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REDUCTIVE METABOLISM AND ACTIVATION OF BENZNIDAZOLE*

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Abstract—Benznidazole (Bz) (*N*-benzyl-2-nitro-1-imidazole-acetamide) is a drug used against Chagas' disease. Rat liver microsomal and cytosolic fractions, but not mitochondria, exhibited Bz nitroreductase activity under anaerobic conditions in the presence of NADPH.

Microsomal nitroreductase activity was enhanced by FAD and was inhibited totally by oxygen and partially by carbon monoxide. Liver cystosol fraction was able to reduce Bz nitrogroups in the presence of either *N*-methylnicotinamide or hypoxanthine as substrates. These enzyme activities were inhibited by menadione or allopurinol respectively. Under every experimental condition leading to enzymatic reduction of Bz nitrogroups and its inhibition or enhancement, reactive metabolites that bind covalently to proteins were also produced. This covalent binding was effectively prevented by reduced glutathione. Results suggest the participation of cytochrome P-450 and cytochrome *c* reductase in liver microsomal processes and of xanthine oxidase and aldehyde oxidase in liver cytosolic processes of Bz nitroreduction and activation to reactive metabolites that bind covalently to proteins. Possible pharmacological and toxicological implications of the described observations were discussed.

American Trypanosomiasis (Chagas' disease) is an endemic disease affecting at least 10–12 million people in Latin America [1]. Despite the major importance of the problem, no treatment of Chagas' disease is completely satisfactory. In effect, no drug presently available is both effective and safe [2].

Benznidazole (Bz) (*N*-benzyl-2-nitro-1-imidazole-acetamide) is one of the drugs used in Latin America against Chagas' disease. Its established adverse reactions include skin rashes, peripheral polyneuropathy, and blood dyscrasias [3].

Little is known about Bz metabolism in the body [4] although that information would be critical for the understanding of both pharmacological and toxicological effects of the drug. This work describes our initial studies on Bz in which we analyzed the possible occurrence of a nitroreductive pathway of biotransformation and activation to reactive metabolites that bind covalently to proteins, as was observed with other nitrogroups [4–11].

MATERIALS AND METHODS

Chemicals. Bz and Bz with ^{14}C in position 2 of the imidazole (sp. act. 58 μCi/mg) were gifts from F. Hoffmann-La Roche & Co., Ltd. Hypoxanthine, *N*-methylnicotinamide, allopurinol, menadione, FAD, reduced glutathione (GSH), enzymes and cofactors were purchased from the Sigma Chemical Co. (St. Louis, MO). Other chemicals employed were of the best quality available.

Animals. Sprague–Dawley male rats (200–220 g) were used in these studies. The animals were fasted 12–14 hr before use but they had access to water *ad lib.* The animals were killed by decapitation and bled. Their livers were rapidly excised and processed.

Procedures. Microsomal fractions were obtained as follows. Livers were homogenized in a teflon–glass Potter–Elvehjem homogenizer with 4 vol. of 1.15%KCl. The homogenate was centrifuged for 20 min at 9,000 *g*, and the supernatant fraction was further centrifuged for 1 hr at 105,000 *g* to obtain the microsomal pellets.

Pellets were resuspended in 20 mM phosphate buffer, pH 7.4. Highly purified mitochondrial fractions were prepared according to Sacchi *et al.* [12]. Briefly, livers were homogenized with 5 vol. of 0.25 M sucrose–2 mM Tris/HCl (pH 7.6) in a teflon–glass Potter–Elvehjem homogenizer. The homogenate was centrifuged for 15 min at 800 *g*. The precipitate was discarded. The supernatant fraction was centrifuged again for 15 min at 800 *g*, and the precipitate was again discarded. The supernatant fraction was centrifuged for 15 min at 3,000 *g*. The pellet was resuspended with 10 vol. of homogenization medium and the suspension was centrifuged for 15 min at 3,000 *g*. The pellet was the mitochondrial preparation employed in our studies. All these operations were performed at 2–4°. The purity of our mitochondrial preparations was checked by electron microscopy and by determination of endoplasmic reticulum marker enzymes like glucose-6-phosphatase and NADPH–cytochrome *c* reductase. Contamination of endoplasmic reticulum was 2%. Glucose-6-phosphatase activity was determined as described by

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Harper [13] and NADPH–cytochrome *c* reductase activity according to Williams and Kamin [14]. Samples for electron microscopy were prepared as described by Bernacchi *et al.* [15].

To obtain the cytosol fraction, livers were homogenized with 2 vol. of 20 mM Tris/HCl (pH 7.4)–5 mM 0.25 M sucrose-EDTA and centrifuged at 9000 *g* for 20 min. The 9000 *g* supernatant fraction was centrifuged for 1 hr at 105,000 *g*. The supernatant fraction obtained was dialyzed overnight in the cold room against 2 l of the same buffer. All incubations were run in 20-ml septum-stoppered vials at 37°. The vials contained in a final volume of 2.5 ml of 20 mM phosphate buffer (pH 7.4): the subcellular fraction (final concentrations ranged from 2.5 to 3 mg/ml for microsomes and mitochondria and from 7 to 8mg/ml for the cytosol) and NADPH-generating system, 0.05 ml. The NADPH-generating system composition used was: 0.3 M Tris/HCl buffer (pH 7.4), 0.2 ml; 1 M $MgCl_2$, 0.2 ml; isocitric acid dehydrogenase type I from porcine heart, 0.6 ml; *d,l*-isocitric acid trisodium salt, 124 mg; NADP sodium salt, 20 mg; and Bz (final concentration 0.288 mM). Vials containing subcellular fraction suspensions were bubbled with oxygen-free nitrogen or CO for 5 min prior to NADPH and Bz as a dimethylformamide solution (0.05 ml of a 3.75 mg/ml solution of Bz in dimethylformamide). The final concentration of dimethylformamide in the incubation mixture was 2% (v/v). After incubation for 10 min (microsomes) or for 15 min (cytosol or mitochondria), reactions were terminated by addition of 1 ml of 15% zinc sulfate. Incubation mixtures were poured over 2.5 g of NaCl and extracted with 7.0 ml of ethyl acetate.

The organic phase was read in a spectrophotometer at 315 nm. At this wavelength Bz had a peak of maximum absorption. Readings were compared against a calibration curve of increasing concentrations of Bz in ethyl acetate. Recovery of known additions of Bz to incubation mixtures was 97%.

Similar incubation mixtures were employed when using [^{14}C]-Bz. The [^{14}C]-Bz covalently bound to protein in incubation mixtures was determined as previously described [16]. Protein concentrations were determined according to Lowry *et al.* [17].

Under these experimental conditions, Bz nitroreductase activity and covalent binding of Bz reactive metabolites were linear with time and protein content.

Table 1. Benznidazole nitroreductase activity in different cellular fractions*

Fraction	Bz nitroreductase activity [nmoles Bz·min^{-1}·(mg protein)$^{-1}$]
Microsomes	1.837 ± 0.512
Mitochondria	0†
Cytosol	0.215 ± 0.050†

* The different subcellular fractions were incubated at 37° under a N_2 atmosphere with Bz and an NADPH-generating system for 10 min in the case of microsomes and for 15 min in the case of mitochondria and cytosol. Values are the means ± S.D. of three determinations.
† $P < 0.01$.

RESULTS

Benznidazole nitroreductase activity in different cellular fractions. Liver microsomal and cytosolic fractions exhibited Bz nitroreductase activity under anaerobic conditions and in the presence of NADPH (Table 1). Most of the activity was found in microsomes. Mitochondria did not have detectable activity (Table 1).

Microsomal benznidazole nitroreductase activity under different atmospheres. Microsomal Bz nitroreductase activity was abolished completely when nitrogen was replaced by a pure oxygen atmosphere (Table 2). About 67% of the total microsomal Bz nitroreductase activity was inhibited when pure CO replaced nitrogen in incubation mixtures (Table 2).

Effect of FAD on microsomal benznidazole nitro-

Table 2. Microsomal benznidazole nitroreductase activity under different atmospheres*

Atmospheres	Bz nitroreductase activity [nmoles Bz·min^{-1}·(mg protein)$^{-1}$]
N_2	1.837 ± 0.512
O_2	0†
CO	0.596 ± 0.100†

* Incubation mixtures were as in Table 1 except for the different atmospheres. Values are the means ± S.D. of three determinations.
† $P < 0.01$.

Table 3. Effect of FAD on microsomal benznidazole nitroreductase activity*

Experimental condition NADPH	FAD	Bz nitroreductase activity [nmoles Bz·min^{-1}·(mg protein)$^{-1}$]
+	– – –	1.623 ± 0.085
– – –	– – –	0†
+	+	7.735 ± 0.265†
– – –	+	0†

* Incubation was as in Table 1 except for the absence of NADPH or the presence of FAD (1 mM) when indicated. Values are the mean ± S.D. of three determinations.
† $P < 0.01$.

Table 4. Characteristics of cytosolic benznidazole nitroreductase activity*

Substrate	Bz nitroreductase activity [nmoles Bz·min^{-1}·(mg protein)$^{-1}$]
N-Methylnicotinamide	
Control	0.181 ± 0.023
Menadione	0.025 ± 0.010†
Hypoxanthine	
Control	0.162 ± 0.032
Allopurinol	0.066 ± 0.004†

* Incubation was as in Table 1 except that NADPH was omitted and either *N*-methylnicotinamide (2.5 mM) or hypoxanthine (0.25 mM) as substrate was included. Menadione (10 μM) or allopurinol (0.15 mM) was added when indicated. Values are the means ± S.D. of three determinations.
† $P < 0.05$.

reductase activity. FAD addition to incubation mixtures significantly enhanced the NADPH-dependent microsomal Bz nitroreductase activity (Table 3). No Bz nitroreductase activity was observed when only microsomes were employed or when FAD but not NADPH was added to microsomal suspensions (Table 3).

Table 5. Characteristics of microsomal benznidazole activation to reactive metabolites that bind covalently to proteins*

Experimental condition	Bz covalently bound (pmoles/mg protein)
N_2	101.1 ± 10.2
N_2 (No NADPH)	35.6 ± 2.4†
Air	24.0 ± 0.7†
CO	45.7 ± 3.8†
Heated	9.7 ± 1.8†
N_2 (+ GSH)	0.4 ± 0.1†

* Incubations were as in Table 1 except that [^{14}C]Bz was used. Proteins were precipitated with trichloroacetic acid, lipids were removed, and the samples were dried, dissolved in formic acid, and counted by liquid scintillation. Values are the mean ± S.D. of three determinations.
† $P < 0.01$.

Characteristics of cytosolic benznidazole nitroreductase activity. Liver cytosol fraction was able to reduce Bz nitrogroups in the presence of *N*-methylnicotinamide as substrate. This activity was inhibited by menadione (Table 4). Cytosolic Bz nitroreductase activity was also observed using hypoxanthine as substrate. This activity was partially inhibited by allopurinol (Table 4).

Characteristics of microsomal benznidazole activation to reactive metabolites that bind covalently to proteins. Under anaerobic conditions, microsomal fractions in the presence of NADPH and Bz were able to produce reactive metabolites that bind covalently to proteins (Table 5). The extent of the covalent binding observed was reduced significantly by heating, when NADPH was omitted or when air replaced nitrogen (Table 5). The production of Bz reactive metabolites was decreased significantly in the presence of an atmosphere of pure CO (Table 5). Addition of GSH to incubation mixtures effectively

Table 6. Characteristics of cytosolic benznidazole activation to reactive metabolites that bind covalently to proteins*

Experimental condition	Bz covalently bound (pmoles/mg protein)
NADPH	8.9 ± 0.3
NADH	9.9 ± 3.0
NADH + GSH	3.6 ± 0.5†‡
Hypoxanthine	45.2 ± 3.6†
Hypoxanthine + allopurinol	23.9 ± 1.6†‡
Hypoxanthine + GSH	13.0 ± 2.1†‡
N-Methylnicotinamide	2.65 ± 0.13†
N-Methylnicotinamide + menadione	2.00 ± 0.06†‡
N-Methylnicotinamide + GSH	0.64 ± 0.03†‡

* Incubations were as in Table 4 except for the experiments using NADPH or NADH. When added, NADPH concentration was 0.5 mM, NADH concentration was 1 mM, and GSH concentration was 1 mM. Values are the mean ± S.D. of three determinations.
† $P < 0.05$ when compared to NADPH.
‡ $P < 0.05$ for NADH vs NADH + GSH; hypoxanthine vs hypoxanthine + allopurinol or hypoxanthine + GSH; *N*-methylnicotinamide vs *N*-methylnicotinamide + menadione or *N*-methylnicotinamide + GSH.

prevented most of the covalent binding of Bz reactive metabolites to proteins (Table 5).

Characteristics of cytosolic benznidazole activation to reactive metabolites that bind covalently to proteins. Cytosolic fraction was able to activate Bz to reactive metabolites that bind covalently to proteins using either NADPH, NADH, hypoxanthine or *N*-methylnicotinamide as substrates (Table 6). In all these cases, the formation of reactive metabolites was decreased by GSH (Table 6). Hypoxanthine-dependent Bz activation was inhibited by allopurinol, and *N*-methylnicotinamide-dependent Bz activation was inhibited by menadione (Table 6).

DISCUSSION

Previous work by Docampo *et al.* [18–20] evidenced nitro anion radical generation from Nifurtimox and Bz in liver microsomal preparations. This present study shows that the nitro groups of Bz had undergone reduction when incubated with rat liver microsomes. The process required NADPH. No Bz nitroreductase activity was detected in rat liver mitochondria, but the cytosol fraction exhibited some enzyme activity. About 67% of the Bz nitroreductase activity in liver microsomes was inhibited by CO, suggesting participation of cytochrome P-450 (P-450) in the process [5–11]. Moreno *et al.* [18] reported that cytochrome P-450 is not involved in the initial reduction of Bz to its nitro anion radical. Thus, cytochrome P-450 participation would be after this initial step. This behavior has been described for other nitrocompounds [7].

The P-450 requirement of Bz nitroreductase explains, in part, the oxygen sensitivity of the liver microsomal process. In effect, substrate competition with oxygen for reduced P-450 has been proposed as one of the reasons for oxygen susceptibility of nitroreductases [5, 7]. Other factors, such as easy oxidation of intermediate hydroxylamines and re-oxidation of nitro anion free radical to parent nitrocompound, have also been suggested to explain poor performance of nitroreductases under oxygen [5–21]. Participation of cytochrome *c* reductase in Bz nitroreduction might account for the non-P-450-dependent microsomal nitroreductase activity observed in an atmosphere of pure CO. Participation of both P-450 and cytochrome *c* reductase has been reported previously for several other nitrocompounds [5, 7–11, 22–25]. The addition of FAD to NADPH-containing incubation mixtures significantly enhanced the Bz nitroreductase activity in liver microsomes. No enhancement was observed when NADPH was omitted. This sort of behavior has been described for the reduction of other compounds [10, 11, 26–28]. Kamm and Gillette [23] provided evidence that nitroreduction in those cases is performed by $FADH_2$ that is formed from a reduction of FAD with NADPH catalyzed by cytochrome *c* reductase.

We also observed the presence of some cytosolic Bz nitroreductase activity. Part of it proceeds using *N*-methylnicotinamide as substrate and it is inhibited by low concentrations of menadione. This suggests participation of aldehyde oxidase in the process [10, 22, 29, 30]. Another fraction of cytosolic Bz nitroreductase activity was observed using hypoxanthine as substrate. This activity was inhibited by allopurinol. These characteristics indicate probable xanthine oxidase participation in cytosolic Bz nitroreduction [5, 7, 10, 30].

Previous work by Clarke *et al.* [31] showed that xanthine oxidase from butter milk was able to reduce nitrogroups from Bz and other nitroimidazoles. Aldehyde oxidase and xanthine oxidase participation in the reduction of other nitrocompounds has been reported previously by other workers [5, 7–11, 25, 30–33].

Studies in other laboratories have shown that reduction by reductases of nitrogroups in chemicals is accompanied frequently by formation of reactive metabolites that bind covalently to macromolecules [5, 7, 9, 10, 25, 33]. In the case of Bz, a similar behavior is observed. In effect, our present studies suggest that microsomal P-450 and cytochrome *c* reductase or cytosolic aldehyde oxidase and xanthine oxidase mediated Bz reductase lead to the formation of reactive metabolites that bind covalently to proteins. The participation of these enzymes is supported by their response to inhibitors like CO, menadione and allopurinol. Reactive metabolites formed in microsomal or cytosolic incubations were effectively trapped by reduced glutathione (GSH). The reactivity of metabolites from other nitrocompounds with GSH was observed previously by others [10].

Concerning the possible participation of the here described pathways of Bz nitroreduction and activation *in vivo*, in situations *in vivo* aerobic conditions predominate and consequently the oxygen susceptibility of Bz nitroreduction and activation may be of relevance. Studies from our laboratory are underway to clarify this point and will be reported elsewhere.

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