Biochemical Pharmacology, Vol. 31, No. 2, pp. 249–251, 1982.
Printed in Great Britain.

0006-2952/82/020249-03 $03.00/0

SHORT COMMUNICATIONS

Reversal of the effects of isoniazid on hepatic cytochrome P-450 by potassium ferricyanide

(*Received* 15 *March* 1981; *accepted* 30 *June* 1981)

It has been demonstrated that isoniazid, a first line drug in the treatment of tuberculosis, inhibits cytochrome P-450-dependent drug oxidation *in vivo* [1, 2] and *in vitro* [1, 3]. In recent studies we investigated the mechanism of this inhibition in rat liver microsomes. Isoniazid was found to decrease carbon monoxide (CO) binding to reduced cytochrome P-450 and to inhibit aniline hydroxylation and aminopyrine *N*-demethylation [4]. Both of these effects were enhanced if the microsomes were incubated with isoniazid in the presence of NADPH and oxygen. Furthermore, incubation of the microsomes with isoniazid in the presence of NADPH and oxygen resulted in a time-dependent spectral change having an absorbance maximum at 449 nm and a broad absorbance at 493 nm. Both isoniazid effects were transient in nature and dependent upon the NADPH concentration to maintain cytochrome P-450 in the ferrous state. These characteristics were different from the more stable cytochrome P-450 metabolic intermediate (MI) complexes previously reported for methylenedioxybenzene derivatives [5, 6] and nitrogenous amines [7–9].

Potassium ferricyanide is the only agent reported which can dissociate the nitrogenous MI cytochrome P-450 complexes [7, 8]. The hypothesis that the decrease in available cytochrome P-450 by isoniazid is due to the formation of a cytochrome P-450 complex, as detected by the appearance of the absorbance maximum at 449 nm and the broad absorbance shoulder at 493 nm, would be greatly strengthened if these changes were reversed by the addition of ferricyanide. Accordingly, we have examined the effects of ferricyanide on each of the isoniazid effects including the decreased CO-binding to cytochrome P-450, the formation of the absorbance at 449 nm, and the *in vitro* inhibition of microsomal *N*-demethylation of aminopyrine.

Materials and methods

Male Sprague–Dawley rats (*ca.* 250 g) were maintained on Purina Lab Chow. All animals received sodium phenobarbital (0.15 g/100 ml) in their drinking water and were allowed to drink *ad lib.* for 5–7 days before being killed. Fresh liver microsomes were prepared as described previously [10] except that EDTA (0.1 mM) was included in the washing medium to minimize endogenous lipid peroxidation produced during incubation of the microsomes with NADPH [11]. Protein concentrations were determined by the method of Lowry *et al.* [12]. All incubations were carried out at 37°. Cytochrome P-450 was measured by difference spectrophotometry according to the method of Omura and Sato [13]. An extinction co-efficient between 450 and 490 nm of 91 $mM^{-1}\,cm^{-1}$ was used to quantitate the carbon monoxide binding to sodium dithionite reduced microsomes. Spectral studies were performed with an Aminco DW-2 spectrophotometer in the split beam mode. Glutathione (1 mM) was added to the microsomal suspension since we found it stabilized cytochrome P-450 levels during prolonged incubations [4]. Microsomal aminopyrine *N*-demethylation was determined by the method of Nash [14], quantitating the amount of formaldehyde produced. Isoniazid and aminopyrine were purchased from the Aldrich Chemical Co., Inc., Milwaukee, WI. Results are expressed as means ± S.E.M. Student's *t*-test was used to calculate the significance of the differences.

Results and discussion

Incubations of rat liver microsomes with isoniazid in the presence of NADPH (220 μM) and oxygen produced a spectral change having an absorption maximum at 449 nm and broad shoulder at 493 nm (Fig. 1A), as well as an absorption minimum at 418 nm (not shown in this figure). The onset of the disappearance of these spectral changes could be delayed when higher NADPH concentrations were used [4]. Thus, the NADPH concentration was increased to 375 μM to maintain the spectral complex and delay the onset of reversal to approximately 15 min. After the formation of the isoniazid spectral species (Fig. 1B), ferricyanide was added to the reaction at 7.5 min. Both the 449 nm peak and the 493 nm shoulder disappeared (Fig. 1B). The absorbance formed below 430 nm was generated by the reduction of ferricyanide, as reported previously by Schenkman *et al.* [7] and Buening and Franklin [8].

The ferricyanide effect supports our contention that the spectral species at 449 nm was produced by the formation of a metabolic intermediate complex (MI) between isoniazid and cytochrome P-450. This spectral response is distinct from both the oxidized binding spectrum and the dithionite reduced spectrum. The interaction between isoniazid and oxidized cytochrome P-450 produced a typical type II difference spectrum, having an absorbance maximum and minimum at 426 nm and 392 nm respectively [4]. If the microsomes were reduced with sodium dithionite, isoniazid interacted with ferrous cytochrome P-450 to produce an absorption maximum in the Soret region at 444 nm, but lacking the shoulder at 493 nm.

Addition of isoniazid to the liver microsomes produced an immediate decrease (*ca.* 20 per cent) in the binding of CO to the reduced cytochrome P-450 [4], by the direct interaction of isoniazid with the ferrous cytochrome. Incubation of the microsomes with NADPH produced an additional decrease in the CO binding (*ca.* 44 per cent). This decrease was transient and dependent upon the presence of oxygen and of NADPH. Thus, the possible participation of the MI complex in the decreased CO binding was investigated.

Treatment of the microsomal suspension by ferricyanide (Fig. 2) in the absence of isoniazid did not alter the binding of CO to cytochrome P-450. However, addition of ferricyanide eliminated the transient decrease in cytochrome P-450 observed when the microsomes were incubated with isoniazid and NADPH. Similarly, the participation of the MI complex was evaluated in the inhibition of drug metabolism.

Microsomes were preincubated with isoniazid and NADPH, washed, and then used to determine aminopyrine *N*-demethylation and the CO binding to cytochrome P-450 (see legend of Table 1). Both reactions were decreased significantly in the isoniazid microsomes as compared to control microsomes (Table 1A). Following ferricyanide addition and washing, the CO binding to cytochrome P-450 returned to control values (Table 1B). Although the

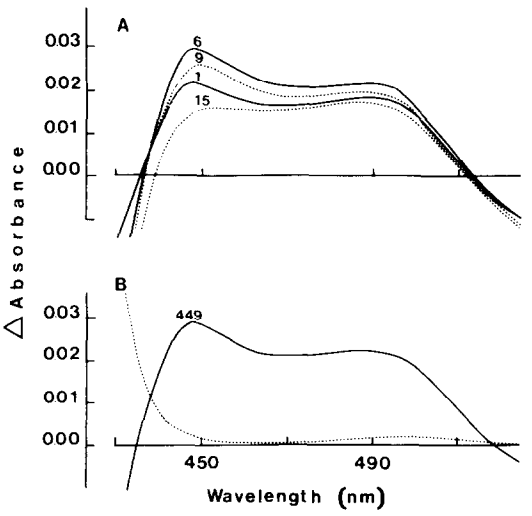

Fig. 1. Formation of a spectral species due to incubation of isoniazid with microsomes in the presence of NADPH and its abolition by ferricyanide. (A) Hepatic microsomes were suspended at 2 mg/ml (3.46 μM cytochrome P-450) in an oxygenated buffer solution, pH 7.4, containing 50 mM Tris–Cl, 150 mM KCl, 10 mM $MgCl_2$ and 1 mM glutathione. The microsomal suspension was reduced with NADH (250 μM) and divided equally into two cuvettes. (This eliminated the influence of reduced cytochrome b_5 on the spectral recording). After establishing a baseline of equal light absorbance, isoniazid (1 mM) and NADPH (220 μM) were added to the sample cuvette. The difference spectrum was then recorded after various periods of time. The figure shows only the spectrum after 1, 6, 9 and 15 min as denoted by numbers. (B) An experiment was performed as described above except that the concentrations of the microsomal protein and NADPH were 1.5 mg/ml and 375 μM respectively. After formation of the 449 nm spectral species (7.5 min, solid curve) ferricyanide (1 mM) was added to the sample and the reference cuvettes. The dotted curve represents the difference spectra obtained after the subsequent addition of ferricyanide.

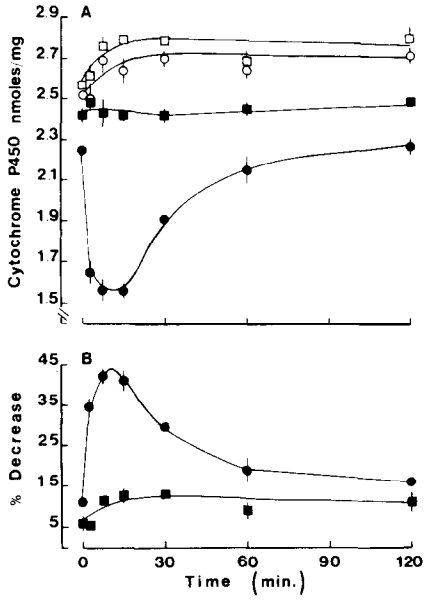

Fig. 2. Reversal of the *in vitro* isoniazid-induced loss of carbon monoxide reactivity of cytochrome P-450 by potassium ferricyanide. Hepatic microsomes (1 mg/ml) were suspended in 50 mM Tris–Cl buffer solution, pH 7.4, containing 150 mM KCl and 10 mM $MgCl_2$ in the presence of NADPH (250 μM) and glutathione (1 mM). The microsomal suspension was then incubated for various periods of time in the absence (open symbols) or in the presence of 1 mM isoniazid (closed symbols). At the end of the incubation period, sodium dithionite was added either immediately or after the addition of ferricyanide (1 mM). The microsomal suspension was then divided between two cuvettes, and carbon monoxide binding to cytochrome P-450 was determined. Circles and squares represent cytochrome P-450 measurements in the absence and in the presence of ferricyanide respectively. Values are means ± S.E.M. of three separate experiments performed in duplicate. Where not shown, the S.E.M. was smaller than the radius of the symbol.

Table 1. Abolition by ferricyanide of the inhibition of aminopyrine *N*-demethylation in isoniazid-pretreated microsomes*

	Aminopyrine *N*-demethylation (nmoles/mg/min)	Cytochrome P-450 (nmoles/mg)
(A) Without ferricyanide		
Control microsomes	20.9 ± 3.6	2.04 ± 0.30
Isoniazid-pretreated microsomes	13.0 ± 1.8†	1.45 ± 0.20†
	(63.5 ± 3.9)	(71.2 ± 3.9)
(B) With ferricyanide		
Control microsomes	15.0 ± 2.9	1.91 ± 0.36
Isoniazid-pretreated microsomes	15.4 ± 3.1	1.81 ± 0.30
	(101 ± 0.5)	(94.8 ± 1.3)

* Microsomes were suspended at 6 mg/ml in the buffer previously described (see legend to Fig. 2) in the presence of NADPH (1.5 mM) and glutathione (1 mM). The microsomal suspension was then incubated for 7.5 min either in the absence (control microsomes) or in the presence of 1 mM isoniazid (isoniazid-pretreated microsomes). At the end of the incubation, potassium ferricyanide (2.7 mM) or buffer was added. The microsomal suspension was transferred to ice and diluted with ice-cold buffer. The microsomes were then centrifuged at 105,000 *g* for 30 min and the supernatant fraction was discarded. The pellets were rinsed three times with buffer and resuspended in fresh buffer at 10 mg/ml. Aminopyrine *N*-demethylation and cytochrome P-450 were determined as described in Materials and Methods. Values are means ± S.E.M. of three separate animals. Numbers in parentheses indicate percentage of the respective controls.

† Significant difference ($P < 0.025$) compared to the appropriate control.

ferricyanide treatment decreased the rate of *N*-demethylation in the control microsomes, the isoniazid inhibition of aminopyrine *N*-demethylation was reversed. The ferricyanide decrease in drug metabolism in control microsomes has been reported previously, but has not been characterized [8].

The results reported here for isoniazid differ from experimental findings reported for phenelzine (phenylethylhydrazine). In the presence of NADPH, phenelzine produced a concomitant decrease in the binding of CO to cytochrome P-450 and in the heme content of the microsomes [15]. The spectral characteristics of the isoniazid complex differ from those formed by the alkyl hydrazine derivatives reported by Hines and Prough [16]. Thus, the various hydrazines and hydrazides can interact differently with the cytochrome P-450 system.

In summary, ferricyanide reversal of the isoniazid inhibition of microsomal activity strongly suggests that isoniazid forms a metabolic intermediate (absorption maximum at 449 nm) in the presence of NADPH and oxygen which binds to cytochrome P-450. As with other metabolic intermediate complexes, a decrease in the functionally available cytochrome P-450 results and the rate of drug metabolism can, in turn, be diminished due to the decreased terminal oxidase.

Acknowledgement—The authors would like to acknowledge the generous support of Dr. Samual P. Bessman.

Department of Pharmacology and Nutrition
University of Southern California School of Medicine
Los Angeles, CA 90033
U.S.A.

SAMAR F. MUAKKASSAH
WAYNE R. BIDLACK*
WILLIAM C. T. YANG

* To whom correspondence should be addressed.

REFERENCES

1. H. Kutt, R. Brennan, H. Dehejia and K. Verebeley, *Am. Rev. resp. Dis.* **101**, 377 (1970).
2. G. Sutton and J. Kupferberg, *Neurology* **25**, 1179 (1975).
3. H. Kutt, K. Verebeley and F. McDowell, *Neurology* **18**, 706 (1968).
4. S. F. Muakkassah, W. R. Bidlack and W. C. T. Yang, *Biochem. Pharmac.* **30**, 1651 (1981).
5. M. R. Franklin, *Xenobiotica* **1**, 581 (1971).
6. P. V. Parke and H. Rahman, *Biochem. J.* **123**, 9 (1971).
7. J. B. Schenkman, B. J. Wilson and D. L. Cinti, *Biochem. Pharmac.* **21**, 2373 (1972).
8. M. K. Buening and M. R. Franklin, *Drug Metab. Dispos.* **4**, 244 (1976).
9. J. Werringloer and R. W. Estabrook, *Life Sci.* **13**, 1319 (1973).
10. D. G. Wolff and W. R. Bidlack, *Biochem. biophys. Res. Commun.* **73**, 850 (1976).
11. Y. Masuda and T. Murano, *Biochem. Pharmac.* **26**, 2275 (1977).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
13. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
14. T. Nash, *Biochem. J.* **55**, 416 (1953).
15. S. F. Muakkassah and W. C. T. Yang, *Fedn Proc.* **40**, 719 (1981).
16. R. N. Hines and R. A. Prough, *J. Pharmac. exp. Ther.* **214**, 80 (1980).

Biochemical Pharmacology, Vol. 31, No. 2, pp. 251–254, 1982.
Printed in Great Britain.

0006-2952/82/020251-04 $03.00/0

Retention of phosphate transport function of rat renal brush border membranes isolated from frozen cortex

(*Received* 16 *April* 1981; *accepted* 2 *July* 1981)

Extensive studies of the transport properties of isolated luminal brush border membrane (BBM) vesicles from the renal proximal tubule have led to identification of specific Na^+-dependent transport systems for glucose, amino acids, and various organic and inorganic ions including inorganic phosphate (P_i) [1, 2]. The P_i transport system in BBM represents a rate-limiting step in transtubular P_i reabsorption and is of particular interest because it is altered in response to several hormones and drugs administered *in vivo*. Examples of these agents are parathyroid hormone, growth hormone, 1,25-dihydroxyvitamin D_3, dibutyryl cyclic AMP, triamcinolone, nicotinamide, and diphosphonates [3, 4]. A combination of *in vivo* and *in vitro* methods provides an extremely useful approach [5–7] for elucidating the mode of action of agents which regulate renal P_i transport. The procedure is to study first with clearance techniques the effects of administered agents on renal function and on transtubular P_i transport. Then, at the end of these experiments, the kidneys are removed, BBM vesicles are prepared from the cortex, and P_i uptake by the vesicles is measured to provide direct determination of any changes in the P_i transport system in the BBM of the proximal tubule. A major disadvantage of this experimental design is that completion without interruption requires an extended period of time (about 15 hr). This problem would be avoided if, following the clearance studies, the renal cortex could be stored until the next day (or longer) before preparing a BBM vesicle fraction. Evidence is presented here that the P_i transport function of rat renal BBM is well preserved in renal cortical tissue stored frozen by liquid nitrogen. Further, differences in P_i transport induced *in vivo* by low P_i diet or nicotinamide are retained in BBM vesicles prepared from frozen cortex.

Rats were anesthetized with ether, kidneys were removed, decapsulated, and rinsed in ice-cold buffered saline (154 mM NaCl, 1 mM Tris–HEPES*, pH 7.5), and the cortex was dissected free from the medulla. The cortex

* HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.