. [20] showed that nodular cells, in non-stimulated assays, had a labeling index of around 40%, which is twice as much as is seen with normal cells.

That the present findings may have relevance to the loss of growth control in liver nodules is suggested from previous findings of Eriksson et al. [38]. They investigated the effect of apotransferrin, diferric transferrin and ferricyanide on S-phase DNA synthesis of rat hepatocytes in primary cultures exposed continuously to [^{3}H]thymidine. The addition of diferric transferrin but not transferrin increased the 48-h labeling index from 23 to 45% in serum-free Williams medium, which contains sufficient iron for strong proliferative responses to epidermal growth factor [33]. The stimulatory effect of diferric transferrin was dose-dependent (0.1–30 μM). Both diferric transferrin and transferrin were toxic and inhibitory at 100 μM. Ferricyanide, which is reduced to ferrocyanide by a transplasma membrane NADH-dependent reductase [27], stimulated hepatocyte proliferation to the same degree and in the same concentration range as diferric transferrin. They concluded that diferric transferrin is mitogenic for hepatocytes in vitro and that this effect was mediated by ferric iron acting as an electron acceptor for the transplasma membrane redox system of the cell. The results are important for

the general concept of growth regulation and fundamental for understanding of the behavior of iron-depleted, transferrin-receptor-enriched preneoplastic cells and their progression in the carcinogenic process. If the oxidase and growth control are related, the findings may very well have relevance to an understanding of the carcinogenic progression in response to 2-acetylaminofluorene feeding. In any event, the findings point to the interesting possibility that the coupling of NADH oxidase to hormone and growth factor receptors is reduced or lost in parallel to the loss of growth control that is characteristic of neoplastic transformation in the liver and in other transformation systems.

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