A NOVEL TYPE OF SUPEROXIDE GENERATING SYSTEM IN NUCLEAR MEMBRANES FROM HEPATOMA 22a ASCITES CELLS

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1. Introduction

The NADPH-dependent redox chain of microsomes has been known to generate hydrogen peroxide [1–3] and superoxide radicals [3,4] the latter being possibly precursors of the former. This reaction is believed to involve autooxidation of NADP-specific flavoprotein dehydrogenase (the so-called NADPH–cytochrome *c* reductase) [4,5] and dissociation of oxycomplex of cytochrome P450 [5–7]. The NADPH-dependent generation of $O_2^{\cdot-}$ and H_2O_2 by nuclei from Ehrlich-Lettré ascites tumor cells has been reported [8].

Here we show that nuclear membranes from ascites tumor hepatoma 22a possess a novel type of an enzymatic $O_2^{\cdot-}$ generating system. This system differs from those described previously in that:

(i) It can use both NADH and NADPH as electron donors;

(ii) It is highly sensitive to inhibition by cyanide and azide.

These features are remarkably similar to those of the microsomal fatty acid desaturase activity (reviewed [9,10]). Possible involvement of cyanide-sensitive cytochrome b_5 autoxidation in $O_2^{\cdot-}$ production by hepatoma 22a nuclei is tentatively suggested.

2. Methods

2.1. *Chemicals*

Adrenaline and NaN_3 were obtained from Serva, Heidelberg; NaCN was from BDH Chemical Ltd., Poole; superoxide dismutase was purified from rat liver [11], alternatively, the commercial product from Sigma Chemical Co., St Louis, MO, was used; NADPH was purchased from Boehringer, Mannheim, and NADH was obtained from Reanal, Hungary.

Abbreviations: CSF, cyanide sensitive factor; SOD, superoxide dismutase

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2.2. *Preparations*

Hepatoma 22a ascites tumor was grown in male CBA × C 57 Bl_6 mice. 7 days after intraperitoneal transplantation, the cells were collected washed and disrupted as in [12]. Nuclei were isolated [13], and the membrane fraction was prepared according to [14]. Rat liver microsomes were isolated according to [15]. Protein was determined by the Lowry method.

2.3. *Assays*

NAD(P)H-dependent adrenaline co-oxidation to adrenochrome was measured at 480 nm as in [4] in a Unicam SP-8000 spectrophotometer at 30°C.

3. Results

As shown in fig.1, addition of NADPH (trace 1) or NADH (trace 2) to an aerobic suspension of nuclear membranes from hepatoma 22a ascites cells initiates active $O_2^{\cdot-}$ generation, measured, as adrenaline oxidation to adrenochrome, which is inhibited by >90% upon addition of SOD (1.7 μg/ml). Boiled SOD was not inhibitory (not shown). The oxidation of adrenaline could not be induced by succinate + antimycin, nor was it affected by rotenone, antimycin and protamine when initiated by NADH or NADPH (not shown, [16]); these controls rule out the possibility of contaminating mitochondria being the source of $O_2^{\cdot-}$ radicals [17,18].

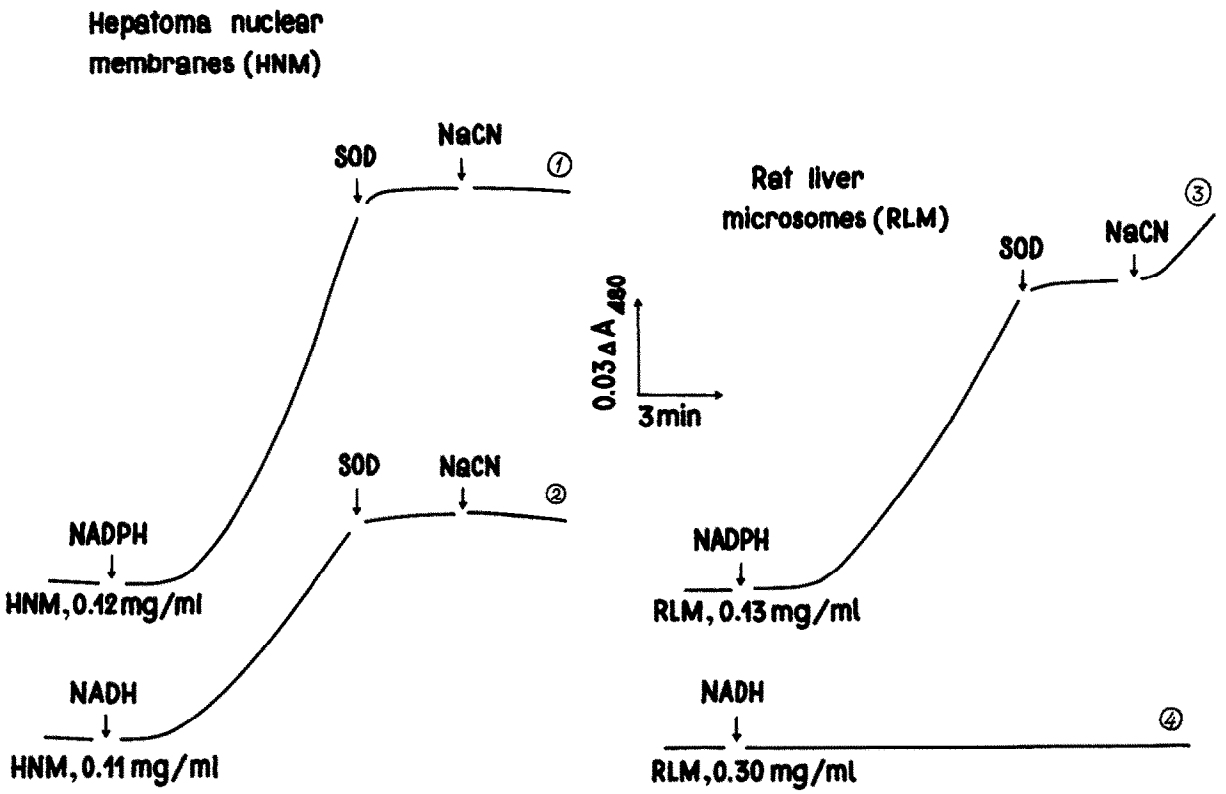


Fig.1. NADH- and NADPH-dependent superoxide generation by nuclear membranes from hepatoma 22a ascites cells [1,2] and rat liver microsomes [3,4]. The reaction medium containing 0.15 M potassium phosphate (pH 8.5), 0.1 mM EDTA and 0.5 mM adrenaline was supplemented with hepatoma 22a nuclear membranes (HNM) or rat liver microsomes (RLM) at the protein concentrations indicated in the figure, and adrenaline co-oxidation was initiated by addition of 0.4 mM NADPH (1,3) or 0.2 mM NADH (2,4). Other additions: SOD, 1.7 μg/ml; NaCN, 1 mM.

The corresponding experiments with rat liver microsomes are shown for comparison in fig.1. As one can see, adrenaline co-oxidation is observed with NADPH (trace 3) but not with NADH (trace 4) in this case. It is noteworthy that app. K_m values for both NADPH- and NADH-dependent adrenaline co-oxidation by the hepatoma 22a membranes were $\sim 10^{-5}$ M [16], which makes unlikely the possibility of an unspecific oxidation of NADH via the NADPH-linked redox chain.

Addition of cyanide to microsomes after the NADPH-dependent $O_2^{\cdot-}$ production has been suppressed by SOD results in a high rate of adrenaline oxidation resotred (fig.1, trace 3), which is evidently due to inactivation of SOD. Surprisingly, with nuclear membranes from hepatoma 22a, the inhibition of $O_2^{\cdot-}$ generation by SOD was not released by cyanide (fig.1, traces 1,2).

On trying to resolve this discrepancy we found that the adrenaline co-oxidase activity of the hepatoma 22a nuclear membranes was itself inhibited by cyanide with both NADPH and NADH as substrates, as shown in fig.2 (traces 1,3,4). Inhibition by 50% was observed at ~10 μM cyanide added, the concentration dependences being exactly the same for the effects of NaCN on the NADPH- and NADH-supported $O_2^{\cdot-}$ generation [16]. Azide has been found to be another inhibitor of the NAD(P)H-dependent production of superoxide by hepatoma 22a nuclei membranes (fig.2, traces 2,5; [16]).

Appropriate controls showed that the NADPH-linked adrenaline co-oxidase of rat liver microsomes as well as the NAD(P)H–cytochrome *c* reductase activities of hepatoma 22a nuclei were not affected appreciably by cyanide or azide under the conditions of our experiments (not shown, [16]).

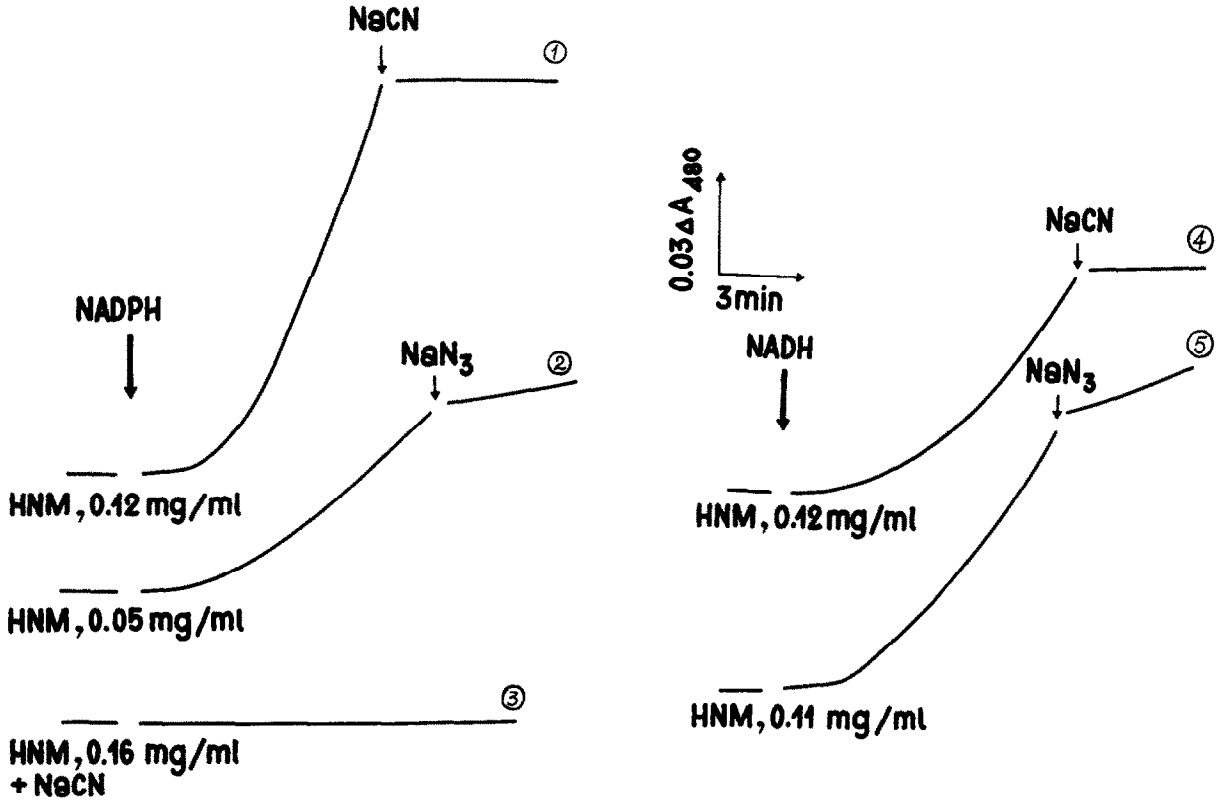


Fig.2. Inhibition of the NAD(P)H-dependent $O_2^{\cdot-}$ generation in hepatoma 22a nuclei membranes by cyanide and azide. Conditions, as in fig.1. Hepatoma 22a nuclear membranes were added at protein concentrations indicated: NaN_3, 1 mM; NaCN, 1 mM.

4. Discussion

Our observation that nuclear membranes from hepatoma 22a ascites cells generate $O_2^{\cdot-}$ radicals actively, corroborates the data in [8] who described the NADPH-dependent $O_2^{\cdot-}$ production by whole nuclei from Ehrlich-Lettré ascites tumor.

From the present results it is clear, however, that the $O_2^{\cdot-}$-yielding system of hepatoma 22a nuclei is entirely different from that of Ehrlich-Lettré cell nuclei as well as from that of liver microsomes; the latter require specifically NADPH as an electron donor and are considered to involve autoxidation of the NADPH–dehydrogenase flavoprotein (NADPH–cytochrome *c* reductase) [4,5] with a possible contribution of cytochrome P450 oxycomplex dissociation [5–7].

At the same time, the features of the $O_2^{\cdot-}$-generating system of nuclear membranes from hepatoma 22a ascites cells, viz. the ability to utilize both NADH and NADPH and the sensitivity to cyanide and azide, are strikingly similar to the properties of the fatty acid desaturase activity [9,10]. So far, we are not aware whether fatty acid desaturase is present in ascites cell nuclei and whether this enzymatic system can yield $O_2^{\cdot-}$ radicals.

The nature of the oxygen-activating component of hepatoma 22a nuclei $O_2^{\cdot-}$-generating system poses a fascinating problem. One could possibly suggest that it is a component similar to the so-called 'cyanide-sensitive factor' (CSF) involved in the microsomal fatty acid desaturase activity [9,10,19–21]. At this point we would note that CSF functioning as an electron acceptor for b_5 or an electron donor to O_2 has been never shown directly. In fact, the presence of 1 Fe atom of non-heme nature/molecule of isolated CSF [20] can hardly substantiate the hypothesis that this lipoprotein is a cyanide- and azide-sensitive terminal oxidase.

On the other hand, the reactivity toward dioxygen,

cyanide and azide is a well-established property of hemoproteins. We would like therefore to suggest that the cyanide- and azide-sensitive oxygen-activating component of microsomal fatty acid desaturase and of hepatoma 22a nuclei is indeed a hemoprotein, e.g., cytochrome b_5 which forms superoxide upon autoxidation (scheme 1).

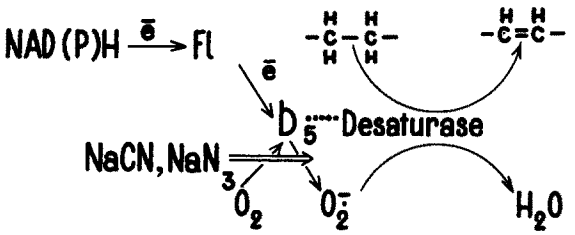
That cytochrome b_5 is present in nuclei from Ehrlich-Lettré ascites cells has been shown [22] and our preliminary experiments revealed a b_5-type cytochrome in hepatoma 22a nuclear membranes [16]. It is, however, known that, although ferrocytochrome b_5 can react with molecular oxygen yielding superoxide radicals [23], the autoxidation reaction per se is fairly slow ($k_v = 2 \times 10^{-3}$ $s^{-1}/4 \times 10^{-2}$ s^{-1} [23–25]) and insensitive to heme ligands [23,25], and may in fact occur by an outer-sphere mechanism [23,26]. In this context, the role of terminal desaturase (CSF) might comprise:

(1) Specific binding of acyl-CoA [21];
(2) Conferring the cyanide-sensitive autoxidizability to cytochrome b_5, e.g., by allosteric interaction which fascilitates the low-spin→high-spin transition of this hemoprotein;
(3) Utilization of $O_2^{\cdot-}$ released by b_5 for oxidative insertion of a double bond into the bound fatty acid residue. Incidentally, participation in the latter reaction would be possibly a more appropriate catalytic function for the non-heme iron of CSF [20] than activation of molecular oxygen.

Any impairment of the coupling between the b_5-activating and $O_2^{\cdot-}$-utilizing functions of terminal desaturase would result in an escape of superoxide radicals, as it is observed with hepatoma 22a nuclear membranes.

Finally, generation of $O_2^{\cdot-}$ radicals via a specifically triggered cytochrome b_5 autoxidation might be implicated in a variety of oxidative processes requiring 'active oxygen'; such a reaction was also postulated in [27] to be involved in hormonal signal processing in the cell.

Scheme 1
Possible involvement of cytochrome b_5 controlled autoxidation in $O_2^{\cdot-}$ production and fatty acid desaturation



Terminal desaturase (CSF) is suggested to form a specific allosteric complex with b_5 so that acyl-CoA binding to CSF confers cyanide-sensitive autoxidizability to ferrocytochrome b_5. $O_2^{\cdot-}$ formed would normally be trapped by the non-heme iron of terminal desaturase [20] and utilized for desaturation of bound acyl-CoA

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