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Nifurtimox biotransformation to reactive metabolites or nitrite in liver subcellular fractions and model systems

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Abstract

Liver microsomal (mic); nuclei (N) and mitochondria (mit) anaerobically nitroreduce Nifurtimox (Nfx) in the presence of NADPH generating system. Simultaneous formation of small amounts of nitrite was observed in microsomes and nuclei but not in mitochondria. The microsomal nitroreductase activity was enhanced by the presence of flavine-adenine-dinucleotide disodium salt (FAD), was not inhibited by CO and was significantly inhibited by diphenyleneiodonium (DPI). In the microsomal NADPH-dependent fraction nitrite formation was null in the presence of FAD, DPI and under air and was partially inhibited by pure CO. Pure human cytochrome P450 reductase in the presence of NADPH significantly nitroreduced Nfx and produced small amounts of nitrite. The nitroreductive process was significantly enhanced by FAD but the nitrite formation became null. FAD itself was able to chemically nitroreduce Nfx without production of nitrite. NADPH generating system enhanced the FAD nitroreductive effect and led to small production of nitrite. Formation of reactive metabolites and nitric oxide during Nfx metabolism might contribute to its toxicity.

Keywords: Nifurtimox; Nitroreductase activity; Nitric oxide and peroxynitrite formation; Liver nuclei or microsomes or mitochondria

1. Introduction

Chagas' disease (American Trypanosomiasis), is an endemic parasitic disease limited to the tropical and subtropical countries of Latin America (Pinto Diaz, 1984). More than 20 million people are infected with the etiologic agent of the disease, *Trypanosoma cruzi* and it has been estimated more than 120 million people are at risk of getting the infection via either vector insects or infected blood transfusions (Moncayo, 1997, 1999).

Two drugs are in use today, Nifurtimox (Nfx) and Benznidazole, and both have a relative value (Stoppani, 1999). They possess toxic side effects which are a serious drawback to their widespread use in the treatment of the acute phase of the disease (Castro and Toranzo, 1988; Castro, 2000; Docampo and Moreno, 1985).

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Nifurtimox (3-methyl-4-[(5-nitrofurfurylidene) amino] thiomorpholine-1, 1-dioxide (Nfx) (Fig. 1) exhibits particularly serious toxic effects including anorexia, weight loss, polyneuropathy, insomnia, headache, hepatic intolerance, skin allergies and other (Castro and Toranzo, 1988). However, those of greater concern are the mutagenic, carcinogenic, reproductive and endocrine effects reported (Castro and Toranzo, 1988; Castro, 2000). These deleterious effects were generally attributed to Nfx nitroreductive biotransformation to a nitroanion radical, redox cycling, generation of reactive oxygen species or lipid peroxidation (Docampo and Moreno, 1985). However, not all the features of the toxic process are fully understood. Recent preliminary studies by Carrizo et al. (2000), opened the possibility for the production of peroxinitrite radicals later detected as nitrite during a liver microsomal NADPH dependent anaerobic process. Parallel preliminary studies from our laboratory found other nitrite-forming processes and confirmed the findings of the former author (Diaz et al., 2000). In the present study the characteristics of the enzymes and their potential presence in other liver cell organelles are described.

2. Materials and methods

2.1. Chemicals

Nfx was a gift from Bayer A.G. Leverkusen, Germany. $NADP^+$, isocitric acid dehydrogenase from porcine heart, sodium isocitrate and flavine-adenine-dinucleotide disodium salt (FAD) were from Sigma Chemical Company. Human liver microsomal P450 reductase was from Gentest Corp., USA. Nitrogen (ultra high purity) was from AGA (Argentina) and carbon monoxide was form Matheson Co. Both gases were further deoxygenated by bubbling them through a solution containing 0.05% 2-anthraquinone sulfonic acid sodium salt and 0.5% $Na_2S_2O_4$ in 0.1 N NaOH. All other chemicals employed were of analytical grade.

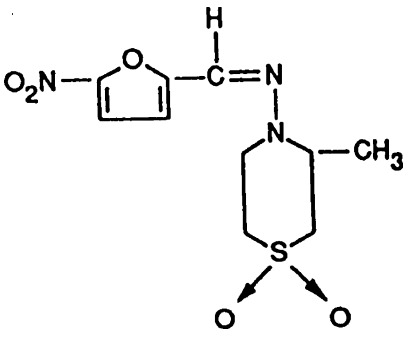

Fig. 1. Chemical structure of Nfx.

2.2. Animals

Non-inbred Sprague–Dawley male rats of 200–300 g body weight were bred in our laboratory. Rats were maintained in a controlled room on a 12 h:12 h light:dark cycle (light phase 07:00–19:00). Temperature in the animals room was 23 ± 2 °C and the relative humidity was between 35 and 65%. Animals were fasted 12–14 h with free access to water before they were euthanized by decapitation. Their livers were rapidly excised and processed.

2.3. Isolation of cellular fractions

All procedures were performed at 0–4 °C. Livers were homogenized in a Teflon-glass Potter-Elvehjem homogenizer with 4 volumes of 1.15% KCl. The homogenates were centrifuged at $9000 \times g$ for 20 min. The resulting supernatants were centrifuged at $100\,000 \times g$ for 1 h and the microsomal pellets were recovered (Castro et al., 1989). Highly purified liver cell nuclei were isolated as described by Diaz Gomez et al. (1999) and Diaz Gomez et al. (2000). These nuclear preparations were assessed for purity on the basis of their lack of activity of marker enzymes for mitochondria (isocitric acid dehydrogenase), for cytosol (lactic dehydrogenase) and by electron microscopy in order to obtain a definitive assessment of the purity of nuclear preparations. These criteria were recently considered as the most suitable to assess that nuclei were free of detectable contamination from other organelles (Diaz Gomez et al., 1999, 2000). Highly purified liver mitochondrial fractions were prepared according to Villarruel et al. (1987). These mitochondrial preparations had only 2% of contamination with endoplasmic reticulum

and cytoplasm by electron microscopy criteria and the use of enzymatic markers (Villarruel et al., 1987).

2.4. Enzymatic and chemical determinations

All incubations were run in 20 ml septum sealed flasks with agitation at 150 oscillations/min in a covered Dubnoff shaker (to ensure darkness) at 37 °C. The incubation mixtures were gassed for 5 min with oxygen-free N_2 or CO. In aerobic conditions the flasks were not sealed. The incubation mixtures contained the following when indicated: (a) 20 mM potassium phosphate buffer pH 7.4; 0.2 mM Nfx in *N*,*N*-dimethylformamide. (b) 0.5 mM NADPH-generating system that contained 0.3 M Tris–HCl buffer pH 7.4 0.2 ml; 1 M $MgCl_2$ 0.2 ml; isocitric acid dehydrogenase type IV from porcine heart, 0.6 ml; DL-isocitric acid trisodium salt, 124 mg and NADP sodium salt 20 mg. (c) The cellular fraction, microsomes (final concentration 1.1–1.9 mg protein/ml), mitochondria (final concentration 2.0 mg protein/ml) and nuclei (final concentration 3.0 mg protein/ml). (d) 1 mM FAD. (f) 10 μM diphenylene iodonium chloride (DPI). Model systems, not containing cellular fractions, were incubated for 1 h, microsomes for 15 min, nuclei for 75 min and mitochondria for 60 min. Placing the flasks on ice interrupted the reaction. In these studies Nfx nitroreductase activity or Nfx consumption in model systems was followed by substrate disappearance. This was calculated by the difference in Nfx concentration at the beginning and at the end of the reaction.

2.5. Nifurtimox concentration measurement by HPLC

An aliquot (250 μl) of the incubation mixture was added to 2 volumes (500 μl) of CH_3OH. The incubation mixtures containing cellular fractions were centrifuged at $15\,000 \times g$ for 10 min. The supernatants were filtered through nylon filter membranes (pore size 0.45 μm) prior to HLPC analysis. Sample (10 μl) was analyzed at 40 °C using a liquid chromatograph with a Hewlett Packard ODS Hypersil column (20 cm × 2.1 mm ID, 5 μm particle size) and diode array detector. The mobile phase, consisting of 60% methanol–water, was delivered at a constant flow-rate of 0.2 ml/min. The column effluent was monitored at 400 nm. Quantification was by peak-area ratio with reference to standards treated identically.

2.6. Nitrite anion (NO_2^-) assay

An aliquot (1.5 ml) of the incubation mixture was added to 0.15 ml of acetic anhydride. The mixture was left for 5 min at room temperature and then mixed with 1.5 ml of 1% (w/v) sulfanilamide in 20% (w/v) HCl, followed by the addition of 1.5 ml of 0.02% (w/v) *N*-(1-naphtyl) ethylenediamine dihydrochloride. After diazo-coupling for 20 min at room temperature, 0.15 ml of 1% (w/v) amonium sulfamate (freshly prepared) was added. In the presence of a cellular fraction the mixture was centrifuged and left for 5 min at room temperature. Absorbance was measured at 540 nm in a spectrophotometer. Readings were compared against a calibration curve of increasing concentrations of nitrite (Asroba and Takahashi, 1998).

2.7. Determination of protein concentration

Protein concentration were determined using the method of Lowry et al. (1951) with bovine serum albumin as standard.

2.8. Statistics

The significance of the difference between two mean values was assessed using the Student's *t*-test as described by Gad and Weil (1982).

3. Results

3.1. Enzymatic nitroreduction of Nfx under different experimental conditions

Liver microsomes in the presence of NADPH generating system and under N_2 significantly nitroreduced Nfx (e.g. 98 times more than with the generating system in the absence of the liver

Table 1
Rat liver microsomal Nfx nitroreductase activity and nitrite (NO_2^-) formation under different experimental conditions

Experimental conditions	Nfx consumed nmol/min/mg protein	NO_2^- formed nmol/min/mg protein
Mic (N_2)	0.07±0.06	0.10±0.07
Mic+NADPH gen sys (N_2)	5.87±0.45	0.31±0.03
Mic+FAD (N_2)	0.80±0.13*	0*
Mic+NADPH gen sys+FAD, (N_2)	6.91±0.45***	0*
Mic+NADPH gen sys+DPI, (N_2)	0.67±0.60*	0*
Mic+NADPH gen sys (CO)	5.37±0.45	0.22±0.02**
Mic+NADPH gen sys (air)	0*	0*
Mic+FAD (air)	1.92±0.19*	0*
Mic+NADPH gen sys+FAD, (air)	1.31±0.15*	0*

Incubations containing 0.2 mM Nfx microsomes (Mic) and NADPH generating system (NADPH gen sys) were bubbled with oxygen-free N_2 or CO when stated.
In some experiments 1 mM FAD was included. In one experiment 10 μM DPI was added. Concentrations of remaining Nfx after incubations was determined by HPLC. NO_2^- was measured by the Griess reaction. See Section 2 for details. Results are the mean±SD of three determinations. Significantly different from Mic+NADPH gen sys (N_2).
* $P < 0.001$.
** $P < 0.01$.
*** $P < 0.05$.

microsomes) (Tables 1 and 4). Nitrite formation under these circumstances was also increased (e.g. ~8.6 times more than without microsomes). The total nitroreductive effect of liver microsomes in the absence of generating system was negligible but it was enhanced slightly by FAD (N_2). Further, microsomes in the presence of NADPH+FAD did not exhibit synergistic but only additive effects (Table 1). No nitrite formation was observed with FAD either in the presence or absence of NADPH (N_2) (Table 1). The NADPH dependent microsomal pathway of Nfx nitroreduction was fully inhibited by DPI (N_2) but not by pure CO (Table 1). In the DPI experiment no nitrite was produced. However, when the pure CO was present in the incubation mixture nitrite formation was observed to an extent to that formed under N_2 (~71.4%, Table 1).

Under air, there was no consumption of Nfx in the presence of NADPH generating system and no nitrite production was observed (Table 1). However, aerobic liver microsomes in the presence of FAD led to significant increases in Nfx consumption (2.4 times more than the equivalent experiment under N_2). Further, aerobic incubations of liver microsomes with FAD and NADPH generating system were 5.27 times less efficient to consume Nfx than the equivalent experiment under N_2 (Table 1). No nitrite formation was observed when experiments were performed under an air atmosphere, irrespectively of whether FAD was present or not (Table 1).

3.2. NADPH dependent anaerobic nitroreductive ability of different subcellular fractions

As shown in Table 2 there was a nitroreductive ability of liver microsomes, in the presence of NADPH, leading to Nfx consumption and nitrite formation. These abilities were significantly smaller in highly purified nuclear fractions (17 times smaller for total nitroreduction and 31 times smaller for nitrite formation). The liver mitochon-

Table 2
Rat liver Nfx nitroreductase activity and nitrite (NO_2^-) formation in different subcellular fractions

Experimental conditions	Nfx consumed nmol/min/mg protein	NO_2^- formed nmol/min/mg protein
N+NADPH gen sys (N_2)	0.35±0.03	0.010±0.004
Mit+NADPH gen sys (N_2)	0.31±0.05	0
Mic+NADPH gen sys (N_2)	5.87±0.45	0.31±0.03

Mixtures containing either rat liver nuclei (N), or mitochondria (Mit) or microsomes (Mic) were incubated as in Table 1.
Concentration of undegraded Nfx from incubation mixtures was determined by HPLC. NO_2^- was measured by the Griess reaction. Results are the mean±SD of three determinations. Values for microsomal fractions were included for comparison.

Table 3
Human P450 reductase activity and nitrite (NO_2^-) formation from Nfx under different experimental conditions

Experimental conditions	Nfx consumed nmol/min /mg protein	NO_2^- formed nmol/min /mg protein
P450 red	0	0
P450 red+NADPH gen sys (N_2)	32.2±8.6	2.62±2.14
P450 red+FAD (N_2)	137.3±2.2*	0*
P450 red+FAD +NADPH gen sys (N_2)	139.0±2.2*	0*

Reaction mixtures containing 0.2 mM Nfx, human P450 reductase (P450 red) (0.1 mg prot/ml), NADPH generating system (NADPH gen sys) when indicated and 1 mM FAD when stated was incubated for 15 min at 37 °C (under a N_2 atmosphere).
Concentration of degraded Nfx was determined by HPLC. NO_2^- was monitored spectrophotometrically. Results are the mean±SD of three determinations. Significantly different from P450 red+NADPH gen sys (N_2).
* $P < 0.001$.

drial fraction, in presence of NADPH, was able to nitroreduce Nfx to a similar extent as the nuclei but not to form detectable nitrite (Table 2).

3.3. Nifurtimox nitroreduction and nitrite formation by pure human P450 reductase

Pure human P450 reductase was able to nitroreduce Nfx and to produce nitrite in the presence of NADPH generating system under a nitrogen atmosphere (Table 3). The addition of FAD to incubation mixtures increased 4.26–4.31 times the nitroreduction ability of P450 reductase in the presence or absence of NADPH respectively but no nitrite formation was observed (Table 3).

3.4. Nifurtimox reduction in model systems

In Table 4 the total reduction of Nfx to products and specifically to nitrite by model systems is shown. The NADPH generating system under N_2 was able to nitroreduce Nfx to a very small extent, about 60% of which was converted to nitrite. Nfx in the presence of FAD, under N_2, was also nitroreduced 4.5 times more intensively, however, no detectable nitrite was produced in this case (Table 4).

When both FAD and NADPH generating system were present (N_2) a far more intense reduction of Nfx was observed than with each one of them separately. For example, 23 times compared with the generating system alone or 5.15 times compared with FAD alone. In this case, however, nitrite was formed but to a smaller extent than with NADPH generating system (N_2) alone ($\sim$58% of it). If FAD/NADPH generating system reacted with Nfx under air, then, the synergistic effect between both of them observed under N_2 completely disappeared (Table 4).

4. Discussion

In agreement with previous studies from others and our laboratory, the liver microsomal fraction exhibited a Nfx nitroreductase activity able to proceed under nitrogen and requiring NADPH (Docampo and Moreno, 1985; Castro and Tor-

Table 4
Effect of different experimental conditions on Nfx reduction and nitrite (NO_2^-) formation in model systems

Experimental conditions	Nfx consumed nmol/min /mg protein	NO_2^- formed nmol/min /mg protein
NADPH gen sys (N_2)	0.06±0.04	0.04±0.003
FAD (N_2)	0.27±0.05***	0*
NADPH gen sys+FAD (N_2)	1.39±0.09*	0.02±0.002**
NADPH gen sys+FAD (air)	0.21±0.10	0.03±0.01***

Mixtures were incubated as in Table 1.
The concentration of remaining Nfx after incubations was determined by HPLC. NO_2^- was measured by the Griess reaction. Results are the mean±SD of three determinations. Significantly different from NADPH gen sys (N_2).
* $P < 0.001$.
** $P < 0.01$.
*** $P < 0.05$.

anzo, 1988; Castro, 2000). The process is not mediated by any cytochrome P450 (P450) form as evidenced by the lack of inhibitory effect of pure CO, a very well known inhibitor of P450 mediated processes (Omiecinski et al., 1999). In contrast, DPI, and inhibitor of flavoenzymes such as NADPH P450 reductase (McGuire et al., 1998; O'Donnell et al., 1993) strongly inhibited the process pointing to P450 reductase as the putative microsomal enzyme involved. P450 reductase is a flavoenzyme having both FMN and FAD in its active center (Müller, 1987; Strobel et al., 1995). Furthermore, chemical model systems containing just FAD or FAD and NADPH, significantly nitroreduced Nfx and though that effect decreased in the presence of oxygen, it was not fully inhibited (Table 4). In fact, even in the absence of NADPH, liver microsomes increased their almost null activity to nitroreduce Nfx in the presence of FAD and almost completely metabolized Nfx in the presence of FAD + NADPH. These experiments suggest the formation of a flavine semiquinone free radical species from either NADPH reduced FAD in model systems or in P450 reductase as previously evidenced by our laboratory in the activation by both pure FAD or P450 reductase of other chemicals, such as ethanol, to 1-hydroxyethyl radicals (Diaz Gomez et al., 2000) or methanol to hydroxymethyl radicals (Castro et al., 2002). The electron transfer process might be visualized as follows: electron transfer would proceed from NADPH to FAD to FMN. It would involve the existence of several unpaired electron-containing semiquinone forms, e.g. [FAD$^{\bullet}$-E-FMN]; [FAD-E-FMNH$^{\bullet}$]; [FADH$^{\bullet}$-E-FMNH$^{\bullet}$]; [FADH$^{\bullet}$-E-FMH_2] and [$FADH_2$-E-FMNH$^{\bullet}$] (Opsian and Coon, 1982; review by Müller, 1987). These semiquinones might be involved in the generation of the nitro anion radical of Nfx by P450 reductase. When pure FAD is used, e.g. Table 4, then FMN is not present as it occurs with P450 reductase and then the semiquinone form of FAD is the only one that might be involved.

Under air, FAD also increased ability of liver microsomes to metabolize Nfx by nitroreduction despite the observed full inhibitory effect of oxygen. This may suggest the presence of another flavoenzyme in the microsomal fraction able to perform the nitroreductive process.

In agreement with previous preliminary observations from our laboratory, highly purified liver nuclei exhibited some Nfx nitroreductive capacity (Toranzo et al., 1997). Purified liver mitochondria exhibited a far smaller ability to nitroreduce Nfx of the same order than that of nuclei. The presence of Nfx nitroreductase activity in liver nuclei should not be surprising in light of the fact that, the outer membrane of nuclei is continuous with the endoplasmic reticulum (Alberts et al., 1983) and that in previous studies from others and of our laboratory, the presence of P450 reductase in the nuclear fraction was demonstrated (Diaz Gomez et al., 1999; Kasper, 1971). All these nitroreductive metabolic processes should lead to reactive moieties (e.g. hydroxylamines; hydroxilamino radicals; nitroanion radicals), which may attack macromolecules and spark directly or via GSH depletion a lipid peroxidation processes having deleterious effects on the cells (Docampo and Moreno, 1985; Castro and Toranzo, 1988; Castro, 2000). Other additional source of potentially deleterious metabolites of Nfx, such as nitric oxide (NO), were also preliminarily reported by others for the microsomal fraction (Carrizo et al., 2000) and by our laboratory for the cytosolic one (Diaz et al., 2000).

In the present studies, it is shown that liver microsomes under nitrogen, even in the absence of NADPH, have some ability to produce nitrite (Table 1). That effect is further enhanced by the presence of NADPH and is fully inhibited by DPI and partially inhibited by pure CO. Under air, all these processes are not observed (Table 1).

The pure CO inhibitable fraction suggests that, cytochrome P450 itself plays some role in the overall nitrite forming process since CO is a well known inhibitor of P450-mediated processes (Omiecinski et al., 1999). This is not unexpected, for P450 is known to bioactivate other nitroderivatives to NO (Sakai et al., 2000). Further, the complete inhibition of all nitrite formation from Nfx by DPI (including the fraction that is mediated presumably by P450 itself) might be interpreted as showing that, the reduced form of P450 is the one activating Nfx to $^{\bullet}$NO. The other fraction would be activated directly by P450

reductase itself, as shown by our studies employing pure human P450 reductase (Table 3). However, and in contrast to what occurs with the Nfx nitroreductase activity, no enhancement of Nfx transformation to nitrite was found when FAD is present (Table 3). The activity is completely suppressed by FAD irrespectively of the presence of NADPH or not (Table 3).

Since some degree of inhibition of nitrite formation from Nfx by FAD is also observed in the studies on chemical model systems (Table 4), it is our hypothesis that this FAD inhibitory effect on nitrite formation might be due to a $^{\bullet}$NO trapping effect of FAD. This, however, remains to be established.

Concerning the potential biological consequences of $^{\bullet}$NO formation from Nfx, some considerations could be made. On one hand, this reactive moiety interacts with virtually every component: proteins, lipids, sugars, nucleic acids and with many low molecular weight oxidants and reductants (Arteel et al., 1999; Sies and Groot, 1992; Beckman and Koppenol, 1996). Its formation in the liver endoplasmic reticulum can destroy P450 (Minamiyama et al., 1997). Its formation near or within the nuclei may act to transduce molecular signals regulating diverse processes (Lane and Gross, 1999; Gudi et al., 2000). Further, the simultaneous biotransformation of Nfx to its nitroanion radical, followed of formation of superoxide anion by redox cycling (Docampo and Moreno, 1985; Castro and Toranzo, 1988) and the production of $^{\bullet}$NO as here reported, might lead to peroxynitrite formation with relevant potential toxic effects (Halliwell et al., 1999). Concerning the pathophysiological significance of these findings we are not in position to reach a definitive conclusion. In effect, on one hand the process leading to $^{\bullet}$NO only occurs under oxygen free N_2 and that might cast doubts about its possibility to significantly operate under in vivo conditions. Further, the quantitative significance of this Nfx activation pathway when compared to that leading to Nfx consumption via biotransformation to its nitroanion radical, redox cycling and promotion of lipid peroxidation (Docampo and Moreno, 1985; Castro and Toranzo, 1988) appears to be much smaller. These facts cast doubts about the possibility that this $^{\bullet}$NO forming pathway were critical in Nfx toxicity to humans.

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