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INFLUENCE OF HYPOXIA ON THE METABOLISM AND EXCRETION OF MISONIDAZOLE BY THE ISOLATED PERFUSED RAT LIVER—A MODEL SYSTEM

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Abstract—The isolated perfused rat liver was evaluated as a model system for the characterization of misonidazole metabolism under hypoxic conditions. Misonidazole metabolism by livers perfused under aerobic conditions was also examined. The clearance of misonidazole was more than three times greater under anaerobic compared to aerobic conditions (4.94 ± 1.56 vs 1.27 ± 0.22 ml/min; x ± S.D., N = 3). Misonidazole metabolites were detected only in the bile. Analysis of these metabolites by reverse-phase high performance liquid chromatography (HPLC) demonstrated that misonidazole metabolism was also qualitatively changed when anaerobic conditions were employed. Misonidazole β-glucuronide was the major metabolite detected under aerobic conditions, but it was a minor metabolite in anaerobically perfused livers. The three major metabolites produced under anaerobic conditions were not characterized, but desmethyl misonidazole (RO-07-9963) and the 2-amino-imidazole derivative of misonidazole (1-[2-aminoimidazol-1-yl]-3-methoxy-2-propanol) were excluded as possible structures.

Misonidazole (3-methoxy-1-[2-nitromidazol-1-yl]-2-propanol) is a nitrosubstituted imidazole derivative that is undergoing clinical trials as a radiation sensitizing agent [1]. Recent evidence suggests that in hypoxic tissue misonidazole also potentiates chemotherapeutic agents or may even have toxic properties of its own. Misonidazole was found to enhance the cytotoxicity of several alkylating anticancer agents in hypoxic Chinese hamster V-79 cells [2]. The activities of alkylating antitumor drugs in tumor-bearing mice were also enhanced by misonidazole [3]. It has also been demonstrated that misonidazole is toxic and/or mutagenic to cultured cells [4–8]. This toxicity is restricted to or intensified by hypoxic conditions.

Evidence has been presented that hypoxic cell toxicity by misonidazole is the consequence of reductively-generated reactive metabolites that subsequently bind covalently to cellular constituents. Varghese and Whitmore [9] reported that radioactivity became associated with acid-insoluble precipitate from CHO cells and KHT murine fibrosarcoma cells incubated with [2-^{14}C]misonidazole. They also demonstrated that the associated radioactivity increased 4-fold if the incubations were carried out under anaerobic conditions. Furthermore, others have shown that rat liver microsomes convert misonidazole to products that covalently bind to protein and that the binding was inhibited by oxygen or glutathione [10].

Although metabolism under hypoxic conditions appears to be required for misonidazole binding, the nature of this metabolism or the products have not been characterized. Metabolism of misonidazole has been studied using subcellular fractions or cultured cells [10–12], but information concerning misonidazole metabolism and the influence of hypoxia on this metabolism in the intact tissue is largely lacking. The objective of this study was to determine the feasibility of the perfused rat liver as a model system to characterize the metabolism of misonidazole under hypoxia. Although the liver is not a target for misonidazole-induced toxicity *in vivo*, this organ is metabolically very active, and it should be evident if reactive drug products are formed.

METHODS

Chemicals. Misonidazole (RO-07-0582) and desmethyl misonidazole (RO-07-9963) were supplied by Dr. W. E. Scott from Hoffmann-LaRoche, Inc. (Nutley, NJ). The amino-derivative of misonidazole (1 - [2 - aminoimidazol - 1 - yl] - 3 - methoxy - 2 - propanol) was prepared by catalytic reduction of misonidazole as previously reported [13]. High performance liquid chromatography (HPLC)-grade solvents were purchased from Fisher Scientific (Fairlawn, NJ). All other chemicals and reagents were the highest grade commercially available and were obtained from reliable sources.

Animals. Adult male Sprague–Dawley CD rats were supplied by Simonsen Laboratories (Gilroy, CA) and were maintained *ad lib.* on Wayne's Lab-blox rodent feed and tap water.

Liver perfusion. The animals were anesthetized using diethyl ether, and the livers were surgically prepared for perfusion as previously reported [14], except that both the portal and hepatic veins were cannulated. An apparatus was constructed from Lexan plastic for perfusing livers under conditions where the oxygen content in the tissue could be carefully monitored and controlled. A YSI model 53 monitor (Yellow Springs Instrument Co., Yellow Springs, OH) was used to measure the oxygen concentration in the efferent and afferent perfusion media. The livers were perfused only through the

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portal vein using a recirculating system. The flow rate of perfusion fluid through the livers was maintained at 4 ml per min per g tissue in order to deliver sufficient oxygen to the tissue using cell-free medium [15]. The perfusion medium was Krebs–Ringer bicarbonate buffer fortified with glucose (5 mM). In aerobic experiments, the tissue oxygen concentration was maintained at 0.1 mM or greater and was determined by measuring the oxygen concentration in the perfusion fluid emerging from the hepatic vein [14]. The perfusion medium was aerated by passing it through a column (2.5 × 32 cm) of glass beads (4 mm) through which the appropriate gas mixture was flowing. The gas mixture was composed of oxygen or nitrogen and enough carbon dioxide to maintain the perfusion medium pH at 7.4. Gas flow was controlled by Gilmont flowmeter-micrometer valve assemblies (Cole–Parmer, Chicago, IL). The gas flow rate was 1 litre/min.

Misonidazole (10 mg; 50 μmoles) was solubilized in perfusion medium (20 ml) by sonication and poured into the apparatus (containing 80 ml of medium) to produce a 100 ml total volume and a 0.5 mM initial drug concentration.

Bile was collected at 20-min intervals, and perfusion medium aliquots (200 μl) were removed at various times to quantitate the misonidazole.

At the end of the experiments, the liver was homogenized in 50 ml of water–methanol (2:1 v/v). This procedure was carried out quickly to minimize metabolism of misonidazole remaining in the tissue. The protein was removed by centrifugation, and an aliquot (1 ml) of the supernatant fraction was concentrated to dryness under nitrogen. The residue was taken up in 0.1 ml of methanol for chromatographic analysis.

Plasma clearance by the perfused livers was calculated by dividing the misonidazole dose by the area under the perfusion medium concentration curve as outlined by Rowland *et al.* [16].

Chromatography. Samples were analyzed for misonidazole and its derivatives using a Varian Instruments (Walnut Creek, CA) model 5000 HPLC fitted with a Varian MCH-10 reverse-phase column. The column was eluted with a water (solvent A): methanol (solvent B) gradient. Both solvents contained acetic acid (1%, v/v) for pH control. The gradient began with 100% A, changed to 10% B during the next 30 min, and then progressed to 30% B in the following 20 min. The flow rate was 1 ml/min, and the column effluent was monitored at 254 or 330 nm with a Varian model VUV-10 detector. Under these conditions, misonidazole had a retention time of 42.5 min.

Perfusion medium and bile samples (20 or 1 μl respectively) were chromatographed directly without workup. Extracts of liver homogenate (10 ml) were concentrated to dryness and reconstituted in water (1 ml), and 20 μl samples were chromatographed. The misonidazole content in the samples was determined by comparing the height of the misonidazole absorbance peak with a standard curve prepared by chromatography of standard solutions.

Enzymatic hydrolysis of conjugated metabolites. Bile samples (10 μl) were added to sodium phosphate buffer (50 μl, 0.1 M, pH 5.0) containing 5000 units of β-glucuronidase (type B-10, Sigma Chemical Co., St. Louis, MO). The resulting solution was incubated at 37° for 60 min, and a 10 μl aliquot was chromatographed directly.

RESULTS

Clearance of misonidazole from the circulation under aerobic and anaerobic conditions. Three experiments were carried out in which oxygenated, perfused livers were given misonidazole (0.5 mM), and the rate of drug disappearance was monitored. Twenty minutes after the misonidazole addition, the gas mixture aerating the perfusion medium was changed to N_2:CO_2 (95:5). This change was followed by a decrease in the O_2 content of the perfusion medium to unmeasurable levels (<0.01 μmole/ml). This decrease was accompanied by an immediate acceleration in the rate of misonidazole removal from the circulation (Fig. 1).

Experiments were subsequently carried out in order to more quantitatively characterize the increase in drug disappearance under anaerobic compared to aerobic conditions. In these experiments, livers were perfused with misonidazole (0.5 mM initial concentration) for 60 min under aerobic or anaerobic conditions. The drug concentration in the perfusion medium during this period was determined as shown in Fig. 2. The results from the individual experiments were plotted on semilog paper, and plasma clearance rates were calculated. Aerobically perfused livers cleared misonidazole from the circulation at a rate of 1.27 ± 0.22 ml/min ($\bar{x}$ ± S.D., N = 3). In anaerobically perfused livers, on the other hand, misonidazole was cleared more than three times faster. The clearance rate under anaerobic conditions was 4.94 ± 1.56 ml/min ($\bar{x}$ ± S.D., N = 3). Furthermore, no detectable misonidazole remained in the tissue at the end of the experiment, and little unchanged drug was excreted in the bile. These observations demonstrate that the increased

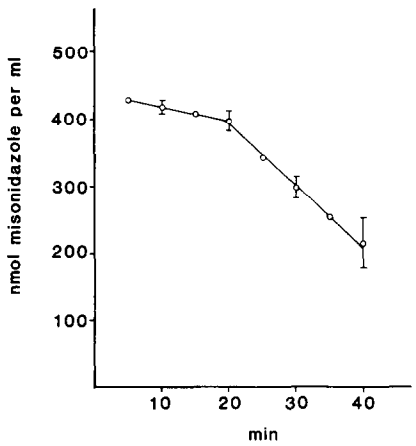

Fig. 1. Clearance of misonidazole by livers perfused under aerobic followed by anaerobic conditions. The perfusion medium was aerated with O_2:CO_2 (95:5) for the first 20 min and N_2:CO_2 (95:5) for the following 20 min. The points are the average of three experiments and the bars represent the standard deviations.

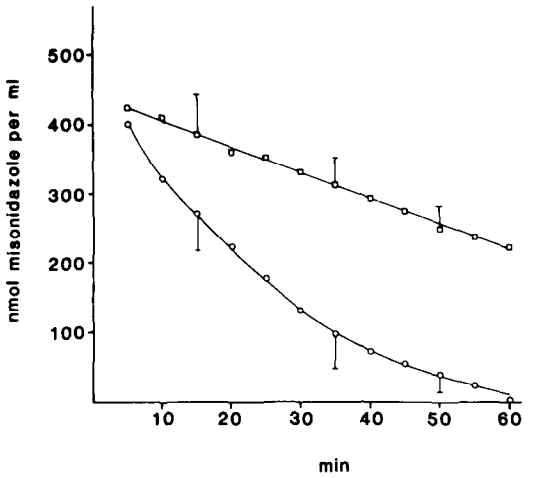

Fig. 2. Clearance of misonidazole by livers perfused for 60 min under aerobic (□) or anaerobic (○) conditions. These results are the average of three experiments, and the bars represent one-half the S.D. Typical or maximum standard deviations are shown (others were omitted for clarity).

misonidazole clearance rate under anaerobic conditions was due to increased misonidazole metabolism and was not the result of enhanced tissue accumulation or biliary excretion of unchanged drug.

Qualitative differences in misonidazole metabolism by aerobically versus anaerobically perfused livers. Misonidazole metabolites were not detected in the perfusion medium under aerobic or anaerobic conditions (Fig. 3A). In the bile, however, clear differences could be seen in the drug metabolite profile from aerobically perfused livers compared to those from livers perfused under anaerobic conditions (Fig. 3, B and C). Aerobically perfused livers excreted a β-glucuronide conjugate of the parent drug (metabolite **1**) as the major metabolite and three unidentified minor metabolites (**3**, **4** and **5**, Fig. 3B). In the bile from anaerobically perfused livers, however, the reverse was seen (Fig. 3C): the misonidazole β-glucuronide was a minor metabolite, and the unidentified metabolites **3**, **4** and **5** were predominant.

Although metabolites **3**, **4**, and **5** were not identified, two possible structures were excluded by chromatographic analysis. Both desmethyl misonidazole and the amino-derivative of misonidazole had retention times (14.5 and 10.5 min respectively) that were shorter than those of unknown metabolites **3**, **4**, and **5**. In addition, none of the unknown metabolites were nitro-substituted imidazoles as evidenced by their lack of measurable absorbance at 330 nm. Bile from untreated livers perfused under aerobic or anaerobic conditions contained no u.v.-absorbing materials that correspond to the misonidazole metabolites.

Identification of misonidazole β-glucuronide. Bile from aerobically perfused livers given misonidazole was subjected to hydrolysis by β-glucuronidase. HPLC analysis demonstrated that this treatment resulted in the complete conversion of the major peak (R_t = 33 min) to misonidazole (Fig. 4). Similar

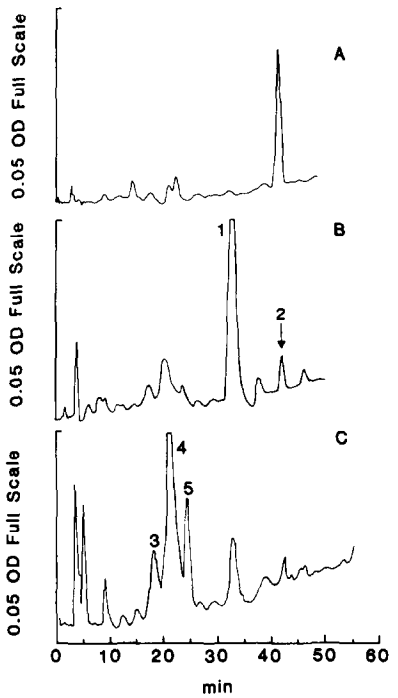

Fig. 3. (A) Chromatographic analysis of the perfusion medium (20 μl) containing misonidazole. This panel depicts a sample taken after 20 min of perfusion under aerobic conditions. Samples taken at other times or from livers perfused under anaerobic conditions were similar but contained greater or lesser amounts of misonidazole. The column effluent was monitored at 254 nm (see Methods for chromatographic details). (B) Chromatographic analysis of a bile sample aliquot (2 μl) collected between 20 and 40 min from a liver perfused under aerobic conditions. Peak **1** is misonidazole β-glucuronide, and peak **2** is misonidazole. (C) Chromatographic analysis of a bile sample aliquot (2 μl) collected between 20 and 40 min from a liver perfused under anaerobic conditions. Major peaks **3**, **4**, and **5** were unidentified. Misonidazole β-glucuronide was a minor metabolite under these conditions.

treatment of bile but without β-glucuronidase did not change the metabolite profile. These results allow the tentative identification of the major biliary metabolite of misonidazole under aerobic conditions as misonidazole β-glucuronide.

Treatment of bile from livers perfused under anaerobic conditions with β-glucuronidase had no effect on unknown metabolites **3**, **4**, or **5**.

DISCUSSION

It is evident from our results that the rate of misonidazole metabolism by the isolated perfused rat liver is strongly influenced by the tissue oxygen levels. Under anaerobic conditions, the rate of misonidazole metabolism was clearly increased compared to that seen in oxygenated tissue. Examination of the biliary metabolites using HPLC demonstrated that misonidazole metabolism was also qualitatively changed by lowering the tissue oxygen levels. These changes in metabolism may be related to the toxicity of misonidazole to hypoxic cells.

In agreement with a recent report by McManus

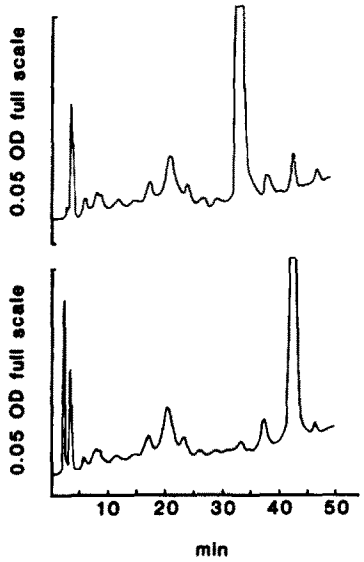

Fig. 4. Treatment of a bile sample containing misonidazole β-glucuronide (upper figure) with β-glucuronidase, resulting in the liberation of free misonidazole (lower figure). See Methods for experimental details.

et al. [17], desmethyl misonidazole was not a major metabolite in isolated perfused rat livers. We did not detect this metabolite in either the bile or the perfusion medium under aerobic or anaerobic conditions. This observation is suggestive that either misonidazole is not *O*-demethylated or that, if this reaction does occur, the product is rapidly metabolized to other derivatives. In support of the first explanation, glucuronide conjugates of *O*-demethylated misonidazole were not detected in this study.

It appears that the bile is a major excretion route for misonidazole metabolites in perfused rat livers. Although misonidazole was the only material detected in the perfusion medium, more sensitive methods will probably reveal additional products that may have little or no u.v. absorbance above 250 nm.

The major biliary metabolite under aerobic conditions was misonidazole β-glucuronide. This derivative was a minor metabolite in anaerobically perfused livers. The major biliary metabolites in anaerobically perfused livers were not identified, but desmethyl misonidazole and the amino-derivative of misonidazole were excluded because the metabolites did not co-chromatograph with authentic samples of these materials. More sensitive analytical techniques will probably detect the amino-derivative of misonidazole, particularly under anaerobic conditions, as this material is the terminal misonidazole reduction product. It is not likely that we would have detected this amino-derivative in the study described here due to its limited optical absorbance at 254 or 330 nm.

We have shown in this study that the perfused rat liver is a useful tool for detecting drug biotransformation reactions that occur predominantly under hypoxic conditions. We have also demonstrated that misonidazole metabolism is both quantitatively and qualitatively influenced by the presence or absence of oxygen in the tissue. The recent synthesis of high specific activity [^{3}H]misonidazole in our laboratory will allow more definitive characterization of misonidazole metabolism [18]. We are currently pursuing the identities of the metabolites produced predominantly under anaerobic conditions, as this information may provide insight into the nature of toxic misonidazole derivatives formed in hypoxic tissue.

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