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Mechanism of impairment of cytochrome P450-dependent metabolism in hamster liver during leishmaniasis

Timir B. Samanta,[a,*] Nilanjana Das,[a] Manika Das,[a] and Radharani Marik[b]

[a] *Department of Microbiology, Bose Institute, Kolkata 700054, India*
[b] *Laboratory of Immunology, National Institute of Aging, National Institutes of Health, Baltimore, MD 21224, USA*

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Abstract

The mechanism of impairment of cytochrome P450 (P450)-dependent metabolism in hamster liver during leishmaniasis is reported. A significant decrease in the level of P450 was observed on the 20th day of infection when the parasite load in the liver was maximum. The decrease in P450 level was accompanied by a significant increase in the level of marker enzymes of liver and degeneration of liver tissue. The impairment was isozyme-specific and concomitant with the induction of nitric oxide synthase. The results of in vitro experiments with generated nitric oxide and with scavengers demonstrated that the impairment is mediated by NO. Treatment of the infected animals with a combination therapy showed reduction in parasite load, reversal of P450 impairment, and recovery of liver enzymes and tissue close to normal.

Keywords: Leishmaniasis; Cytochrome P450 impairment; Nitric oxide; Mediator

Dedication

During the Summer of mid-1980s I met Professor I.C. Gunsalus (Gunny to his colleagues) in a group meeting of his associates. He was discussing research problem on the monoxygenase system degrading *p*-cymene. For me it was a journey from eukaryotes to procaryotes because prior to joining his lab I was examining the monoxygenase system of fungal microsomes. I started to grow *Pseudomonas*, the pet bug in Gunny's lab. It had a long lag phase of growth that became very difficult to overcome. I had a long hard battle growing this bug, but without success. In fact, I was planning to change the problem and sought Gunny's advice. He surprised me by saying that he would respond the next day. After a long discussion, 24 mixtures were tried for growth, but they did not work. The next day using the best growth as inoculum another 4 combinations were tried. To my surprise, I found 2 of them were fast growing, the 8-h lag phase reduced to less than 2 h. Looking at the growth curve Gunny said, "It's incredible, but this is biology; if you ask me the reason, I don't know." This is Gunny, who is so modest. Although a biochemist by training, he has insights into the domains of chemistry and biology which make him the doyen of P450 research.

Introduction

Cytochrome P450 (P450) enzymes include a large, but closely related, superfamily of distinct gene products with different substrate specificities [1]. Each protein has characteristic molecular properties and catalytic specificity [2,3]. The drug-metabolizing enzymes, viz., P450 (phase I enzyme) and glutathione-*S*-transferase (GST, phase II enzyme), play crucial roles, in drug metabolism within the system. Evidently, alterations in the level of these enzymes will have a serious effect on residual drug management which might lead to adverse effects. White et al. [4] observed a marked difference in the pharmacokinetics of quinine in normal and malaria-infected people. Clearance of quinine was reduced, resulting in increased plasma concentration in acute falciparum malaria and was significantly lowered in cerebral cases. Similarly, impairment of microsomal drug metabolizing

* Corresponding author. Fax : +91-33-2334-3886.
E-mail address: timir@bic.boseinst.ernet.in (T.B. Samanta).

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activity was observed during amoebiasis, filariasis, *Fasciola hepatica* infection, and leishmaniasis [5–9]. Hamsters infected with *Leishmania donovani* (*L. donovani*) were reported to suffer loss in P450 and cytochrome b_5 in membranes, leading to a decreased rate of P450-mediated reactions. Despite wide acceptance that parasitic infection can inhibit hepatic drug metabolism, the mechanism of this effect remains unknown, particularly in leishmaniasis. Our study reveals that nitric oxide (NO), produced in hamster liver infected with *L. donovani*, mediates the impairment of P450 enzymes in vitro and in vivo, which can be protected against by a combination therapy of the infected animals.

Materials and methods

Experiments utilized age and sex-matched animals obtained from Organon Research Centre, Kolkata, India. The strain *Leishmania donovani* (*L. donovani* 2302) was used for infection of animals.

Reagents

Ethylenediamine tetraacetic acid (EDTA), dithiothreitol (DTT), protein A (PA), sulfosalicylic acid, glucose 6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide phosphate (NADP), phenobarbital (PB), L-N^{ω}-methyl arginine methyl ester (L-NAME) and gentamycin were purchased from Sigma-Aldrich Corporation, USA; sodium stilbogluconate (SB) was from Gluconate Health Limited, Kolkata, India; and other chemicals and reagents were purchased from Merck (India).

Culture of parasites

The flagellated promastigotes were maintained on N-N-N medium prepared by mixing 9 g NaCl, 10 g agar, 50 mg gentamycin, and 1000 ml distilled water, pH 7.2–7.4. The medium was distributed in test tubes (3.75 ml each) and sterilized, and defibrinated rabbit whole blood (1.25 ml) was then aseptically added to the molten agar in each tube.

The amastigote form of *L. donovani* was maintained by serial passage through a host hamster. The spleen of the infected animal was dissected out and homogenized in normal saline by passing through a fine wire mesh. The homogenate was made free of particulate matter and was injected intracardially to infect the experimental animals. The number of parasites in an organ was estimated by an impression smear stained with Geimsa.

Isolation of liver microsomes

Hamsters were sacrificed by decapitation. The liver was dissected out, washed to make it free of blood, and placed in ice-cold Tris–acetate buffer (50 mM, pH 7.5) (5 ml/g liver) supplemented with glycerol (20%, v/v), EDTA (1 mM), and DTT (10 mM) (Buffer A), homogenized, and centrifuged at 22,000*g* for 20 min. The supernatant was again centrifuged at 100,000*g* for 1 h. The pellet was suspended in phosphate buffer (0.2 M, pH 7.4) containing glycerol (20%, v/v), EDTA (1 mM), and DTT (10 mM) (Buffer B) for future use.

Parasite load estimation

The parasite burden was expressed as percentage parasitemia. The impression smear of the infected liver or spleen was stained with Geimsa and the ratio of nucleated cells to the parasites was determined.

Enzyme assays

Aniline hydroxylation. Aniline hydroxylation (AH) was measured according to the method of Coombs et al. [9] in the presence of an NADPH-regenerating system. The reaction mixture (total volume 1.5 ml), containing microsomal protein (3 mg), glucose 6-phosphate (4 mM), NADP (0.4 mM), glucose-6-phosphate dehydrogenase (4 U), and aniline hydrochloride (8 mM) as substrate all in Buffer B, was incubated at 37 °C on a rotary shaker for 30 min. The reaction was stopped by the addition of trichloroacetic acid (TCA, 0.5 ml). The precipitated protein was removed by centrifugation at 18,000*g* for 5 min. The supernatant layer (1 ml) was transferred to a test tube, and 0.5 ml of 2% phenol in 0.2 N NaOH was added, followed by 0.5 ml of Na_2CO_3 (1 M). After incubation at 37 °C for 30 min the color developed was read at 630 nm, and the value was compared with a standard curve. The activity was expressed as nmol/min/mg protein.

Aminopyrine demethylation. Aminopyrene demethylation (APD) activity was measured by the method of Ndifor et al. [10] exactly in the same way used for assay of aniline hydroxylation. The formaldehyde formed was trapped with semicarbazide hydrochloride (5 mM) and was measured with the Nash reagent.

Estimation of serum nitrate/nitrite. The serum nitrate/nitrite content was determined after deproteinization. Serum (500 μl) was mixed with sulfosalicylic acid (35%, 100 μl) and allowed to react at room temperature (25 °C) for 10 min with intermittent shaking followed by centrifugation at 10,000*g* for 15 min. The supernatant layer was then mixed with NH_4Cl (5%, 300 μl) and NaOH (5%, 60 μl) and the mixture was passed through a copper–cadmium column for conversion of nitrate to nitrite. The color was developed with Greiss reagent [11] and the amount of nitrite formed was calculated from a standard curve obtained with known nitrite.

Estimation of marker enzymes of the liver. Serum glutamic-oxalacetic transaminase (SGOT) activity was determined by the method of Reitman and Frankel [12] using α-ketoglutarate and L-aspartate as substrates. The absorbance of the dinitrophenylhydrazone derivative of α-ketoglutarate was measured at 510 nm. The activity was expressed as μmol of pyruvate formed/min/liter serum.

The serum glutamic-pyruvic transaminase (SGPT) activity was measured and expressed identically except that alanine was used as substrate instead of aspartate. Serum alkaline phosphatase (ALP) and serum acid phosphatase (ACP) activity were measured using *p*-nitrophenyl phosphate as substrate [13]. The pH values of the reaction mixtures were maintained at 9.3 and 5, respectively. The actvity was expressed as mmol *p*-nitrophenol formed/min/liter serum.

Histological examination of liver tissue. The livers were taken from each group of animals and passed through graded alcohol (50–90%) followed by xylene, and the tissues were then embedded in paraffin. Sections were cut with a microtome, stained with eosin, and hematoxylin, fixed with DPX, and examined under the microscope.

Effect of nitric oxide producer and/or scavengers on P450 activity in vitro. Liver microsomes (0.4 mg protein) isolated from hamster (non-infected) taken in Tris–HCl buffer (50 mM, pH 7.6, 1 ml) supplemented with $MgCl_2$ (5 mM) and NADPH (0.33 mM) received sodium nitroprusside (SNP) at varying concentrations (0.01–0.5 mM) separately. After exposure to SNP on ice for 30 min, the reaction mixture received substrate, i.e., aniline or aminopyrene, and was incubated at 37 °C for 30 min. After the incubation was over, the reaction was stopped by addition of trichloroacetic acid (20%). It was then centrifuged and the supernatant was assayed for aniline hydroxylase and aminopyrine demethylase activity individually as described above.

In another set of experiments, the reaction mixtures contained diethylamine (DEA), hemoglobin (Hb), or myoglobin (Mb) (each 0.01 mM) in conjunction with sodium nitroprusside and were incubated as described above. The aniline hydroxylation and aminopyrine demethylation activities were then measured.

Effect of drug treatment. In view of the induction of nitric oxide synthase (i-NOS) and involvement of nitric oxide (NO) in impairment

of P450 in hamster liver infected with *L. donovani*, a combination therapy was tried. During the drug regimen, the animals were divided into six groups. Animals in group 1 were uninfected controls, while animals in groups 2–5 were all infected with *L. donovani*. Those in group 3 received stilbogluconate at 10 mg Sb^{iv}/kg for 7 days, those in group 4 received protein A at 50 mg/kg/week for 5 weeks, those in group 5 received SB and protein A as in group 3 and 4, and those in group 6 received phenobarbital at 80 mg/kg for 3 days and SB as in group 3 and L-NAME. Animals receiving L-NAME were given the drug ad libitum in their drinking water (10 mM), beginning 4 days prior to killing. The levels of parasitemia, P450, and nitric oxide were determined with the microsomes of the sacrificed animals, and the results were compared with the respective controls.

Protein estimation. Protein was estimated by the method of Lowry et al. [14] using bovine serum albumin as standard.

Determination of cytochrome P450. P450 was measured by the spectrophotometric method recommended by Omura and Sato [15].

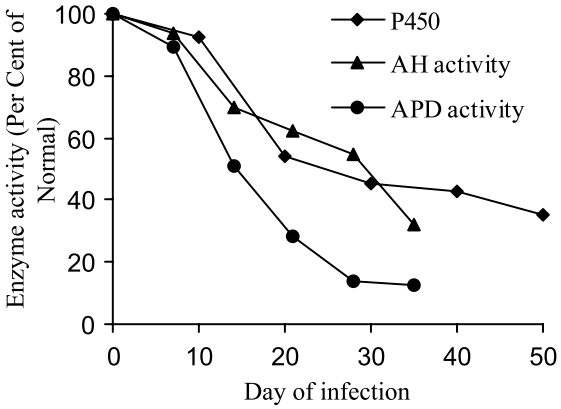

Fig. 2. Impairment of P450 activity during infection. Data are from pooled liver microsmes of 5 infected animals of group 2.

Results

The results of the experiment on the kinetics of infection are presented in Fig. 1. The animals showed an increase in parasite count in the liver after 7 days of infection, which reached a maximum on day 20, after which there was a gradual decrease. In contrast, the parasite load in the spleen increased rapidly after the third week of infection, and it remained unabated until death ensued (Fig. 1). The P450 profile of the infected control animals (Fig. 2) revealed that the level of the enzyme fell in the first 2 weeks of the disease. It was as low as 54% of the normal control by the third week. Although the level of the enzyme continued to decrease with the progress of the disease, the rate of decrease was much slower in later stages. The lowest level recorded before the onset of death was 38% of the normal control. The first rapid phase of decrease in the enzyme level was found to be associated with the increase in the liver parasitaemia. A similar correlation between parasite load and P450 level in the infected animal has been observed by others [9,16]. The assay of microsomal P450 activity in liver of the infected animals with aniline or aminopyrine as substrate showed a decrease in both the cases. At the end of the 5th week of infection, the aminopyrine demethylase activity was 12% of normal, while the aniline hydroxylation was 32%, indicating that the impairment was isozyme-specific. It was interesting to note that the impairment of P450 was accompanied by induction of nitric oxide synthase (data not shown). The level of nitrate/nitrite in the serum was found to be 53.3 μmol/L on the 20th day of infection. However, the level started to decrease after 30 days, indicating that the impairment of P450 and nitric oxide synthase induction is similar.

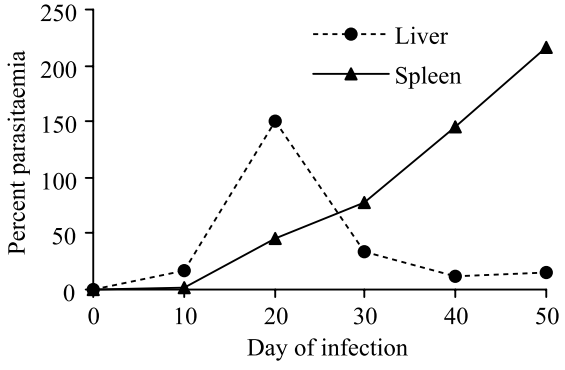

Fig. 1. Kinetics of infection. The extent of parasitemia in liver and in spleen tissue of hamsters after infection with 2×10^7 amastigotes of *L. donovani* is shown as a function of time. Data are from pooled tissues of 5 infected animals of group 2.

Histological studies

Histological examination of liver tissue collected from normal (uninfected) hamsters showed radial distribution of hepatocytes around the central vein. This arrangement was found to be altered in *L. donovani*-infected animals as a result of massive infiltration of mononuclear and other cells around the central vein.

Liver marker enzymes

The degeneration of liver tissue of the infected animals was reflected by a 2- to 3-fold increase in SGOT, SGPT, ALP, and ACP after 20 days of infection compared to the uninfected control.

Studies in vitro

Experiments in vitro done with microsomes obtained from uninfected control animals using sodium nitroprusside exhibited a decrease in both aniline hydroxylation and aminopyrine demethylation activity in a dose-dependent manner. On the other hand, nitric oxide scavengers, viz., diethylamine, hemoglobin, and myoglobin, reversed the deleterious effect of nitric oxide, albeit to varying extents. Hemoglobin exhibited better

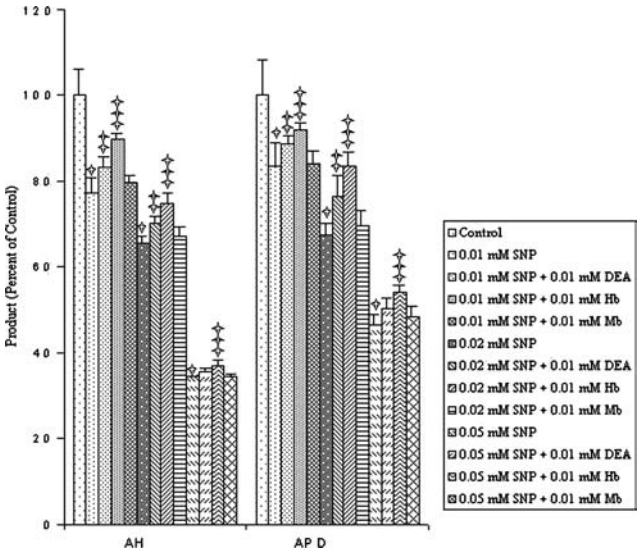

Fig. 3. P450 activity in vitro in the presence of nitric oxide production and scavengers (AH, aniline hydroxylation; APD, aminopyrine demethylation). Data are means ± SD ($n = 3$). ☆ denotes significant inhibition of activity relative to control ($P < 0.01$); ☆☆ denotes significant increase in activity in presence of DEA ($P < 0.01$); ☆☆☆ denotes significant increase in activity in presence of Hb.

protection at all concentrations of sodium nitroprusside used (Fig. 3); the optimal concentration of the scavenger used was 0.01 mM.

Effect of drug treatment

In view of the impairment of P450 vis-à-vis induction of nitric oxide synthase due to infection, the animals were subjected to a combination therapy. The results are presented in Fig. 4. Both stilbogluconate and protein A either individually or in combination reduced parasitemia quite considerably. In fact, the combination showed an infection that was 27% of the infected control, while stilbogluconate alone could reduce 85% of the infection. On the other hand, treatment of infected animals with phenobarbital alone (data not shown) or with stilbogluconate and L-NAME did not exhibit reduction in parasite count. Together they showed a high level of infection, indicating the importance of achieving a balance in the drug administered. The effect on the marker enzymes of liver was almost identical with that of the infected control. This might be due to the inhibition of nitric oxide production by L-NAME present in the combination. Although partial recovery of P450 was observed in all of the treatments, the maximum benefit in terms of reversal of impairment of P450 was achieved with the combined treatment of stilbogluconate and protein A. Moreover, stilbogluconate in combination with protein A resulted good recovery of SGOT, SGPT, ALP, and ACP of liver of the infected animal close to normal. This was reflected in the histological examination of the liver of drug-treated animals, which exhibited a more compact cellular arrangement of hepatocytes filled with intracellular materials like that of the uninfected control.

Discussion

The results with the parasite model reported here reflect a fundamental mechanism regulating the expression of a very important mammalian enzyme system. The occurrence of similar effects in humans would have severe implications for individuals infected with leishmania parasites. Decreased hepatic xenobiotic-metabolizing capacity would result in altered drug

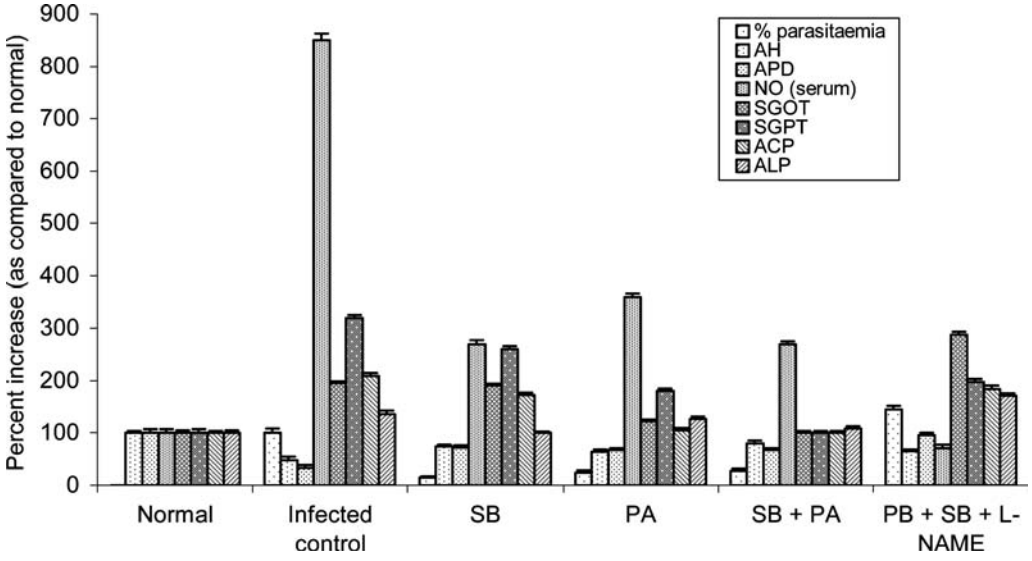

Fig. 4. Effects of combination therapy on hamsters infected with *L. donovani*.

clearance rates and so increase adverse drug effects [17–19].

Evidently the mechanism of impairment of P450-dependent metabolic activity, also known as mixed function oxidase (MFO) activity, is very important with respect to both drug design and detoxification. It is believed that more than one mechanism's involved in the impairment of MFO system during parasitic infection. In diseases such as facioliasis, schistosomiasis, and hepatic amoebiasis, the damage to the liver caused by peroxides, superoxide, and hydrolytic enzymes produced during the infection had been reported to be responsible for the impairment of MFO system [16]. In the present study, the results of histological examination of liver tissues vis-à-vis the level of marker enzymes of infected hamsters liver are complementary to previous reports. Immunomodulators such as insect venom, Freund's adjuvant, and *Mycobacterium butyricum* depress the MFO system by enhancing the activity of heme oxygenase—an enzyme responsible for inactivation of P450 via degradation of heme component [20]. Again, immunostimulants such as lipopolysaccharide (LPS), interferon-γ, interleukins, and TGF-α are potent suppressors of P450 expression [21]. It is known that infection triggers the synthesis of immunostimulants, which in turn switch on the synthesis of i-NOS. In the present study, both reduction in parasite load and reversal of impairment of P450 in infected animals by protein A treatment provide further support for the previous observations. Presumably the phenomena of induction of i-NOS during leishmaniasis, reversal of impairment of P450 by protein A, and nitric oxide scavengers clearly suggest that NO is a mediator of decrease in P450-dependent metabolism in leishmania-infected animals.

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