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Role of Reactive Oxygen Intermediates in the Interferon-mediated Depression of Hepatic Drug Metabolism and Protective Effect of *N*-Acetylcysteine in Mice[1]

Pietro Ghezzi,[2] Marina Bianchi, Luigi Gianera, Santo Landolfo, and Mario Salmona

Laboratory for Enzyme Research, Istituto di Ricerche Farmacologiche "Mario Negri," 20157 Milan [P. G., M. B., L. G., M. S.], and Istituto di Microbiologia, Università degli Studi di Torino, 10126 Torino [S. L.], Italy

ABSTRACT

Interferon (IFN) and IFN inducers are known to depress hepatic microsomal cytochrome P-450 levels, and the liver toxicity of IFN was reported to be lethal in newborn mice.

We have observed that administration to mice of IFN and IFN inducers caused a marked increase in liver xanthine oxidase activity. Because this enzyme is well known to produce reactive oxygen intermediates and cytochrome P-450 was reported to be sensitive to the oxidative damage, we have tested the hypothesis that a free radical mechanism could mediate the depression of cytochrome P-450 levels by IFN.

Administration to mice of the IFN inducer polyinosinic-polycytidylic acid (2 mg/kg i.p.) caused a 29 to 52% decrease in liver cytochrome P-450. Concomitant p.o. administration of the free radical scavenger, *N*-acetylcysteine (as a 2.5% solution in drinking water), or the xanthine oxidase inhibitor, allopurinol (100 mg/kg), protected against the IFN-mediated depression of P-450 levels. The results suggest that an increased endogenous generation of free radicals, possibly due to the induction of xanthine oxidase, is implicated in the IFN-mediated depression of liver drug metabolism.

The relevance of these data also extends to cases in which this side effect is observed in pathological situations (*e.g.*, viral diseases and administration of vaccines) associated with an induction of IFN.

INTRODUCTION

The depression of hepatic microsomal cytochrome P-450 after the administration of various inducers of IFN-α[3] and IFN-β was first reported by Renton and Mannering (29). Subsequent studies with purified IFN preparations including highly purified recombinant IFN showed that this impairment of liver drug metabolism was mediated via IFN (27, 33). Similar results were recently obtained by Sonnenfeld *et al.* (34, 35) with the induction or passive transfer of IFN-γ (type II, immune). The mechanism(s) by which IFN depresses liver cytochrome P-450 are still unknown although the study of the effects of the administration of IFN inducers on the heme-metabolizing enzymes and the incorporation of radiolabeled glycine and δ-aminolevulinic acid into cytochrome P-450 has led to the hypothesis that an increased degradation of the cytochrome, rather than a decreased synthesis, was involved (7). We have recently reported that administration to mice of various IFN inducers [poly(I-C), tilorone, and bacterial lipopolysaccharide] and 2 different IFN preparations markedly increased the levels of XO in the liver as well as in several other tissues (11). XO is well known for its ability to generate reactive oxygen intermediates, particulary superoxides and hydrogen peroxide, as reduction products of molecular oxygen (9, 23). Reactive oxygen intermediates are known to be implicated in tissue injury, especially through the peroxidation of membrane lipids (5, 6, 10). Since microsomal cytochrome P-450 is particularly sensitive to the peroxidative attack (14, 18), we have hypothesized that an increased generation of reactive oxygen intermediates resulting from the increase of XO activity might play a role in the IFN-mediated depression of liver drug-metabolizing enzymes. To test this hypothesis, we have used as tools a free radical scavenger, NAC, and the specific inhibitor of XO, AP. AP was recently used to investigate the role of XO and superoxides in intestinal ischemia (28). NAC has been used as a tool for studying the role of reactive intermediates in the destruction of liver microsomal cytochrome P-450 by cyclophosphamide (1). It has proved particularly useful because it does not simply act as a free radical scavenger *per se* but also raises the intracellular levels of glutathione by increasing its synthesis through the γ-glutamyl cycle (36).

MATERIALS AND METHODS

Animals and Tissue Preparation. Adult male CD-1 mice (22 to 25 g; Charles River, Italy) were allowed free access to food and water during the course of the experiments. Animals were killed by cervical dislocation 24 h after a single dose of IFN or IFN inducers, except in time course experiments. Livers were removed, rinsed in cold 0.9% NaCl solution (saline), and homogenized with a Potter Teflon homogenizer in 4 volumes of 0.05 M sodium phosphate buffer, pH 7.4. Microsomes were prepared according to the method of Kato and Takayanaghi (17) and resuspended in 0.05 M phosphate buffer, pH 7.4. Serum was prepared from blood samples taken by cardiac puncture under light ether anesthesia.

Biochemical Assays. Protein concentration (21), cytochrome P-450 (26), and ethoxycoumarin *O*-deethylase (15) were determined as described previously. Non-heme (trichloroacetic acid-soluble) iron was measured with a commercially available kit (Boehringer, Mannheim, Germany).

XO activity was measured on the cytosolic fraction. The supernatant was dialyzed overnight against a large excess of buffer at 4°C and assayed for XO activity. Briefly, 10 μl of liver homogenate, diluted 1:50 with saline, were incubated for 10 min at 37°C with 5 μl of 0.2 M Tris-Cl (pH 7.8) and 5 μl of [8-^{14}C]hypoxanthine (53 mCi/mmol, diluted to 9 μCi/

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[2] Training fellow of C.N.R. (National Research Council). To whom requests for reprints should be addressed.

[3] The abbreviations used are: IFN, interferon; AP, allopurinol; NAC, *N*-acetylcysteine; poly(I-C), polyinosinic-polycytidylic acid; SOD, superoxide dismutase; XO, xanthine oxidase.

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ml; Amersham). The reaction was stopped by the addition of 10 μl of 1 M perchloric acid, and the mixture was centrifuged. Five μl of supernatant were applied onto thin-layer chromatography-cellulose plastic sheets (Polygram; Macherey and Nagel) along with 2.5 μl of a carrier solution of hypoxanthine, xanthine, and uric acid (10^{-2} M each). Thin-layer chromatography was developed with butanol:methanol:water:25% NH_4OH (60:20:20:1), purines were located under UV, and the radioactivity of the spots was measured by liquid scintillation counting in 10 ml of Filter Count (Packard). XO activity was expressed as nmol of hypoxanthine oxidized to xanthine + uric acid/min/g liver.

Animal Treatments. Poly(I-C) (sodium salt; Sigma) was given i.p. at a dose of 2 or 10 mg/kg. LPS from *Escherichia coli*, strain 055B5 (Difco), was given i.p. at the dose of 10 mg/kg. All compounds were given in 0.2 ml of sterile, pyrogen-free saline. Tilorone (Richardson Merrel, Cincinnati, OH) was given p.o. in 0.2 ml of water at a dose of 75 mg/kg. Purified mouse IFN-α,β (10^7 units/mg protein) was a kind gift from Dr. M. Chirigos (National Cancer Institute, Frederick, MD). IFN, in McCoy's medium with 2% fetal calf serum, was given i.p. at a dose of 1.5×10^4 or 5×10^4 units/mouse, appropriately diluted with saline. Controls received medium alone diluted the same way and also containing 2% fetal calf serum. AP (Welcome, Pomezia, Rome, Italy) was given p.o. 1 h before and 6 h after poly(I-C) at a dose of 50 mg/kg in 0.2 ml of water. Controls received water alone. NAC (Zambon S.p.A., Bresso, Milan, Italy), was given in drinking water as a 2.5% solution, neutralized with 1 N sodium hydroxide, for 2 days starting 1 day before treatment with IFN or poly(I-C). A fresh solution of NAC was prepared every day to minimize the risk of oxidation.

IFN Titration. Mixture of mouse fibroblast (β) and leukocyte (α) IFN, kindly provided by Dr. De Mayer, Paris, France, was elicited with Newcastle disease virus in monolayer cultures of Swiss mouse C-243 cells as described (22). IFN assays were performed by microtitration of cytopathology of vesicular stomatitis virus on mouse L-929 fibroblasts (20). In order to test the effect of NAC or AP on IFN-induced antiviral resistance, L-929 cells were incubated with different drug concentrations ranging from 1 to 100 mM. After 24 h, 81 units of IFN-α,β were added to the cell monolayers and tested for the capability to induce antiviral resistance.

RESULTS

Effect of IFN on Liver XO and Cytochrome P-450. Table 1 shows that the administration to mice of 3 structurally unrelated IFN inducers [poly(I-C), lipopolysaccharide, and tilorone] and of a partially purified mouse IFN-α,β at the doses tested effectively induced liver XO and depressed the liver microsomal cytochrome P-450.

Denaturation of Cytochrome P-450 by XO *in Vitro*. The ability of purified bovine milk XO to denature cytochrome P-450 *in vitro* in mouse liver microsomes is shown in Table 2. The reversal of this effect by SOD (1000 units/ml) demonstrates that the *in vitro* denaturation of cytochrome P-450 is due to the generation of superoxides by XO. SOD denatured by boiling was not effective in reversing this effect (data not shown). NAC (10 mM) had a protective effect comparable to that obtained with SOD, thus confirming the ability of this free radical scavenger to protect cytochrome P-450 against reactive metabolite-induced damage. SOD and NAC alone had no effect on cytochrome P-450.

***In Vivo* Effect of NAC and AP on the IFN-mediated Depression of Liver Cytochrome P-450.** To investigate the effect of NAC on the depression of liver P-450 levels after a single i.p. dose of poly(I-C), we have used a submaximal dose of poly(I-C) (2 mg/kg). In 5 different experiments with this dose, hepatic cytochrome P-450 levels were reduced by 29, 37, 32, 32, and 52%. Administration of NAC p.o. provided consistent protection against the depression of liver P-450 levels by poly(I-C) without reversing the induction of XO (Table 3). NAC *per se* only slightly affected basal cytochrome P-450 levels. The protective effect of NAC was observed not only on microsomal cytochrome P-450 content but also when ethoxycoumarin *O*-deethylase activity was measured in whole-liver homogenate.

Table 1

XO and cytochrome P-450 in mouse liver 24 h after administration of IFN or IFN inducers

Treatment	XO (nmol/min/g liver)	P-450 (nmol/mg protein)
Control (saline i.p.)	18.0 ± 4.0[a]	1.04 ± 0.05
Poly(I-C) (10 mg/kg i.p.)	100.0 ± 26.5[b]	0.57 ± 0.04[c]
Lipopolysaccharide (10 mg/kg i.p.)	116.0 ± 24.5[b]	0.49 ± 0.04[c]
IFN-α,β (50,000 units/mouse i.p.)	67.5 ± 12.7[b]	0.54 ± 0.03[c]
Control (water p.o.)	11.7 ± 1.7	1.38 ± 0.13
Tilorone (75 mg/kg p.o.)	83.3 ± 4.4[c]	0.92 ± 0.09[b]

[a] Mean ± SE from experiments with at least 4 animals/group.
[b] $P < 0.05$ *versus* control by Dunnett's test.
[c] $P < 0.01$ *versus* control by Dunnett's test.

Table 2

Effect of SOD and NAC on XO-induced cytochrome P-450 in mouse liver microsomes in vitro

Microsomes (5 mg protein/ml) were incubated for 30 min at 37°C in 0.05 M phosphate buffer containing 0.01 M hypoxanthine. Additions were: XO, 0.2 unit/ml; SOD, 1000 units/ml; NAC, 0.01 M.

Addition	Absorbance (450–480 nm)	Cytochrome P-450 (% of control)
None	0.129 ± 0.001[a]	100 ± 1
XO	0.076 ± 0.013	59 ± 10[b]
XO + SOD	0.128 ± 0.014	99 ± 11
XO + NAC	0.117 ± 0.002	90 ± 2

[a] Mean ± SE.
[b] $P < 0.01$ *versus* control (no addition) by Dunnett's test.

Table 3

Effect of NAC on the depression of liver cytochrome P-450 and ethoxycoumarin O-deethylase and the induction of XO by poly(I-C)

Treatment[a]	Cytochrome P-450 (nmol/mg microsomal protein)	XO (nmol/min/g liver)	Ethoxycoumarin *O*-deethylase (nmol/min/g liver)
Saline	0.93 ± 0.06[b] (100)[c]	13.1 ± 1.4	6.8 ± 0.4
Poly(I-C)	0.60 ± 0.04[d] (64)	25.6 ± 2.2	4.7 ± 0.4
Saline + NAC	0.86 ± 0.08 (92)	11.3 ± 0.4	6.9 ± 0.3
Poly(I-C) + NAC	0.81 ± 0.08 (87)	27.2 ± 2.1	6.3 ± 0.5

[a] Poly(I-C) was given i.p. at the dose of 2 mg/kg, with or without NAC (2.5% solution in drinking water).
[b] Mean ± SE obtained from 3 separate experiments with at least 4 animals/group.
[c] Numbers in parentheses, percentage of untreated controls.
[d] Significantly different ($P < 0.01$) from untreated controls ($P < 0.01$) and from poly(I-C) + NAC ($P < 0.05$) by Duncan's test.

Table 4

Effect of NAC on the depression of liver cytochrome P-450 and the induction of XO by IFN-α,β

Treatment[a]	Cytochrome P-450 (nmol/mg microsomal protein)	XO (nmol/min/g liver)
Vehicle	0.91 ± 0.11[b] (100)[c]	8.3 ± 0.7
IFN	0.67 ± 0.07[d] (74)	15.3 ± 2.7
Vehicle + NAC	0.85 ± 0.11 (93)	12.7 ± 2.1
IFN + NAC	0.94 ± 0.21 (103)	24.1 ± 3.1

[a] IFN was given i.p. at the dose of 15,000 units/mouse, with or without NAC (2.5% solution in drinking water).
[b] Mean ± SE of experiment with 4 animals/group.
[c] Numbers in parentheses, percentage of untreated controls.
[d] $P < 0.05$ *versus* untreated controls by Duncan's test.

Table 5

Effect of AP on the depression of liver cytochrome P-450 and the induction of XO by poly(I-C)

Treatment[a]	Cytochrome P-450 (nmol/mg microsomal protein)	XO (nmol/min/g liver)
Saline	0.87 ± 0.09[b] (100)[c]	23.0 ± 3.8
Poly(I-C)	0.41 ± 0.04 (48)[d]	42.0 ± 12.7
Saline + AP	0.62 ± 0.07 (72)	6.2 ± 2.4
Poly(I-C) + AP	0.63 ± 0.13 (73)	4.8 ± 2.1

[a] Poly(I-C) was given i.p. at a dose of 2 mg/kg with or without AP [50 mg/kg p.o., 1 h before and 6 h after poly(I-C)].
[b] Mean ± SE of experiment with 5 animals/group.
[c] Numbers in parentheses, percentage of untreated controls.
[d] $P < 0.01$ *versus* untreated controls by Duncan's test.

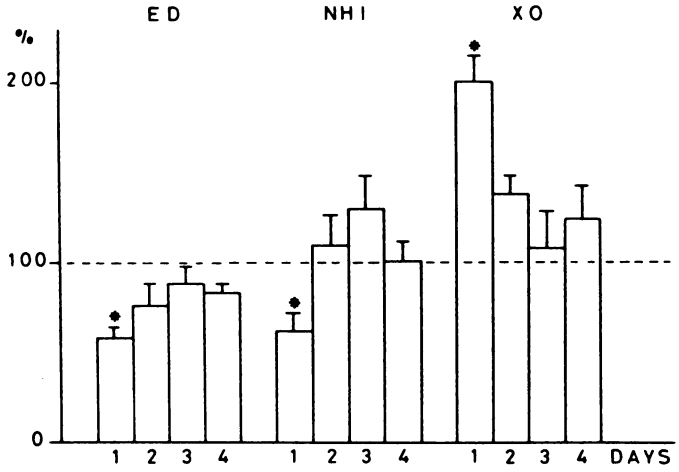

Chart 1. Ethoxycoumarin *O*-deethylase (*ED*) activity, XO activity, and non-heme iron (*NHI*) levels in liver homogenate of mice 1, 2, 3, and 4 days after a single dose of poly(I-C) (10 mg/kg i.p.). Data are given as percentage of control values (control values were: ethoxycoumarin *O*-deethylase, 5.5 ± 0.4 nmol/min/g tissue; XO, 78.2 ± 7.5 nmol/min × g tissue; non-heme iron, 66 ± 10 μg/g tissue). *Bars*, SE. *, $P < 0.05$ *versus* control.

Table 6

Effect of NAC on the depression of liver non-heme iron content by poly (I-C)

Treatment[a]	Non-heme iron (μg/g liver)
Saline	83 ± 4[b] (100)[c]
Poly(I-C)	69 ± 4[d] (83)
Saline + NAC	72 ± 4 (86)
Poly(I-C) + NAC	55 ± 1[d] (66)

[a] Experimental design as in Table 3.
[b] Mean ± SE obtained from 2 separate experiments with 4 animals/group.
[c] Numbers in parentheses, percentage of untreated controls.
[d] Significantly different ($P < 0.01$) from respective control by Duncan's test.

NAC also protected against the decrease of cytochrome P-450 levels caused by 1.5×10^4 units of partially purified IFN per mouse (Table 4). In the same experiment, NAC did not reverse the induction of XO by IFN. Table 5 shows the results of an experiment with AP. Although in the reported experiment AP completely protected against the depression of cytochrome P-450 by poly(I-C), in other cases the protection was only partial. In all cases, AP *per se* significantly depressed cytochrome P-450 levels. As shown in Table 5, this dose resulted in a complete inhibition of XO activity.

Effect of Poly(I-C) on Liver Non-Heme Iron Content. Chart 1 shows that administration of poly(I-C) to mice caused a decrease in liver non-heme iron content concomitantly with the depression of ethoxycoumarin deethylase and the induction of XO. As shown in Table 6, the depression of liver non-heme iron by poly(I-C) was not reversed by NAC, by the same treatment schedule that gave effective protection against the reduction of liver cytochrome P-450.

Effect of NAC and AP on the Induction of IFN by Poly(I-C) and the *in Vitro* Antiviral Activity of IFN. IFN titer was measured in serum of mice treated with poly(I-C) and NAC or AP with the same schedule used for studies on cytochrome P-450.

Blood was taken 24 h after poly(I-C) treatment, since i.p. administration of poly(I-C) was reported to result in a long-lasting IFN induction, by contrast with the i.v. route of administration (4). AP and NAC did not affect the induction of IFN by poly(I-C) (data not shown).

The possibility that NAC or AP might affect the *in vitro* antiviral activity of IFN was also investigated. Addition of NAC or AP at concentration as high as 0.1 M did not impair the IFN-induced antiviral resistance in L-929 cells (data not shown).

DISCUSSION

The protective effect of NAC on the IFN and poly(I-C)-mediated depression of liver cytochrome P-450 reported here (Tables 3 and 4) supports the hypothesis that an increased generation of reactive oxygen intermediates, possibly resulting from the induction of XO by IFN, is responsible for this "toxic" effect of IFN and IFN-inducers. The ability of NAC to prevent the destruction of cytochrome P-450 by reactive oxygen was confirmed *in vitro* with mouse liver microsomes, utilizing the hypoxanthine:XO superoxide generating system (Table 2). In aggrement with this hypothesis, AP also reversed the depression of cytochrome P-450 after poly(I-C), although these data were blurred by the fact that AP *per se* markedly reduced liver cytochrome P-450 levels when administered at the dose of 2 × 50 mg/kg, required for complete inhibition of XO.

The depressant effect of poly(I-C) on liver non-heme iron reported here confirms the findings of Sonnenfeld *et al*. (34) with IFN-γ. The induction of XO might provide an explanation for the effect of IFN on liver iron. In fact, XO (particularly the dehydrogenase form of this enzyme) has been proposed as participating in the mobilization of iron, and its inhibition was reported to result in an increase in the non-heme iron content of the liver (37).

Since NAC did not prevent this effect of poly(I-C), it seems clear that the effect of poly(I-C) on cytochrome P-450 is not a direct consequence of the lowering of non-heme iron levels. It should be noted that NAC *per se* lowered liver non-heme iron, possibly through its iron-chelating activity. Our data are in agreement with those of El-Azhary *et al*. (7), suggesting that the IFN-mediated depression of cytochrome P-450 levels proceeds through increased degradation rather than inhibition of its synthesis.

Since NAC is widely used in humans as a mucolytic agent and its very low toxicity permits the use even of high doses (32), our data suggest a possible use of this compound as a chemoprotective agent against some of the toxic effects of IFN.

Oxidative damage has been proposed as mediating Adriamycin cardiotoxicity (24), paraquat-induced pulmonary damage (8), carbon tetrachloride-induced hepatotoxicity including depression of liver cytochrome P-450 (38) and, more obviously, oxygen toxicity (3). It is therefore conceivable that free oxygen radicals, and possibly XO, might mediate not only the effect of IFN on cytochrome P-450 but also other toxic effects of IFN, including

toxicity in newborn mice (16) and cardiotoxicity (25). In fact, experiments with animals fed a vitamin E-deficient diet have raised the possibility that free radicals have a role in endotoxicosis (31), and endotoxin, like all the other IFN inducers tested, proved effective both in inducing XO and in depressing cytochrome P-450 (Table 1).

In conclusion, we have suggested that IFN and its inducers destroy liver cytochrome P-450 via the generation of free radicals, while the hypothesis that other toxic effects of IFN may also be due to an exacerbation of oxygen toxicity requires further investigation.

Depression of liver cytochrome P-450 has been proposed as a property common to most immunostimulating agents (13, 29, 33); Parkinson *et al*. (27), testing different IFN hybrids produced by recombinant techniques, showed that the ability of IFN preparations to depress cytochrome P-450 parallels their effectiveness as antiviral agents, *i.e.*, that this "toxic" effect cannot be dissociated from the pharmacological activity, at least with the IFN species tested. In this respect, the use of a free radical scavenger such as NAC might prove useful for dissociating such effects *in vivo* and, if confirmed in other experimental models (*e.g.*, cardiotoxicity, toxicity in newborns, antiviral and antitumor activity *in vivo*), then an improvement in the therapeutic index of IFN may well be within reach. In addition, these data suggest a possible use of free radical scavenger not only in cases in which IFN is administered directly (*i.e.*, exogenously) but also when an alteration of hepatic drug metabolism is observed in pathological conditions (*i.e.*, viral infection and administration of vaccines) associated with IFN-induction (2, 19, 30).

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