Biochimica et Biophysica Acta, 1116 (1992) 17–23

BBAGEN 23638

Effect of phenobarbital and 3-methylcholanthrene treatment on NADPH- and NADH-dependent production of reactive oxygen intermediates by rat liver nuclei

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(Received 15 August 1991)

Key words: Nuclei; NAD(P)H; Oxygen radical production; Phenobarbital; 3-Methylcholanthrenus

The effect of inducing the rat liver nuclear mixed-function oxidase system by phenobarbital or 3-methylcholanthrene on NADPH- and NADH-dependent production of reactive oxygen intermediates was evaluated. The inducing agents produced a 2-fold increase in cytochrome *P*-450, a 50 to 70% increase in NADPH-cytochrome *c* reductase activity, and a 20 to 30% increase in NADH-cytochrome *c* reductase activity. Associated with these increases was a corresponding increase in NADPH- and NADH-dependent production of hydroxyl radical (·OH)-like species and of H_2O_2. Rates of ·OH production were inhibited by catalase and partially sensitive to superoxide dismutase. The increase in nuclear production of ·OH-like species after drug treatment appears to be due a corresponding increase in H_2O_2 generation. In contrast to H_2O_2 and ·OH generation, production of thiobarbituric acid-reactive material by nuclei was not increased by the phenobarbital or 3-methylcholanthrene treatment. Redox cycling agents such as menadione and paraquat increased oxygen radical generation to similar extents in the control and the induced nuclei. These results indicate that induction of the nuclear mixed-function oxidase system by phenobarbital or 3-methylcholanthrene can result in a subsequent increase in production of reactive oxygen intermediates in the presence of either NADPH or NADH.

Introduction

The presence of a carbon monoxide binding pigment in the nucleus was first shown by Omura and Sato [1], and characterized by Rogan and Cavalieri [2,3]. Bresnick et al. [4–6] demonstrated by immunohistochemical techniques the presence of cytochromes *P*-448 and *P*-450 in the nuclei. The nuclear cytochrome *P*-448 was shown to be immunochemically identical to the microsomal enzyme [7] and to be an intrinsic protein localized on the cytoplasmic face of the outer membrane of the nuclear envelope [8]. A variety of studies have shown that nuclear cytochrome *P*-450 can be increased 2-4-fold by treatment with phenobarbital or 3-methylcholanthrene [2–4,9–114]. In addition to cytochrome *P*-450, the nucleus also contains cytochrome b_5, and the flavoprotein reductases, NADH- and NADPH-cytochrome *c* reductase (reviewed in Ref. 14). The activity of NADPH-cytochrome *P*-450 reductase has been reported to increase after phenobarbital treatment [15,16].

The nuclear NADPH- and NADH-dependent electron transfer systems have been shown to produce superoxide and H_2O_2, and in the presence of various iron complexes, nuclei undergo lipid peroxidation and generate hydroxyl radical-like species [17–24]. Nuclei can catalyze the reduction of ferric complexes such as ferric-EDTA and ferric-ATP in the presence of either NADPH or NADH [24]. In contrast to microsomes, NADH-dependent generation of reactive oxygen intermediates by nuclei is greater than the NADPH-dependent reactions [24]. The nuclear electron transfer systems can activate redox cycling agents such as adriamycin, daunomycin, bleomycin, paraquat and menadione, with the subsequent production of superoxide, H_2O_2, and generation of ·OH-like species and thiobarbituric acid-reactive components [25–29]. NADPH is usually more effective than NADH in promoting the

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ability of certain redox cycling agents, e.g., menadione and paraquat to catalyze nuclear generation of reactive oxygen species [29]. The effect of induction of cytochrome *P*-450 and/or the flavoprotein reductases on oxygen radical generation by nuclei has not been determined. The current study was carried out to evaluate the effect of phenobarbital or 3-methylcholanthrene on NADH- and NADPH-dependent generation of reactive oxygen intermediates by isolated rat liver nuclei.

Materials and Methods

Male Sprague Dawley rats, weighing 150–170 g, were treated i.p. either with 25 mg/kg 3-methylcholanthrene or 80 mg/kg phenobarbital for 3 days. Controls received an equal volume of saline or corn-oil. All rats were starved overnight prior to the preparation of the nuclei.

Rat liver nuclei were prepared by the method of Spelberg et al. [30] as previously described [24]. The final pellet of purified nuclei was resuspended (1 mg per g of liver) in 25% glycerol in 10 mM Tris-HCl, 1 mM $MgCl_2$ (pH 7.9) and frozen at $-70\,^{\circ}C$ until used. Samples of frozen nuclei were thawed out and centrifuged at $800 \times g$ for 20 min and resuspended and washed with 125 mM KCl prior to experimentation. Protein was determined by the method of Lowry et al. [31]. Contamination by microsomes as assessed by glucose-6-phosphatase activity did not exceed about 3%; rates of production of ·OH or H_2O_2 by the nuclei were about 10% that found with microsomes, which exceeds this level of contamination.

The production of ·OH-like species was routinely assayed by measuring the generation of ethylene gas from 2-keto-4-thiomethylbutiric acid (KMB) or the production of formaldehyde from dimethyl sulfoxide (DMSO) [32]. The basic reaction system consisted of 100 mM potassium phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM sodium azide, 0.05 mM ferric-EDTA, about 0.3 to 0.5 mg nuclear protein, either 10 mM KMB or 33 mM DMSO and either 2 mM NADH or an NADPH-generating system consisting of 0.4 mM NADPH, 10 mM glucose 6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase in a final volume of 1 ml. Production of ethylene was determined by head space gas chromatography [32] and formaldehyde by the Nash reaction [33].

The production of hydrogen peroxide was determined by measuring the generation of formaldehyde from the oxidation of methanol by the catalase-compound I complex [34]. Reactions were carried out as previously described [24]. The production of thiobarbituric acid-reactive components was measured as an index of lipid peroxidation [35]. Reactions were carried out as previously described in the presence of 0.05 mM ferric-1 mM ATP as the iron catalyst [24].

TABLE I

Effect of treatment with phenobarbital or 3-methylcholanthrene on the nuclear content of cytochrome P-450 and activities of NADPH- and NADH-cytochrome c reductase

Rats were treated with either phenobarbital or 3-methylcholanthrene or their respective controls (saline, corn-oil) and the various activities assayed as described in Materials and Methods. Results are from three experiments.

Treatment	Cytochrome *P*-450 content (nmol/mg)	NADPH-cytochrome *c* reductase (nmol/min per mg)	NADH-cytochrome *c* reductase (nmol/min per mg)
Saline	0.037 ± 0.002	5.7 ± 0.4	18.9 ± 1.4
Phenobarbital	0.075 ± 0.008 (+102%)	8.7 ± 0.5 (+53%)	22.8 ± 1.7 (+21%)
Corn oil	0.024 ± 0.005	5.0 ± 0.7	16.6 ± 1.4
3-methylcholanthrene	0.052 ± 0.004 (+116%)	8.4 ± 0.9 (+68%)	22.1 ± 3.1 (+31%)

The content of cytochrome *P*-450 was determined by the method of Omura and Sato [1] and the activities of NADPH-cytochrome *c* reductase and NADH-cytochrome *c* reductase were determined by the method of Phillips and Langdon [36] in 300 mM phosphate buffer (pH 7.4).

The ferric-EDTA (1:2) or ferric-ATP (1:20) complexes were prepared by dissolving ferric ammonium sulfate in 0.1 M HCl, and then diluting with the respective chelator to the appropriate stock solution. All reagents were of the highest grade available. The buffers and the water used to prepare all solutions were passed through columns containing Chelex-100 resin to remove metal contaminants.

All results are from experiments carried out in duplicate, and replicated with at least two or three different nuclei preparations. Where indicated, values refer to mean ± S.E.

Results

The content of cytochrome *P*-450 in the isolated nuclei was similar in the saline controls and the corn-oil controls. Treatment with phenobarbital or 3-methylcholanthrene resulted in a 2-fold increase in the content of cytochrome *P*-450 (Table I). Accompanying the increase in total *P*-450 content was an increase in the activity of NADPH-cytochrome *c* reductase by the phenobarbital and 3-methylcholanthrene treatments. The activity of NADH cytochrome *c* reductase was about 3-fold higher than that of NADPH cytochrome *c* reductase, and was only slightly increased by the treatment with either phenobarbital or 3-methylcholanthrene (Table I).

To evaluate the effect of phenobarbital or 3-methylcholanthrene treatment on the generation of ·OH-like

TABLE II

Effect of treatment with phenobarbital or 3-methylcholanthrene on the production of ·OH-like species by rat liver nuclei

The oxidation of DMSO to formaldehyde or of KMB to ethylene gas by rat liver nuclei was determined as described in Materials and Methods. Results are from three to four experiments.

Treatment	Rate of formaldehyde production from DMSO (nmol/min per mg protein)		Rate of ethylene production from KMB (nmol/min per mg protein)	
	NADPH	NADH	NADPH	NADH
Saline	0.41 ± 0.02	1.06 ± 0.11	0.19 ± 0.06	0.60 ± 0.10
Phenobarbital	0.79 ± 0.07 (+92%)	1.63 ± 0.18 (+54%)	0.41 ± 0.08 (+115%)	1.12 ± 0.39 (+83%)
Corn oil	0.52 ± 0.03	1.03 ± 0.08	0.21 ± 0.04	0.77 ± 0.12
3-Methylcholanthrene	0.85 ± 0.06 (+63%)	1.75 ± 0.15 (+70%)	0.34 ± 0.03 (+62%)	1.51 ± 0.12 (+96%)

species, the production of ethylene from KMB or of formaldehyde from DMSO by the nuclei was evaluated in the presence of ferric-EDTA as the iron catalyst. Ferric-EDTA has been shown to be very reactive in promoting NADPH- and NADH-dependent production of ·OH-like species by nuclei [24,29]. With NADPH as the reductant, the oxidation of both KMB and DMSO was increased by the phenobarbital (about 2-fold) and by the 3-methylcholanthrene (about 60%) treatments (Table II). With NADH as the reductant, an increase in nuclear oxidation of KMB and DMSO was also observed by the treatment with the two inducers (Table II). Thus, with either KMB or DMSO as the scavenger substrate, treatment with phenobarbital or 3-methylcholanthrene resulted in an increase in nuclear generation of ·OH-like species in the presence of NADPH or NADH as the electron donor. Production of ·OH-like species was dependent on the presence of reductant (NADH or NADPH), nuclei and iron. Reactions were linear with time for 15 to 30 min with all nuclear preparations. Few or no products were produced in zero-time controls in which acid was added to the nuclei prior to the cofactor.

The roles of superoxide radical and hydrogen peroxide in the overall pathway of ·OH generation by the nuclei was evaluated. Catalase was a potent inhibitor of the NADPH- and NADH-dependent oxidation of KMB by control nuclei and induced nuclei (Table III). Superoxide dismutase caused a 30 to 60% decrease in KMB oxidation by the various preparations. DMSO, added as a competing ·OH scavenging agent, produced strong inhibition of KMB oxidation (Table III). In general the NADPH- and the NADH-dependent systems showed similar sensitivities to catalase, superoxide dismutase and DMSO; control nuclei and induced nuclei also showed similar sensitivities to the radical scavengers.

Redox cycling agents such as paraquat and menadione were recently shown to increase nuclear generation of ·OH-like species, when NADPH was the reductant [29]. The effect of paraquat and menadione on the oxidation of KMB by nuclei from control rats and rats

TABLE III

Effect of anti-oxidative agents on the oxidation of KMB by rat liver nuclei

NADPH-dependent or NADH-dependent oxidation of KMB by control or induced nuclei was determined in the absence or presence of the indicated additions. Final concentrations of superoxide dismutase, catalase or DMSO were 43 units per ml, 60 units per ml, and 50 mM, respectively. Results are from two experiments.

Cofactor	Addition	Rate of ethylene production (nmol/min per mg protein) Nuclei			
		saline	phenobarbital	corn oil	3-methylcholanthrene
NADPH	–	0.18	0.52	0.27	0.40
	superoxide dismutase	0.11 (−39%)	0.22 (−59%)	0.16 (−41%)	0.26 (−35%)
	catalase	0.04 (−75%)	0.03 (−95%)	0.002 (−99%)	0.07 (−83%)
	DMSO	0.06 (−66%)	0.07 (−87%)	0.02 (−92%)	0.10 (−75%)
NADH	–	0.72	1.67	0.93	1.53
	superoxide dismutase	0.45 (−38%)	0.66 (−61%)	0.33 (−64%)	0.91 (−40%)
	catalase	0.04 (−94%)	0.15 (−91%)	0.02 (−98%)	0 (−100%)
	DMSO	0.11 (−85%)	0.30 (−82%)	0.01 (−99%)	0.36 (−76%)

TABLE IV

Effect of redox cycling agents on the oxidation of KMB by rat liver nuclei

NADPH-dependent or NADH-dependent oxidation of KMB by control or induced nuclei was determined in the absence or presence of either 2 mM paraquat or 0.1 mM menadione.

Cofactor	Addition	Rate of ethylene production (nmol/min per mg protein) Nuclei			
		saline	phenobarbital	corn oil	3-methylcholanthrene
NADPH	–	0.19 ± 0.03	0.33 ± 0.05	0.20 ± 0.04	0.30 ± 0.06
	paraquat	1.09 ± 0.06 (+473%)	1.62 ± 0.07 (+390%)	0.84 ± 0.34 (+320%)	1.12 ± 0.18 (+273%)
	menadione	1.53 ± 0.27 (+705%)	2.09 ± 0.42 (+533%)	1.45 ± 0.71 (+625%)	1.96 ± 0.73 (+533%)
NADH	–	0.35 ± 0.03	0.58 ± 0.06	0.42 ± 0.04	0.97 ± 0.10
	paraquat	0.42 ± 0.05 (+20%)	0.63 ± 0.06 (+8%)	0.63 ± 0.18 (+50%)	0.95 ± 0.05 (0%)
	menadione	1.01 ± 0.19 (+188%)	1.82 ± 0.20 (+213%)	0.96 ± 0.29 (+118%)	2.18 ± 0.90 (+124%)

treated with either phenobarbital or 3-methylcholanthrene was determined. Paraquat produced a 4–5-fold increase in ethylene generation from KMB with all nuclear preparations when NADPH was the added cofactor, whereas little or no effect by paraquat was found when NADH was the reductant (Table IV). Menadione produced a 6–7-fold increase in KMB oxidation by all nuclear preparations in the presence of NADPH, and a 2–3-fold increase in the presence of NADH (Table IV). Treatment with phenobarbital or 3-methylcholanthrene did not change the interactions of the redox cycling agents with either NADPH or NADH to promote the nuclear generation of ·OH-like species.

To evaluate possible factors which play a role in the increase in production of ·OH-like species by the nuclei after treatment with phenobarbital or 3-methylcholanthrene, the production of H_2O_2 and the rate of reduction of added iron were determined. The potent inhibition of KMB oxidation by catalase suggested that H_2O_2 was a precursor to the oxidant responsible for the oxidation of KMB. Control rates of H_2O_2 production were higher with NADH than NADPH, which probably contributes to the elevated rates of oxidation of KMB or DMSO in the presence of NADH compared to NADPH. In the NADPH-dependent system, treatment with penobarbital or 3-methylcholanthrene resulted in increases of 104 and 68% in the rates of H_2O_2 production (Table V). In the NADH-dependent system, the treatment with inducers resulted in about a 45% increase in the rates of H_2O_2 production (Table V).

Rates of reduction of ferric-EDTA or ferric-ATP were determined in the presence of 2,2′-dipyridyl by following the increase in absorbance at 520 nm of the ferrous-dipyridyl complex. Control rates of ferric-EDTA reduction were 4.8 and 8.3 nmol/min per mg nuclear protein with NADPH and NADH, respectively; control rates of ferric-ATP reduction were 4.1 and 23.4 nmol/min per mg nuclear protein with NADPH and NADH, respectively. Treatment with either phenobarbital or 3-methylcholanthrene did not change the NADPH- or NADH-dependent rates of reduction of ferric-EDTA or ferric-ATP (data not shown).

The production of thiobarbituric-acid reactive material was determined as a measure of nuclear lipid peroxidation. In contrast to rates of production of ·OH-like species and of H_2O_2, rates of production of

TABLE V

Effect of treatment with phenobarbital or 3-methylcholanthrene on H_2O_2 production by rat liver nuclei

The production of H_2O_2 by control and induced nuclei was determined as described in Materials and Methods. Results are from three experiments.

Treatment	Rate of H_2O_2 production (nmol/min per mg protein)	
	NADPH	NADH
Saline	0.71 ± 0.08	1.71 ± 0.16
Phenobarbital	1.45 ± 0.29 (+104%)	2.44 ± 0.24 (+43%)
Corn oil	0.54 ± 0.06	1.33 ± 0.24
3-Methylcholanthrene	0.91 ± 0.15 (+68%)	1.96 ± 0.17 (+47%)

TABLE VI

Effect of treatment with phenobarbital or 3-methylcholanthrene on nuclear generation of TBA-reactive intermediates

The production of TBA-reactive components by control and induced nuclei was determined as described in Materials and Methods. Results are from two experiments.

Treatment	TBA-reactive components (A_{535}/mg protein)	
	NADPH	NADH
Saline	0.100	0.037
Phenobarbital	0.108	0.050
Corn oil	0.087	0.054
3-Methylcholanthrene	0.085	0.040

TBA-reactive material were greater with NADPH than NADH as the added reductant (Table VI). Treatment with either phenobarbital or 3-methylcholanthrene did not increase the NADPH- or the NADH-dependent rates of production of TBA-reactive material (Table VI).

Discussion

Rat liver nuclei contain inducible, functionally active mixed-function oxidase activity, cytochrome *P*-450, NADPH- and NADH-cytochrome *c* reductase activity [14]. Associated with these electron transfer systems are the production of superoxide and hydrogen peroxide, and in the presence of iron catalysts, the generation of more potent oxidizing species capable of causing lipid peroxidation, DNA damage, and oxidation of typical ·OH-scavenging agents [17–29]. While there is much interest in the ability of nuclei, especially after induction, to activate various substrates to reactive intermediates, there have been few studies to evaluate the effect of induction of the nuclear mixed-function oxidase system on the production of reactive oxygen intermediates. In the current report, treatment of rats with phenobarbital or 3-methylcholanthrene was confirmed to increase the content of total cytochrome *P*-450; control values of nuclear cytochrome *P*-450 (0.02 to 0.04 nmol/mg protein) were reported to be increased 2–4-fold (0.04 to 0.08 nmol/mg protein) after treatment with phenobarbital or 3-methylcholanthrene (Table I of Ref. 14). We observed increases of about 2-fold (control values of 0.02 and 0.04 nmol/mg protein; induced values of 0.05 and 0.08 nmol/mg protein) after treatment with these inducing agents, as well as more modest increase in the activities of NADPH- and NADH-cytochrome *c* reductase.

Associated with the increase in components of the nuclear mixed-function oxidase system was an increase in the production of ·OH-like species and of H_2O_2 after treatment with phenobarbital or 3-methylcholanthrene. In general, the increases in oxidation of KMB or of DMSO, or in the production of H_2O_2 were about 50 to 100%, which is similar to the increases in *P*-450 content and reductase activities. Increases were found with both NADPH and NADH as the electron donors, with the NADPH-dependent systems being somewhat more increased than the NADH-dependent systems, e.g. NADPH-dependent H_2O_2 production was elevated 68 to 104% after induction, whereas NADH-dependent H_2O_2 production was increased about 45%. These differences may relate to the effect of the inducers on the activities of the reductases as NADPH-cytochrome *c* reductase was increased 53 to 68%, while NADPH-cytochrome *c* reductase was only increased 21 to 31%. Since rates of iron chelate reduction were not increased, the increase in production of ·OH-like species after treatment with phenobarbital or 3-methylcholanthrene may be due to the increase in H_2O_2 production by the nuclei after induction. Oxidation of KMB (and DMSO) was completely sensitive to catalase implicating a role for H_2O_2 as a precursor of the oxidant responsible for oxidation of the scavenger substrates. With NADPH as the reductant, rates of oxidation of KMB were about 0.2 or 0.3 to 0.5 nmol per min per mg for control or induced preparations, respectively, which is similar to the rates of H_2O_2 generation of about 0.5 to 0.7 or 0.9 to 1.4 nmol per min per mg for control or induced preparations, respectively. By contrast, rates of ferric-EDTA reduction were 4 nmol/min per mg nuclear protein. A similar rate-limiting factor for H_2O_2 generation appears for the NADH-dependent reactions; rates of KMB oxidation (0.4 to 1.6 nmol/min per mg) for the control and induced preparations were similar to rates of H_2O_2 production (1.3 to 2.4 nmol/min per mg). It would appear that the increase in nuclear generation of ·OH-like species after treatment with phenobarbital or 3-methylcholanthrene is due, at least in part, to the increase in H_2O_2 production as a consequence of the induction of components of the nuclear mixed-function oxidase system.

In previous studies with microsomes, production of ·OH-like species was not inhibited by superoxide dismutase; indeed, increases by superoxide dismutase were often observed [37,38]. It was concluded that the production of ·OH-like species was primarily due to a Fenton-type of reaction between H_2O_2 and ferrous iron. With nuclei, superoxide dismutase produced partial inhibition of KMB oxidation, ranging from about 30 to 60% inhibition (Table III). Inhibition was observed for both the NADPH- as well as the NADH-dependent reactions, and for control nuclei as well as induced nuclei. It appears that with the nuclei, generation of ·OH-like species occurs via Fenton-type, as well as iron-catalyzed Haber-Weiss types of reactions.

The production of TBA-reactive material in the presence of either NADPH or NADH was not increased after the treatment with phenobarbital or 3-methylcholanthrene. Under our experimental conditions, nuclear lipid peroxidation is not sensitive to catalase, superoxide dismutase or ·OH scavengers with NADPH as cofactor, and only partially sensitive to these anti-oxidants when NADH is the reductant [24,29]. Therefore, there is little or only a small role for H_2O_2 or ·OH in the overall pathways for production of TBA-reactive material. Hence, increases in H_2O_2 or ·OH production by the nuclei after induction need not necessarily translate to similar increases in lipid peroxidation.

In the presence of NADPH, paraquat and menadione strikingly increased the oxidation of KMB in control and induced nuclei. As described previously

[29], paraquat was ineffective in the NADH-dependent system, while menadione produced a 2–3-fold increase in KMB oxidation. Treatment with phenobarbital or 3-methylcholanthrene did not affect the response of the nuclei to paraquat or menadione, although rates of production of ·OH-like species remained elevated in the induced nuclei. The increase in production of ·OH-like species after addition of paraquat of menadione is largely due to elevated rates of production of H_2O_2 [29].

The toxicity associated with various agents often requires activation to a reactive intermediate by mixed-function oxidase systems. Whereas the microsomal systems are far more reactive, there has been considerable interest in the ability of the nuclei to activate various toxicological agents and carcinogens to reactive intermediates since such intermediates would not be detoxified as readily by cytosolic antioxidants and would be produced in close proximity to the genetic apparatus. Particular significance has been attached to induction of the nuclear mixed-function oxidase system by agents such as phenobarbital and especially 3-methylcholanthrene since induction often causes increased activation of a variety of toxic agents and carcinogens to reactive intermediates, which may escape or overwhelm deactivating defensive systems, and subsequently react with DNA [39,40]. Oxygen radicals also affect the expression of genetic material and interaction of primary and secondary products of reduced oxygen species with DNA can cause mutagenesis and cell injury [41–46]. Reactive oxygen radicals generated by the nucleus would be less susceptible to scavenging by enzymes such as superoxide dismutase, catalase or glutathione peroxidase. The induction of the nuclear mixed-function oxidase system and subsequent increased production of reactive oxygen intermediates by nuclei with either NADPH or NADH as cofactors after treatment with phenobarbital or 3-methylcholanthrene may play a role in the toxicity associated with these inducing agents.

Acknowledgements

These studies were supported by USPHS Grant AA-03312 from The National Institute on Alcohol Abuse and Alcoholism and NSF-INT 8901813 USA-Argentina Cooperative Research Grant from The National Science Foundation. We thank Ms. Lucy Martinez for typing the manuscript.

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