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SPIN TRAPPING OF FREE RADICAL INTERMEDIATES PRODUCED DURING THE METABOLISM OF ISONIAZID AND IPRONIAZID IN ISOLATED HEPATOCYTES

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Abstract—By the use of spin trapping agents phenyl-*t*-butyl nitrone (PBN) and 4-pyridyl-1-oxide-*t*-butyl nitrone (4-POBN) free radical species were detected in isolated hepatocytes incubated with either isoniazid, iproniazid and their respective metabolites acetyl-hydrazine and isopropyl-hydrazine. The addition of bis-nitrophenyl phosphate, an inhibitor of the acylamidase enzymes, to isolated hepatocytes decreased the free radical activation of isoniazid and iproniazid, but not that of acetyl- and isopropyl-hydrazine, confirming that the radical species were originating from the biotransformation of these latter compounds. The ESR spectra were ascribed to the trapping of, respectively, acetyl and isopropyl free radicals on the basis of the analogies of the spectral feature with those of chemically-prepared spin adducts. Comparable ESR spectra were also observed during the metabolism of acetyl- and isopropyl-hydrazines by liver microsomes and their formation was inhibited by the omission of $NADP^+$, anaerobic incubation and enzyme denaturation. In the microsomal preparations inhibitors of the mixed function oxidase system decreased to various extents the free radical formation and a similar effect was also observed following the destruction of cytochrome P-450 induced by pretreating the rats with $CoCl_2$. The addition of reduced glutathione also decreased the radical trapping indicating that GSH can effectively compete with the spin traps for the reaction with the free radicals. The incubation of isolated hepatocytes with isoniazid or acetyl-hydrazine reduced by 20–25% the intracellular GSH content, while a 50% decrease in GSH was present in the cells exposed to iproniazid and isopropyl-hydrazine. In the same hepatocyte preparations stimulation of lipid peroxidation and leakage of LDH were also observed during cell incubation with iproniazid and isopropyl-hydrazine, but not with isoniazid or acetyl-hydrazine and the extent of both phenomena correlated with the susceptibility of the above compounds to the free radical activation.

Isonicotinic acid hydrazide (isoniazid) and isonicotinic acid 2-isopropyl-hydrazide (iproniazid) are structurally related compounds which have found applications in human therapy.

Isoniazid, one of the most active agents against Bacillus Tuberculosis is still widely used both for the treatment and the prophylaxis of tuberculosis. Iproniazid has found application in the treatment of depressive syndromes, but it has been removed from clinical usage because of its toxicity [1]. Both drugs have been reported to cause hepatocellular damage morphologically as well as biochemically similar to viral hepatitis [1].

Metabolic studies have revealed that the toxic effects are consequences of activation processes and that the two compounds are metabolized in the liver through essentially the same pathway [2]. Acetylation is the initial step in isoniazid biotransformation. Acetyl-isoniazid, likewise iproniazid, are then hydrolyzed by arylamidase enzymes with the release of isonicotinic acid and of, respectively, acetyl- or isopropyl-hydrazine [2–4]. These latter compounds are considered as the precursors of the reactive intermediates and display toxic effects comparable to those of the parent drugs [2–4].

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The oxidation of both acetyl- and isopropyl-hydrazine by the microsomal monoxygenase system has been shown to produce electrophilic metabolites able to alkylate liver proteins and to interact with thiols [5]. The adducts produced with cysteine and glutathione have been characterized, revealing that acetyl and isopropyl groups are the alkylating species [5–7].

The possibility that free radicals might be produced from alkyl-hydrazines was first suggested by Prough *et al.* [8] to explain the formation of hydrocarbon gases observed during the microsomal oxidation of these compounds.

More recently, research performed by the use of spin trapping techniques have demonstrated the production of free radical intermediates during the microsomal metabolism of alkyl-substituted hydrazines [9–11]. Furthermore, the peroxidase-catalyzed oxidation of isoniazid, iproniazid and hydralazine similarly results in the formation of free radical species [12, 13].

Since the bio-activation of isoniazid and iproniazid in the liver involves a complex metabolic sequence we have investigated the free radical formation from the two drugs by using isolated hepatocytes. Preliminary studies have demonstrated that liver cell suspensions can effectively activate isoniazid and iproniazid and that free radical species are produced in the process [14].

MATERIALS AND METHODS

Male Wistar rats (250–300 g body wt) were supplied by Nossan (Corezzana, Italy) and fed *ad libitum* with a standard laboratory diet, devoid of antioxidants, prepared by Piccioni (Brescia, Italy). Phenobarbital, 0.1% solution (w/v) was included in the drinking water for at least one week before the sacrifice. Cobalt chloride was injected subcutaneously as a solution containing 6 mg/ml in 0.85% saline 72 and 48 hr before microsome preparation.

Isoniazid, acetyl-hydrazine, phenyl-*t*-butyl nitrone (PBN), 4-pyridyl-1-oxide-*t*-butyl nitrone (4-POBN), 1-nitroisothiocyanate (NITC) were purchased from Aldrich-Europe (Bersee, Belgium). Collagenase Type I, Iproniazid, 2-methyl-1,2-di-3-pyridyl-1-propanone (Metyrapone), *p*-chloromercuri benzoate (pCMB) were supplied by Sigma Chemical Co. (St Louis, MO). Diethylaminoethyl-diphenylamino acetate (SKF 525A) was kindly provided by Smith, Kline & French Ltd. (Welwyn Garden City, Herts, U.K.).

Acetyl-isoniazid was prepared according to the method described by Nelson *et al.* [3]. Isopropyl-hydrazine oxalate was synthetized as reported by Gever and Hayes [15]. The purity of both compounds was checked by thin-layer chromatography and NMR spectroscopy.

Isolated hepatocytes were prepared as described in [16] and 2 ml aliquots of the cell suspensions (7.5×10^6 cells/ml) were incubated at 37° in the presence of the various compounds under study and using 25 mM of either PBN or 4-POBN as spin trapping agents [17].

Liver microsomes were prepared as described by Slater and Sawyer [18], except that the livers were perfused with ice-cold saline to remove the blood before homogenization. For the experiments 0.4 ml of the microsomal suspension (approx. 2 mg proteins) were added to 1.5 ml of an incubation mixture containing 83.5 mM KCl, 37.2 Tris–HCl buffer pH 7.4, 2 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.25 mM $NADP^+$, 10 IU of glucose-6-phosphate dehydrogenase and 25 mM of either PBN or 4-POBN.

The microsomes were incubated for 30 min at 37° in 25 ml bottles closed with screw caps. The various compounds were dissolved in water and added to the incubation mixture to make up the final volume of 2 ml. Carbon monoxide was fluxed for 1 min through the microsomal suspension immediately before to add it to the incubation mixture.

The free radical adducts with the spin traps were extracted from isolated hepatocytes and microsomal suspensions by shaking them with 1 ml chloroform/methanol (2:1, v/v) mixture and the chloroform phase, separated by centrifugation, was used for the ESR analysis [17].

A Bruker 200 D/SCR spectrometer fitted with a variable temperature cavity was used. The instrument settings were as follows: microwave power 10 mW; modulation frequency 100 MHz; modulation amplitude 1 G; scanning field 100 G; sample temperature −50°.

Intracellular glutathione (GSH) content was estimated by using the Ellman's reagent as reported in [19]. Stimulation of lipid peroxidation was assayed by measuring malondialdehyde (MDA) formation in the hepatocyte suspensions according to Poli *et al.* [18]. The structural integrity of isolated hepatocytes was evaluated by monitoring lactate dehydrogenase (LDH) release in the incubation medium as previously reported [19]. All above measurements were performed in the same hepatocyte suspensions (5×10^6 cells/ml) incubated with the various hydrazines, but without the spin traps.

Copper-catalyzed oxidation of acetyl- and isopropyl-hydrazines was performed as described in [9, 10] using a 50 mM solution of Na_2CO_3 in water (pH 10) containing 0.01 mM $CuCl_2$, 1 mM of either acetyl- or isopropyl-hydrazine and 50 mM PBN or 4-POBN. The spin adducts were extracted with 1 ml chloroform–methanol mixture as reported for the experiments in biological systems. The same reaction performed in the absence of the hydrazine did not result in any detectable ESR signal indicating that, under the conditions used, there was no oxidation of the spin traps to nitroxide derivatives.

RESULTS

Isolated hepatocytes prepared from phenobarbital-induced rats were incubated 60 min at 37° with

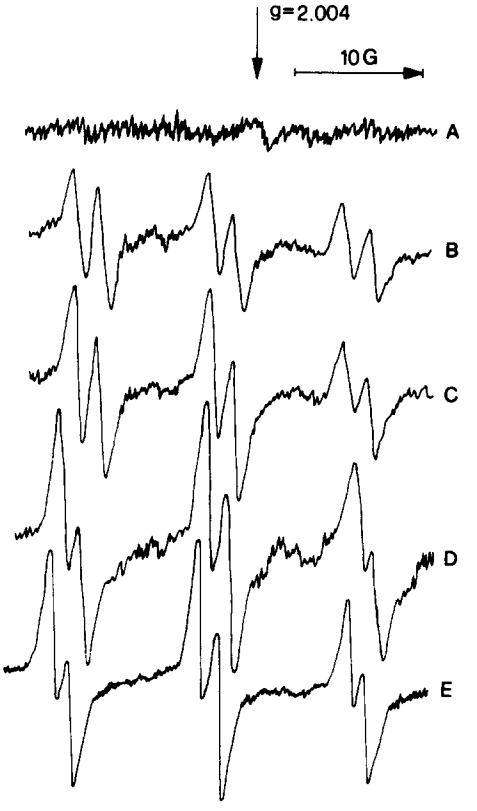

Fig. 1. ESR spectra of the PBN adducts produced in isolated hepatocytes incubated with isoniazid, acetyl-hydrazine, iproniazid and isopropyl-hydrazine. Isolated liver cells were incubated 30 min at 37° with 25 mM PBN alone (trace A) or with PBN and 1 mM of, respectively, isoniazid (trace B), acetyl-hydrazine (traces C) iproniazid (trace D) and isopropyl-hydrazine (trace E). The amplification gain was 10^6 for all traces.

Table 1. Hyperfine splitting constants of the ESR spectra due to the spin adducts produced during biological activation or chemical oxidation of isoniazid, iproniazid and their metaboliltes

Compounds	Hyperfine splitting values of the ESR spectra					
	Isolated hepatocytes		Liver microsomes		Chemical oxidation	
	aN	aH	aN	aH	aN	aH
Isoniazid (PBN)	14.4	2.47	—	—	—	—
Acetyl-isoniazid (PBN)	14.3	2.44	—	—	—	—
Acetyl-hydrazine (PBN)	14.4	2.43	14.3	2.43	14.4	2.53
Iproniazid (PBN)	14.9	2.47	—	—	—	—
Iproniazid (4-POBN)	14.9	1.75	—	—	—	—
Isopropyl-hydrazine (PBN)	14.9	2.49	14.9	2.46	15.1	2.49
Isopropyl-hydrazine (4-POBN)	15.1	1.75	15.1	1.74	14.9	1.73

The values are expressed in Gauss and represent the means of 5–9 different experiments in the case of biological samples and of three experiments in the case of chemical oxidation.

1 mM of either isoniazid and iproniazid and 25 mM PBN. Analysis by ESR spectroscopy of the chloroform extracts revealed the presence of well-detectable ESR spectra (Figs 1b and 1d) showing spectral features consistent with the trapping of carbon-centered free radicals by PBN (Table 1). No appreciable ESR signals were observed when the drugs were omitted from the incubation mixture (Fig. 1a).

The same experiments as above were also performed using either 1 mM acetyl-isoniazid or acetyl-hydrazine, the main metabolites of isoniazid. As shown in Fig. 1 (trace c) and in Table 1 ESR spectra comparable with those produced by isoniazid were observed in hepatocytes receiving the two metabolites, suggesting that essentially the same free radical species was trapped. Likewise, liver cells incubated with 1 mM isopropyl-hydrazine produced PBN-free radical adducts identical to those formed in the presence of the parent drug, iproniazid (Fig. 1e; Table 1).

Liver microsomes similarly activated acetyl-hydrazine and isopropyl-hydrazine giving PBN-free radical adducts with spectral features comparable to those observed in isolated hepatocytes (Table 1). The free radical production required the presence of $NADP^+$, and was completely inhibited by incubation under nitrogen atmosphere or by enzyme denaturation (Table 2).

A recent report by Ortiz de Montellano and coworkers [10] has pointed out that PBN can interfere with the microsomal activation of alkyl-hydrazines by interacting with cytochrome P-450. Thus, we decided to repeat the above experiments replacing PBN with 4-POBN, which, according to these authors, did not affect alkyl hydrazine metabolism.

Indeed, both isolated hepatocytes and liver microsomes incubated with 25 mM 4-POBN and in the presence of 1 mM iproniazid or isopropyl-hydrazine produced ESR spectra almost twenty times more intense than those observed using PBN (Fig. 2b, c). The measurement of the hyperfine splitting constants of the 4-POBN adducts confirmed that the same free radical species was produced from both iproniazid and isopropyl-hydrazine (Table 1).

Table 2. Effects of different treatments on the intensities of the ESR signals produced by acetyl- and isopropyl-hydrazine in liver microsomes

	ESR signal intensity (arbitrary units)	
	Acetyl-hydrazine	Isopropyl-hydrazine
Control microsomes complete medium	16	180
Control microsomes $-NADP^+$	1 (−94%)	20 (−89%)
Control microsomes anaerobic incubation	1 (−94%)	11 (−94%)
Control microsomes + GSH 4 mM	3 (−81%)	40 (−78%)
Heat-inactivated microsomes	3 (−81%)	21 (−88%)
Phenobarbital-pretreated microsomes	40 (+250%)	472 (+260%)
$CoCl_2$-pretreated microsomes	5 (−69%)	78 (−40%)

Rats pretreatments were performed as described in the Method section. Liver microsomes were incubated 30 min at 37° with, respectively, 1 mM acetyl-hydrazine and 25 mM PBN or 1 mM isopropyl-hydrazine and 25 mM 4-POBN.

The results are expressed in arbitrary units and are means of two experiments.

The values in parentheses represent the percentage changes in respect to controls.

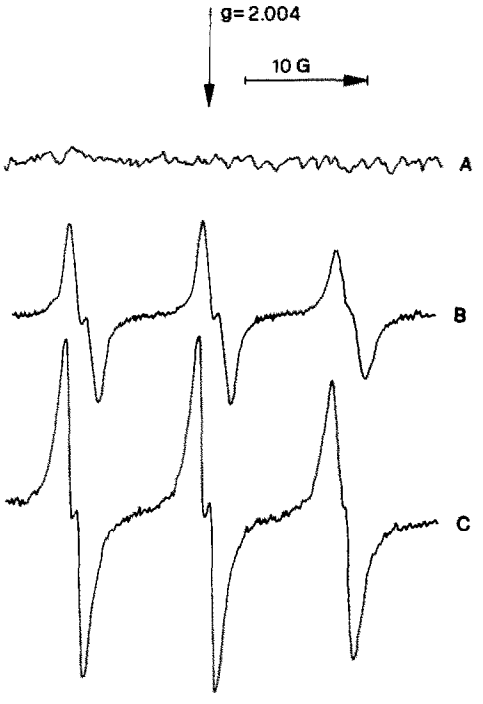

Fig. 2. ESR spectra of the spin adducts formed by 4-POBN with the free radicals originating from iproniazid and isopropyl-hydrazine in isolated hepatocytes. The experimental condition were identical to those reported in Fig. 2, but 25 mM 4-POBN were used instead of PBN. Trace A refers to liver cells incubated with 4-POBN alone, traces B and C to hepatocyte preparations exposed to iproniazid and isopropyl-hydrazine, respectively. The amplification gain was 10^6 for trace A, 10^5 for traces B and C.

Disappointing results were, instead, obtained using 4-POBN for the trapping of isoniazid-derived free radicals. Unresolved ESR spectra were, in fact, obtained following the incubation of microsomes or isolated hepatocytes with this spin trap and in the presence of, respectively, acetyl-hydrazine, isoniazid and acetylisoniazid (not shown). Therefore, PBN was still used for the experiments concerning these latter compounds, while 4-POBN replaced it in the study of other alkyl-hydrazines.

In isolated hepatocytes incubated with isoniazid, iproniazid and their metabolites the free radical production showed a concentration-dependent variation and an almost linear increase in the ESR signal intensities was observed following the cell incubation up to 60 min (Fig. 3). Both acetyl- and isopropyl-hydrazine produced approximately twice as much spin adducts as compared with equimolar amounts of the parent drugs (Fig. 3).

On the contrary, incubation at 37° of the above compounds with hepatocytes which have been lysed by the addition of 0.5% Triton did not result in any detectable ESR signal (not shown), indicating that free radical formation was not due to a spontaneous decomposition of the chemicals and required cellular integrity.

The identification of the free radical adducts obtained in biological systems was accomplished by comparing the hyperfine splitting constants of the spectra with reference values obtained following copper-catalyzed oxidation of acetyl- and isopropylhydrazines in the presence of either PBN or 4-POBN.

The chemical oxidation of isopropyl-hydrazine gave the following values aN = 15.1, aH = 2.49 G and aN = 14.9, aH = 1.75 G for the PBN and 4-POBN adducts, respectively. In both cases, these values were very close to the hyperfine constants produced during bioactivation of iproniazid and isopropyl-hydrazine, suggesting the trapping of isopropyl free radicals (Table 1).

A similar comparison was also possible for the splitting constants of the PBN adduct resulting from the chemical oxidation of acetyl-hydrazine (aN = 14.4, aH = 2.53 G) and the spectral features observed during the metabolism of isoniazid, acetyl-isoniazid and acetyl-hydrazine (Table 1). Thus, the formation of acetyl free radicals was postulated to take place during the activation of isoniazid and its metabolites.

Early investigations have shown that the hydrolysis of the isonicotinic ring is a critical step in the metabolic activation of isoniazid and iproniazid [3, 4] and that bis-nitrophenyl phosphate (BNPP) inhibits this reaction by acting as a competitive substrate for the arylamidase enzyme [4].

Figure 4 shows that in isolated hepatocytes preincubated for 10 min with 2 mM BNPP the free radical formation from isoniazid, acetyl-isoniazid and iproniazid is lowered by 40, 66 and 75%, respectively, while the activation of acetyl- and isopropylhydrazines was not appreciably influenced (Fig. 4).

Experiments performed with liver microsomes obtained from phenobarbital- or $CoCl_2$-pretreated rats showed that phenobarbital stimulation of cytochrome P-450 increased by 2–3-fold the intensities of the spin adducts due to acetyl and isopropyl radicals, whereas the administration of $CoCl_2$ reduced them by 69 and 40%, respectively (Table 2).

The role played by the mixed function oxidase system in the generation of free radical metabolites from hydrazine compounds was further characterized by incubating phenobarbital-induced microsomes with various inhibitors of the monoxygenase system. As shown in Fig. 5, the addition of 1 mM SKF 525A decreased by approximately 30% the intensity of the ESR signals due to acetyl- and isopropyl-hydrazine, while 0.5 mM metyrapone and 0.2 mM 1-naphthyl-isothiocyanate (NITC) appeared to be more effective as inhibitors reducing the free radical formation by 39–53% and 66–73%, respectively.

Carbon monoxide, a "classical" cytochrome P-450 inhibitor displayed a variable activity. It almost completely blocked the formation of spin adducts from acetyl-hydrazine, but decreased by only 23% the free radical activation of isopropyl-hydrazine (Fig. 5).

The interference in the electron supply to cytochrome P-450 caused by 0.1 mM *p*-chloromercuri benzoate (*p*CMB) also lowered by approximately

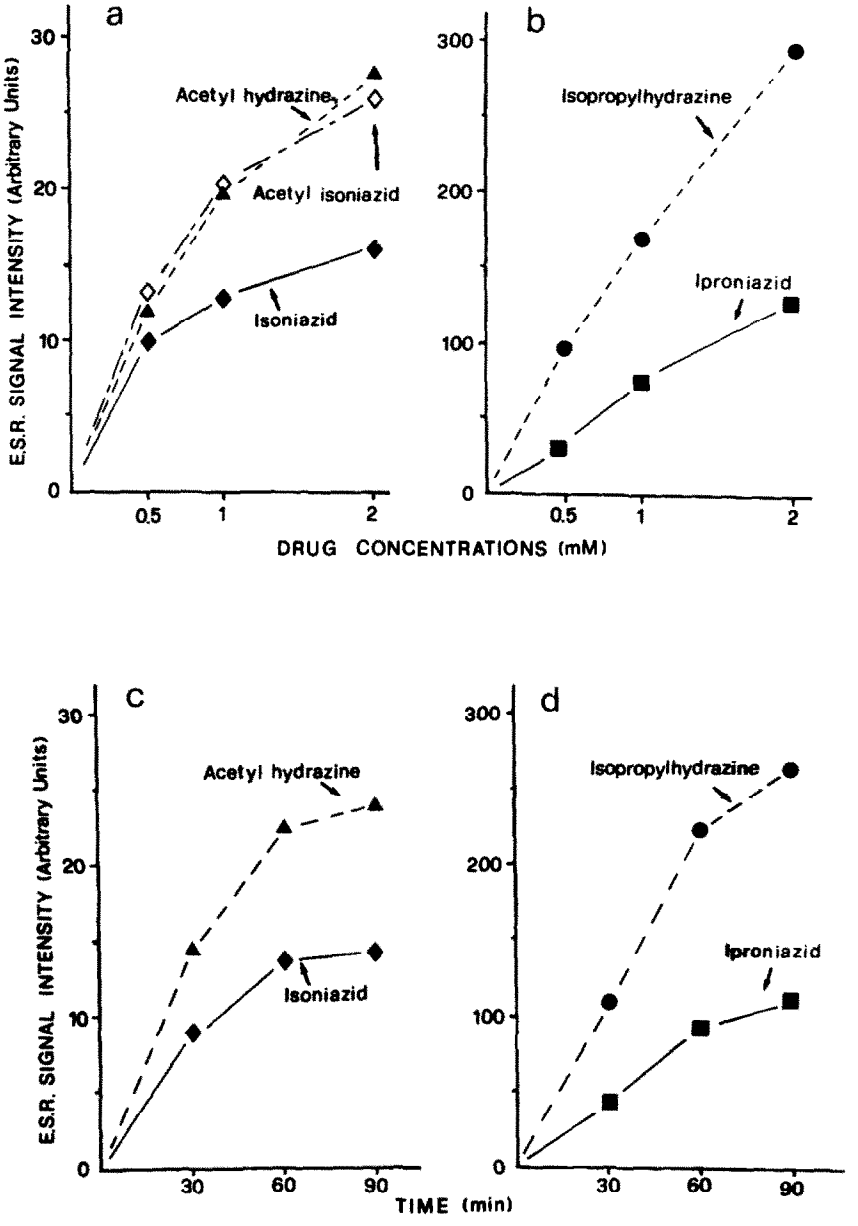

Fig. 3. Time- and concentration-dependent changes in the intensities of the ESR signals produced by isolated hepatocytes exposed to, respectively, isoniazid, acetyl-isoniazid, acetyl-hydrazine, iproniazid and isopropyl-hydrazine. Hepatocyte suspensions were incubated 60 min at 37° with increasing concentrations of the above compounds using PBN (panel a) or 4-POBN (panel b) as spin traps. For time-course experiments liver cell suspensions were incubated up to 90 min at 37° with 1 mM of either isoniazid or acetyl-hydrazine and 25 mM PBN (panel c). 1 mM iproniazid or isopropyl-hydrazine and 25 mM 4-POBN were used for the experiments shown in panel (d). The values are representative of a typical experiment.

40% the intensities of the ESR signals derived from both acetyl- and isopropyl-derivatives (Fig. 5).

The reactive species generated during microsomal activation of alkyl hydrazines are known to readily react with thiols [5, 6, 20]. During spin trapping experiments the addition of physiological concentrations (4 mM) of reduced glutathione (GSH) to liver microsomes decreased by approximately 80% the trapping of either acetyl or isopropyl free radicals (Table 2). This effect was not due to an unspecific reduction of the nitroxide adducts by GSH since the ESR signals were not affected by adding GSH at the end of the incubation period (not shown).

Consistently, isolated hepatocytes incubated with either 2 mM isoniazid or iproniazid showed a time-dependent depletion of GSH (Fig. 6a). A similar effect was also induced by their respective metabolites acetyl- and isopropyl-hydrazine. Following a

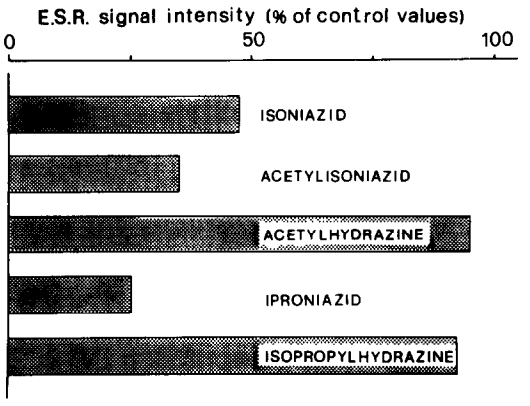

Fig. 4. Effect or acylamidase inhibitor bis-n-nitrophenyl phosphate (BNPP) on the intensities of the ESR signals produced in isolated hepatocytes by isoniazid, iproniazid and their respective metabolites. Isolated liver cells were preincubated 10 min at 37° with 2 mM BNPP before the addition of 1 mM of each hydrazine compound and then further incubated for 30 min. The results are expressed as per cent of the ESR signal intensities obtained in control cell incubated without BNPP. The values are means of two different experiments.

4-hr incubation the cellular content of GSH was lowered by about 25–30% of the control values in the hepatocytes receiving isoniazid and acetyl-hydrazine, while iproniazid and isopropyl-hydrazine decreased it by approximately 50% (Fig. 6a).

The stimulation of lipid peroxidation as a consequence of the free radical production was investigated by monitoring malondialdehyde (MDA) accumulation in the same hepatocyte preparations. As reported in Fig. 6(b), an appreciable increase of lipid peroxidation was present only in the cells exposed to iproniazid and isopropyl-hydrazine, but not in those receiving isoniazid and acetyl-hydrazine. On a molar basis, isopropyl-hydrazine was almost twice as active as iproniazid in stimulating lipid peroxidation, consistently with a higher rate of free radical production observed with the former compound.

Hepatocyte integrity, evaluated by LDH leakage, was not modified in the cells incubated for up to 4 hr with either isoniazid or acetyl-hydrazine (Fig. 6c). On the contrary, isolated hepatocytes exposed to iproniazid and isopropyl-hydrazine showed an appreciable increase in LDH release (Fig. 6c) during the same incubation period.

DISCUSSION

The formation of free radical intermediates during the biotransformation of isoniazid and iproniazid has been demonstrated by the use of the spin trapping technique coupled with ESR spectroscopy. Both liver microsomes and isolated hepatocytes are able to generate free radical species, but the latter have the advantage over the subcellular organelles in being able to activate not only the hydrazine compounds isopropyl- and acetyl-hydrazines, but also their more complex parent drugs.

The trapping of isopropyl free radicals from both iproniazid and isopropyl-hydrazine is supported by several pieces of indirect evidence. Evolution of isopropane has been, in fact, reported to occur concomitantly with the covalent binding of isopropyl residues in microsomes incubated with isopropyl-hydrazine [3]. Moreover, the isopropyl adducts of cysteine and glutathione have been identified by means of mass spectroscopy in microsomal incubations containing isopropyl-hydrazine and the two thiols [7]. More recently, Sinha [12] has detected spin adducts, interpreted as due to isopropyl free radicals, during horse-radish peroxidase and prostaglandin synthetase catalyzed oxidation of iproniazid in the presence of the spin traps DMPO.

The identification of the spin adducts observed during the activation of isoniazid, acetyl-isoniazid

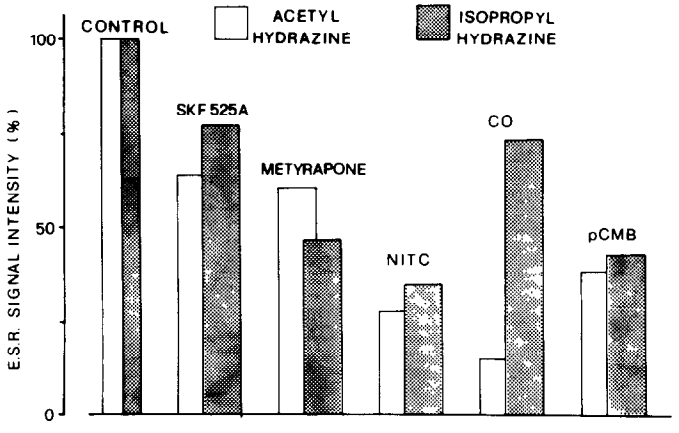

Fig. 5. Effects of various inhibitors of the microsomal mixed function oxidase system on the free radical activation of either acetyl- or isopropyl-hydrazine. Liver microsomes were incubated for 30 min at 37° with, respectively, 1 mM acetyl-hydrazine and 25 mM PBN or 1 mM isopropyl-hydrazine and 25 mM 4-POBN and the following concentrations of inhibitors: SKF 525A 1 mM, metyrapone 0.5 mM, 1-nitroisothiocyanate (NITC) 0.1 mM, *p*-chloromercuribenzoate (*p*CMB) 0.1 mM. The results are means of three different experiments and are expressed as percent of the ESR signal intensities obtained in reference samples incubated without inhibitors.

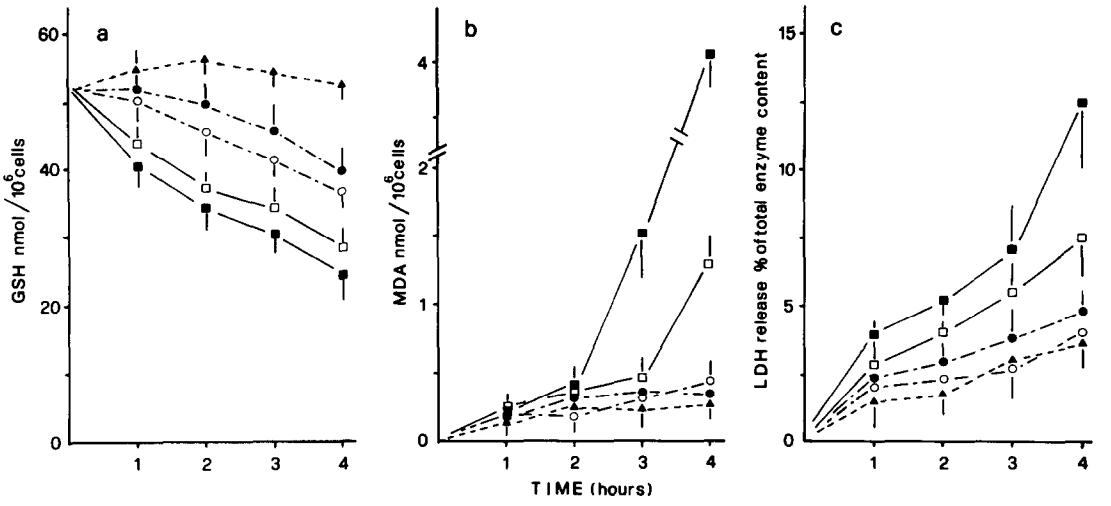

Fig. 6. Time-courses of the depletion of intracellular glutathione (panel a), stimulation of lipid peroxidation (panel b) and appearance of cell damage, as measured by LDH release (panel c) in isolated hepatocytes exposed to isoniazid, iproniazid and their metabolites. For these experiments liver cells (5×10^6/ml) were incubated in 100 ml Erlenmeyer flasks for up to 4 hr at 37° without any additions (▲) or with 2 mM of either isoniazid (○), acetylhydrazine (●), iproniazid (□) or isopropyl-hydrazine (■). The above parameters were determined for each treatment in the same cell suspensions. The results are means of three different experiments ± SD.

and acetyl-hydrazine is not as circumstantial as that of iproniazid.

The hyperfine splitting constants that we have observed are similar to those reported by Augusto *et al.* [9] for the PBN adduct produced following biological or chemical oxidation of acetyl-hydrazine.

Nonetheless, as previously noticed by the same authors these values are not consistent with the spectral features (aN = 14.16, aH = 3.1 G) attributed to the acetyl-PBN adduct [20].

It must be noticed, however, that experiments using liver microsomes incubated with [^{14}C]carbonyl-[^{3}H]-methyl-acetyl hydrazine have shown the covalent binding of the whole acetyl group to microsomal proteins [5]. Moreover *N*-acetylcysteine and *S*-acetyl glutathione have been identified by mass spectroscopy as the reaction products between, respectively, cysteine and glutathione and the reactive metabolite produced during microsomal oxidation of acetyl-hydrazine [6, 7].

This latter finding is particularly significant since a form of spin trapping, using thiols instead of nitrones, was employed for the identification of the reactive species.

Thus, the available evidence can be taken as sufficient to make a preliminary assignment of the PBN adducts resulting from the metabolic activation of isoniazid and its metabolites as due to the acetyl free radical.

Spin trapping experiments have substantially confirmed previous investigations concerning the metabolic pathways responsible for the activation of isoniazid and iproniazid [2–4]. The inhibition given by BNPP on acylamidase–catalyzed hydrolysis of these drugs [4], decreased the free radical formation as well as the protein alkylation and the toxic effects induced by acetylisoniazid and iproniazid [2–4]. On the contrary, BNPP does not influence the trapping of both acetyl and isopropyl free radicals, neither their covalent binding to hepatic proteins [3, 4].

Nelson's group has established that both rat and human microsomes are capable of generating alkylating metabolites from either acetyl- or isopropyl-hydrazine [5].

Phenobarbital pretreatment of rats increases the rate of free radical formation, while the administration of $CoCl_2$ significantly lowers it and such effects are consistent with the changes induced by the same treatments on the covalent binding of the reactive species [3, 4].

The involvement of the cytochrome P-450-dependent monoxygenase system in the activation process is suggested by the observation that monosubstituted hydrazines and hydrazides interact with cytochrome P-450 giving a type II binding spectrum and cause destruction of the haemoprotein [21]. In our hands, inhibitors of cytochrome P-450 systems, such as SKF 525A, metyrapone and carbon monoxide, depress to various extents the free radical generation. Likewise, 1-nitro-isothiocyanate effectively inhibits the production of radical species from acetyl- and isopropyl-hydrazines, in accordance with the protective effects displayed against the hepatotoxicity of these compounds [2].

Reduced glutathione effectively interacts with the free radical metabolites of acetyl- and isopropyl-hydrazine and decreased both the rates of spin trapping and their covalent binding to microsomal proteins [5]. Two types of reactions are possible between GSH and free radical metabolites: i.e. hydrogen subtraction and thiol conjugation, as recently demonstrated [22]. As a result of these reactions, GSH depletion was observed in isolated hepatocytes incubated with isoniazid, iproniazid and their hydrazine metabolites.

Stimulation of lipid peroxidation as indicated by

malondialdehyde (MDA) accumulation is evident in isolated hepatocytes incubated with iproniazid and isopropyl-hydrazine, but not in those receiving isoniazid and acetyl-hydrazine. This discrepancy could be explained by the differences in the rate of free radical production between the two classes of compounds.

The experiments performed with isolated hepatocytes have also shown that LDH leakage is occurring concomitantly with MDA accumulation. However, these results alone are not sufficient to support a cause–effect relationship.

Isolated hepatocytes, however, failed to show any appreciable cell damage when incubated with isoniazid and its metabolites, most likely because the observation time was not sufficient for the development of massive cell damage.

In conclusion, the data obtained demonstrate that isoniazid and iproniazid are metabolically activated with the formation of free radical species able to interact with GSH and to stimulate lipid peroxidation. It is possible that these effects, together with the alkylation of cellular macromolecules induced by the same free radicals, might be the initial events responsible for the development of liver necrosis.

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REFERENCES

1. H. J. Zimmerman, in *Hepatotoxicity*, p. 487. Appleton-Century-Crofts, New York (1978).
2. J. A. Timbrell, *Drug Metab. Rev.* **10**, 125 (1979).
3. S. D. Nelson, J. R. Mitchell, W. R. Snodgrass and J. A. Timbrell, *J. Pharmac. exp. Ther.* **206**, 574 (1978).
4. J. A. Timbrell, J. R. Mitchell, W. R. Snodgrass and S. D. Nelson, *J. Pharmac. exp. Ther.* **213**, 346 (1980).
5. S. D. Nelson, J. R. Mitchell, J. A. Timbrell, W. R. Snodgrass and G. B. Corcoran, *Nature, Lond.* **193**, 901 (1976).
6. S. D. Nelson, J. A. Hinson and J. R. Mitchell, *Biochem. biophys. Res. Commun.* **69**, 900 (1976).
7. S. D. Nelson, J. R. Mitchell and L. R. Pohl, in *Mass Spectroscopy in Drug Metabolism* (Eds A. Frigerio and E. L. Ghisalberti), p. 237. Plenum Press, London (1977).
8. R. A. Prough, J. A. Wittkop and D. J. Reed, *Archs Biochem. Biophys.* **131**, 369 (1969).
9. O. Augusto, P. R. Ortiz de Montellano and A. Quintanilla, *Biochem. biophys. Res. Commun.* **101**, 1324 (1981).
10. P. R. Ortiz de Montellano, O. Augusto, F. Viola, K. L. Kunze, *J. biol. Chem.* **258**, 8623 (1983).
11. O. Augusto, L. R. Du Plessis and C. L. V. Weingrill, *Biochem. biophys. Res. Commun.* **126**, 853 (1985).
12. B. K. Sinha, *Biochem. Pharmac.* **33**, 2777 (1984).
13. B. Kalyanaraman and B. K. Sinha, *Envir. Health Perspect.* **64**, 179 (1985).
14. E. Albano, A. Tomasi, V. Vannini and M. U. Dianzani, *Biochem. Pharmac.* **34**, 381 (1985).
15. G. Gever and K. Hayes, *J. org. Chem.* **14**, 813 (1949).
16. G. Poli, E. Gravela, E.Albano and M. U. Dianzani, *Exp. molec. Pathol.* **30**, 116 (1979).
17. E. Albano, K. A. K. Lott, T. F. Slater, A. Stier, M. C. R. Symons and A. Tomasi, *Biochem. J.* **204**, 593 (1982).
18. T. F. Slater and B. C. Sawyer, *Biochem. J.* **123**, 815 (1982).
19. E. Albano, G. Poli, E. Chiarpotto, F. Biasi and M. U. Dianzani, *Chem.-Biol. Interact.* **47**, 249 (1983).
20. E.G. Janzen, E. R. Davies and D. E. Nutter, *Tetrahedron Lett.* 3309 (1978).
21. S. J. Moloney, B. J. Snider and R. A. Prough, *Xenobiotica* **14**, 803 (1984).
22. M. E. Spearman, S. J. Moloney and R. A. Prough, *Molec. Pharmac.* **26**, 566 (1984).