NADH OXIDATION DURING PEROXIDASE CATALYZED METABOLISM OF CARCINOGENIC NON-AMINOAZO DYE 1-PHENYLAZO-2-HYDROXYNAPHTHALENE (SUDAN I)

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The peroxidase catalyzed oxidation of 1-phenylazo-2-hydroxynaphthalene (Sudan I) in the presence of NADH results in oxidation to oxidized NAD^+. This oxidation is supposed to be caused by the reduction of reactive Sudan I metabolite(s). Under certain conditions NADH acts as the agent protecting the cellular nucleophilic compounds such as nucleic acids (DNA, tRNA) against modification caused by reactive metabolite(s) formed from carcinogenic Sudan I by the peroxidase/H_2O_2 system. The results are discussed from the point of view of the physiological significance of NADH in the initiation of chemical carcinogenesis.

The induction of cancer is believed to be initiated by metabolism of the xenobiotic carcinogens to electrophilic metabolites, which covalently bind to nucleophilic macromolecules of cells, particularly DNA. Several cytochrome P-450 species are generally considered to be responsible for oxidative metabolic activation of xenobiotic carcinogens leading to formation of the above mentioned electrophilic metabolites. It was, however, found that several tissues that contain the highest levels of cytochrome P-450 are not typical target organs, in which the tumors are induced, but in further organs the processes of chemical carcinogenesis are also observed. Peroxidases are the enzymes, which are widely distributed among several nonhepatic target tissues (e.g. bladder, mammary gland, eosinophils, leukocytes, uterus, thyroid) and, moreover, many carcinogens that cause nonhepatic tumors are also substrates for these peroxidases[1].

Non-aminoazo compound 1-phenylazo-2-hydroxynaphthalene (Sudan I) is a hepatocarcinogen and a urinary bladder carcinogen in rats[2]. Studies on the metabolism of Sudan I as the model of the non-aminoazo carcinogenic compound by liver microsomal enzymes and the formation of Sudan I-metabolite(s)–DNA adducts in vitro suggested that rat liver cytochrome P-450 is responsible for the activation of this carcinogen in liver[3,4]. As Sudan I causes also tumors of the urinary bladder, peroxidase was also studied from the point of view, whether this enzyme is able to convert this compound (Sudan I) into active metabolite(s) binding to nucleic acids in this target organ. Preliminary results indicated that Sudan I is oxidized by the

model peroxidase system (horseradish peroxidase in the presence of hydrogen peroxide) to products, which bind to DNA and tRNA in vitro[5-7].

It was recently shown that the addition of catalytic amounts of various xenobiotics (e.g. arylamines, phenols) results in rapid oxidation of NADH in a peroxidase, H_2O_2 reaction system[1]. In the present study, we have examined whether Sudan I as the substrate of peroxidase could induce NADH-oxidase activity of the peroxidase system as well, and whether, on the other hand, NADH could play the role in the activation of Sudan I by the peroxidase system leading to binding of its metabolite(s) to nucleic acids.

EXPERIMENTAL

Chemicals

Sudan I (1-phenylazo-2-hydroxynaphthalene) (British Drug Houses, Poole, U.K.), NADH, DNA from calf thymus and horseradish peroxidase (Boehringer Mannheim, F.R.G.) and other chemicals (Lachema, Brno, Czechoslovakia) were of analytical grade. Rat liver tRNA was prepared as described by Rogg et al.[8].

^{14}C-Labelled 1-([U-^{14}C]phenylazo)-2-hydroxynaphthalene (^{14}C-Sudan I) (20 MBq $mmol^{-1}$) was synthesized as described in an earlier paper[9] from [U-^{14}C]aniline (The Radiochemical Centre, Amersham, England) and β-naphthol and purified by column chromatography on basic alumina and preparative thin-layer chromatography (TLC) on silica gel. The labelled compound was stored in a methanol solution at −5°C.

Oxidation of Sudan I by the Peroxidase System

The reaction mixtures contained in a final volume of 1·0 ml: 0·1M sodium phosphate buffer pH 8·4, 0·2 mg of horseradish peroxidase, 0·3 mM Sudan I or ^{14}C-Sudan I and 2·25 mM H_2O_2. The mixtures after the suitable period of incubation were twice extracted with ethylacetate (2 × 2 ml). Three methods for the determination of Sudan I concentrations were then used: *1*) The ethylacetate extracts from the reaction mixtures containing radioactive ^{14}C-Sudan I were evaporated under a stream of N_2, dissolved in a minimal volume of methanol and TLC on silica gel (Silufol, Kavalier, Czechoslovakia) as well as the estimation of the amounts of unconverted ^{14}C-Sudan I was performed by the methods described in our previous papers[5,10]. *2*) The ethylacetate extracts obtained with non-radioactive Sudan I in the incubation mixtures, after evaporation and dissolution in methanol (see above) were separated by TLC similarly. The residual Sudan I and Products were separated mechanically, by cutting of thin layer of silica gel. Sudan I was extracted from the layer by ethylacetate (2 × 2 ml). The measuring of absorbance at 475 nm (the absorption maximum of Sudan I in ethylacetate solution) was then used for the estimation of concentrations of Sudan I in these ethylacetate extracts. *3*) The concentrations of Sudan I was estimated by the measuring of the absorbance at 475 nm in the ethylacetate extracts obtained by extraction of the reaction mixture without further operations.

No statistical differences in the determination of Sudan I concentrations by the above mentioned three methods were obtained. Most of the data presented in the paper were obtained by he third simplest method.

Isolation of DNA and tRNA after Modification of ^{14}C-Sudan I Metabolite(s) Formed with the Peroxidase System

Incubation mixtures used for the modification of DNA or tRNA by ^{14}C-Sudan I activated by peroxidase with or without NADH contained in a final volume of 1·5 ml: 50 mM Tris-HCl buffer pH 7·4, 0·2 mg of horseradish peroxidase, 0·5 mM — 1·0 mM H_2O_2, 0·2 mM ^{14}C-Sudan I, 1–2 mg of DNA or tRNA and 0–1 mM NADH. The mixtures after incubation (37°C, 120 min) were twice extracted with ethylacetate (2 × 2 ml), 1 ml of 80% phenol and 1 ml of chloroform were then added to the aqueous layer and the reaction mixtures shaken vigorously and centrifuged. The water phases were transferred into another test-tube and the phenol–chloroform extraction procedure was repeated. DNA and tRNA was then precipitated from water phases by ethanol (5 ml) after addition of 100 µl of 1M NaCl or 100 µl of 1M sodium acetate, respectively. Precipitates of nucleic acids were washed with ethanol, ethanol–diethyl ether (1 : 1), diethyl ether (2×) and dried under a stream of N_2. Nucleic acids were dissolved in 1 ml of distilled water and the ^{14}C-radioactivity of nucleic acids was determined in a 100 µl aliquots by liquid scintillation counting[5,10]. The content of nucleic acids was measured spectrophotometrically as described previously[11].

Measurement of NADH Oxidation

Unless stated otherwise the reaction mixture, in 2 ml of 0·1M potassium phosphate buffer pH 7·7, contained 3 µM Sudan I, 10 µg of horseradish peroxidase, 100 µM NADH and 55 µM H_2O_2. Reactions were started by addition of Sudan I and the disappearance of NADH with time was followed at 340 nm using the Perkin–Elmer Lambda 5 UV/VIS spectrophotometer.

RESULTS AND DISCUSSION

NADH inhibits conversion of Sudan I by the peroxidase/H_2O_2 system under several conditions. At short time of incubation of the reaction mixture, NADH acts as the effective inhibitor. However, practically no inhibition occurs during the longer time

TABLE I

The effect of NADH on the conversion of Sudan I by the peroxidase/H_2O_2 system. Experimental conditions: 0·1M sodium phosphate buffer pH 8·4, 0·3 mM Sudan I, 2·25 mM H_2O_2, 0·2 mg of peroxidase, 0–1 mM NADH; 20°C. The values given are averages of three parallel experiments and standard deviations

Time of incubation min	% of Sudan I converted in the samples	
	without NADH	with 1 mM NADH
5	78·00 ± 0·3	10·66 ± 0·5
10	82·66 ± 0·6	75·33 ± 0·4
20	87·83 ± 0·4	80·00 ± 0·5
40	87·83 ± 0·3	80·00 ± 0·5

of incubations (Table I). It is known that NADH alone is not the substrate of peroxidase[1]. Thus, the inhibition of the reaction by NADH is not caused by the competition of this compound (NADH) with Sudan I for the binding site of the enzyme. This inhibition could be due to reduction of the reactive oxidized Sudan I intermediates by NADH, which is simultaneously oxidized. Decrease of the inhibition after long time of incubation may be explained by depletion of NADH available for oxidation.

An interesting effect of NADH on the conversion of Sudan I was, however, observed in the mixture, which does not contain H_2O_2. In the absence of both NADH and H_2O_2 Sudan I is converted (oxidized) by peroxidase and O_2 only to a small extent (Table II). On the other hand, NADH stimulated the oxidation of Sudan I

TABLE II

The effect of NADH on the oxidation of Sudan I by the peroxidase/O_2 system (without H_2O_2). Experimental conditions: 0·1M sodium phosphate buffer pH 8·4, 0·3 mM Sudan I, 0·2 mg peroxidase, 0–1 mM NADH; 20°C. The values given are averages of three parallel experiments and standard deviations

NADH mmol l^{-1}	% of Sudan I converted during	
	5 min	45 min
0	0·65 ± 0·05	2·42 ± 0·15
0·2	1·80 ± 0·60	3·03 ± 0·17
0·4	2·55 ± 0·15	3·64 ± 0·21
0·8	4·08 ± 0·13	4·24 ± 0·25
1·0	11·23 ± 0·27	13·33 ± 0·22

TABLE III

The oxidation of NADH by the peroxidase/H_2O_2/Sudan I system. Experimental conditions: 0·1M potassium phosphate buffer pH 7·7, 100 μM NADH; 20°C. The oxidation of NADH was measured as decrease of absorbance at 340 nm during the first 3 minutes of the reaction. The values given are averages of three parallel experiments and standard deviations

Sudan I μmol l^{-1}	Peroxidase μg	H_2O_2 μmol l^{-1}	Converted NADH nmol min^{-1}
3	10	55	5·65 ± 0·2
—	10	55	0·11 ± 0·01
3	10	—	0·02 ± 0 002
3	—	55	0

by the peroxidase/O_2 system under these conditions significantly (Table II). This fact is difficult to interpret without taking into account a different mechanism of peroxidase reactions. Formation of catalytic amounts of reduced peroxidase by NADH with subsequent formation of oxyperoxidase (Compound III) in ref.[12] may be responsible for oxidation of Sudan I in this system.

Further experiments confirmed that NADH plays the role in the reduction of Sudan I reactive intermediates as it is oxidized by these compounds. It was, namely, found that Sudan I can act as the catalyst in the oxidation of NADH by hydrogen peroxide and peroxidase. It means that peroxidase and H_2O_2 have the NADH--oxidase activity in the presence of this carcinogenic azo compound. In the absence

TABLE IV
The effect of NADH on the binding of ^{14}C-Sudan I metabolite(s) formed by the peroxidase/H_2O_2 system to nucleic acids. Experimental conditions: 50 mM Tris-HCl buffer pH 7·7, 0·2 mM ^{14}C-Sudan I, 0·5 or 1·0 mM H_2O_2, 0—1 mM NADH, 1 mg of DNA or tRNA, 0·2 mg of peroxidase; 37°C, incubation time, 30 min. The values given are averages of three parallel experiments and standard deviations

NADH mmol l^{-1}	DNA-binding[a]		tRNA-binding[a]	
	0·5 mM H_2O_2	1·0 mM H_2O_2	0·5 mM H_2O_2	1·0 mM H_2O_2
0	2·21 ± 0·31	4·03 ± 0·35	7·4 ± 0·5	7·5 ± 0·61
1	1·05 ± 0·1	3·51 ± 0·2	3·2 ± 0·2	6·2 ± 0·5

[a] nmol of ^{14}C-Sudan I/mg.

TABLE V
The effect of guanosine and DNA on the oxidation of NADH by the peroxidase/H_2O_2/Sudan I system. Experimental conditions: 0·1M potassium phosphate buffer pH 7·7, 3 μM Sudan I, 55 μM H_2O_2, 100 μM NADH, 10 μg peroxidase; 20°C. The oxidation of NADH was measured as described in Table III. The values given are averages of three parallel experiments and standard deviations

Added to reaction mixture	Converted NADH nmol min^{-1}	% of inhibition
Control	5·65 ± 0·2	0
+ 1·77 mM guanosine	3·39 ± 0·2	40·0
+ 1 mg of DNA	2·56 ± 0·1	54·7

of Sudan I the oxidation of NADH by the peroxidase system with H_2O_2 (measured as the decrease of the absorbance at 340 nm) was very slow. However, in the presence of trace amounts of Sudan I, the oxidation of NADH was rapid (Table III).

During the reaction of Sudan I with the peroxidase/H_2O_2 system the reactive intermediates or products are bound to nucleic acids[5-7]. The above described binding of Sudan I metabolite(s) to nucleic acids is proposed to be mainly responsible for the initiation of chemical carcinogenesis induced by this carcinogenic azo dye in the second target tissue (urinary bladder), which is rich in the peroxidase content.

It may be supposed that NADH (the compound of physiological significance), which is oxidized during the peroxidase reaction with Sudan I, could play the protective effect against the binding of reactive form(s) of studied carcinogen to nucleic acids as a compound competing with cell nucleophiles important for normal functions of cells. Indeed, NADH partially decreases the binding of Sudan I metabolite(s) formed in the peroxidase-mediated reaction to DNA and tRNA in vitro (Table IV). Similarly, the nucleophiles such as DNA and guanosine inhibit the oxidation of NADH in the used system (Table V). Thus, the reactive intermediates or products (which normally oxidized NADH) could be trapped by DNA or guanosine as strong nucleophiles. The formation of adducts from these nucleophiles and reactive intermediates or products of Sudan I oxidation thus can cause the decrease of the effective concentrations of these reactive compounds and they, hence, cannot oxidize NADH.

The results presented in this paper are important from the point of view of the role of peroxidases in the activation of carcinogens to their ultimate forms as well as of the role of peroxidase as the NADH-oxidase system in the presence of carcinogenic xenobiotics. The peroxidase/H_2O_2 system is able to convert carcinogenic Sudan I to the ultimate form(s), which bind to nucleic acids only in the presence of H_2O_2 in the reaction mixture. When only O_2 is present, Sudan I is partially oxidized but no binding of metabolites to DNA was, however, observed. The detailed mechanism (giving an answer to the question whether NADH is oxidized only by reactive intermediates and/or products of Sudan I oxidation or whether in this system the active form(s) of oxygen can also be formed, which, moreover, could also participate in the oxidation of NADH) will be studied in the future.

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