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Hypoxia-enhanced reduction and covalent binding of [2-^{3}H]misonidazole in the perfused rat liver*

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The effectiveness of cancer radiation therapy is often limited by the resistance of tumor tissue to such treatment. In some cases, this resistance is thought to be due to the hypoxic conditions in core areas of solid tumors. In normal tissue, oxygen can contribute to radiation-induced damage by combining with the freed electrons to form toxic superoxide radicals [1]. Oxygen can also exacerbate radiation-induced damage to membrane lipids by reacting with lipid radicals to form hydroperoxides [2], a process involved in lipid peroxidation. Several electron-affinic drugs are under development which are designed to mimic this action of oxygen in irradiated hypoxic tissue, and these agents are known as radiation sensitizers. Misonidazole (1-[2-nitroimidazol-1-yl]-3-methoxy-2-propanol) is a 2-nitroimidazole that is undergoing clinical trials as a radiation sensitizer.

Recent investigations have revealed that, in addition to being a radiosensitizer, misonidazole also potentiates the effectiveness of alkylating antitumor agents (see Ref. 3 for review). Furthermore, results from cell culture studies suggest that misonidazole may have toxic properties of its own, particularly under hypoxic conditions where reductive metabolism is believed to play a role [4–7]. Although reductive metabolism appears to be related to the action of misonidazole [8], information concerning its metabolism and the influence of hypoxia on this metabolism in intact mammalian tissue is largely lacking. Therefore, we have implemented the isolated perfused rat liver as a model system to characterize the metabolism of misonidazole [9]. Although the liver is not a target for misonidazole-induced toxicity, it is evident from our results that it can form reactive products from this drug. In this study, we demonstrate that [2-^{3}H]misonidazole undergoes reductive metabolism in hypoxic tissue, and that this metabolism correlates with production of highly reactive derivatives. These products extensively alkylate tissue protein and RNA (but not DNA), and deplete hepatic glutathione.

Materials and methods

Misonidazole (RO-07-0582) was supplied by Dr. W. E. Scott from Hoffmann–LaRoche, Inc. (Nutley, NJ). Misonidazole-amine (1-[2-aminoimidazol-1-yl]-3-methoxy-2-propanol) was prepared by catalytic reduction of misonidazole as previously reported [10]. [2-^{3}H]Misonidazole (8.41 mCi/mmole) was synthesized as described earlier [11].

Adult male Sprague–Dawley CD rats (200 ± 20 g) were anesthetized using diethyl ether, and their livers were surgically prepared and perfused using a cell-free recirculating medium as previously reported [9]. In aerobic experiments, tissue oxygen levels were maintained at 0.1 mM or greater. Misonidazole was solubilized by sonication in 40 ml of perfusion medium and added to the apparatus to produce a 100 ml final volume and a drug concentration of 0.5 or 2.5 mM. Perfusion medium samples (50 μl) were taken at timed intervals for chromatographic analysis of metabolites.

The rate of misonidazole reduction was estimated by measuring the formation of misonidazole-amine. Misonidazole-amine in the perfusion medium and tissue homogenates was quantitated radiometrically after isolation by high performance liquid chromatography (HPLC) (primary solvent system: 0.05 Tris–phosphate buffer, pH 2.75; the elution gradient was 0% methanol for 5 min, then 0–5% methanol during the next 23 min; the retention time of misonidazole-amine was 12.5 min). A Varian Instruments (Walnut Creek, CA) model 5020 HPLC, fitted with a Varian MCH-10 reverse-phase column was used. The flow rate was 1 ml/min, and fractions were collected at 30-sec intervals for radiometric quantitation of metabolites. Bile and perfusion medium samples were chromatographed directly without work-up. Tissue samples were homogenized in 4 vol. of methanol–H_2O (2:1) and centrifuged to remove the precipitated protein. Methanol was removed from the supernatant fraction under reduced pressure prior to chromatography.

Glutathione (GSH) depletion was accomplished as previously described [12] by administering diethyl maleate (DEM) to the rats 45 min before surgical removal of their livers. This treatment diminished hepatic GSH to 1.40 ± 0.21 mM ($\bar{x} \pm$ S.D., N = 3), or about 20% of control concentrations, when measured after the 60-min perfusion period. Hepatic GSH was estimated by measuring tissue nonprotein thiols using the colorimetric Ellman method [13]. Oxidized GSH (GSSG) was measured by the method described by Akerboom and Sies [14].

Covalent binding of misonidazole-derived radioactivity to tissue macromolecules (DNA, RNA, and protein) was determined by the phenol extraction method developed by Brookes and Lawley [15] and by Kuroki and Heidelberger [16].

Results

Misonidazole reduction, as indicated by the rate of misonidazole-amine biosynthesis, was greatly facilitated by hypoxia (Fig. 1). This finding is in accord with our earlier study where misonidazole clearance from the circulation was enhanced about 3-fold when livers were perfused under hypoxic conditions [9]. Extensive covalent binding of misonidazole derivatives to tissue protein was also observed. Importantly, the degree of covalent binding was increased under hypoxic compared to aerobic conditions, and it roughly paralleled the extent of reductive metabolism (Fig. 2A, 2B). GSH appears to play a protective role against the covalent binding, as binding was enhanced in livers depleted of this cofactor with DEM. GSH depletion did not alter the extent of misonidazole reduction, as indicated by the amount of misonidazole-amine produced (Fig. 2D). However, a relationship between misonidazole metabolism and GSH was demonstrated in experiments where the initial misonidazole concentration in the perfusion medium was increased 5-fold to 2.5 mM. This drug concentration represents the upper end of the therapeutic range [17]. The use of a 2.5 mM misonidazole concentration was accom-

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panied by greater reductive metabolism and covalent binding than were seen in livers given 0.5 mM misonidazole (Fig. 2E) and resulted in depletion of hepatic GSH during the 60-min perfusion period to 2.13 ± 0.35 mM ($\bar{x}$ ± S.D., N = 4), which reflected an approximate 70% loss of this tripeptide. GSH concentrations in livers exposed to 0.5 mM misonidazole under aerobic or hypoxic conditions were in the normal range (data not shown). Tissue GSSG concentrations were in the normal range (less than 30 μM [14]) under conditions where GSH depletion occurred; thus, the depletion was not the consequence of GSH oxidation to the disulfide. It appears, therefore, that GSH protected against misonidazole binding by combining with a reactive metabolite, possibly a precursor to misonidazole-amine. Aerobically perfused livers were also given 2.5 mM misonidazole, but normoxia could not be maintained under these conditions (data not shown).

Covalent binding to DNA and RNA was also determined, but only in livers from untreated rats perfused under hypoxic conditions. Covalent binding to RNA (1.38 ± 0.37; $\bar{x}$ ± S.D., N = 3; nmoles/mg dry wt) was similar to the protein binding, but covalent binding to DNA was markedly lower (0.025 ± 0.006; as above).

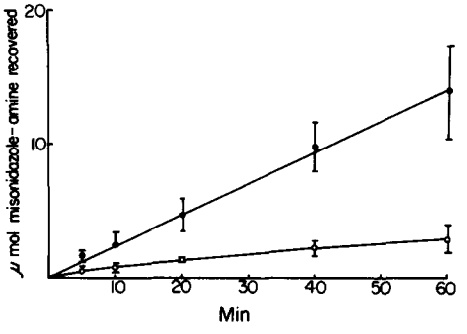

Fig. 1. Reduction of [2-^{3}H]misonidazole to misonidazole-amine (1-[2-aminoimadazole-1-yl]-3-methoxy-2-propanol) by isolated livers perfused under arobic (○) or hypoxic (●) conditions. Each point is the average of three or four experiments, and the bars represent standard deviations.

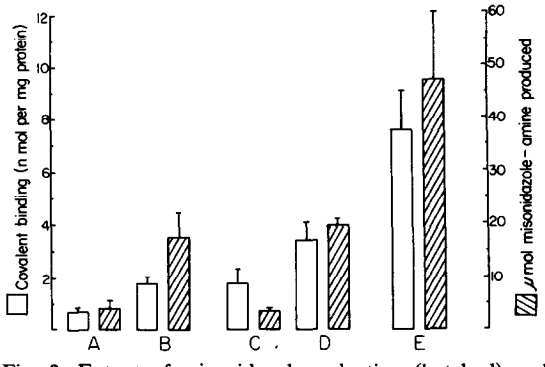

Fig. 2. Extent of misonidazole reduction (hatched) and covalent binding (open) to tissue protein as a function of various experimental conditions. Key: livers from untreated animals perfused under aerobic (A) or hypoxic (B, E) conditions; and livers from diethyl maleate-treated animals perfused under aerobic (C) or hypoxic (D) conditions. The initial drug concentration was 0.5 mM and A–D and 2.5 mM in E. The bars represent one standard deviation (N = three or four experiments). All measurements were taken following a 60-min perfusion period.

Oxidative metabolism of misonidazole (e.g. formation of desmethyl misonidazole) was not detected. In agreement with our earlier report [9], misonidazole β-glucuronide was a major biliary metabolite under aerobic but not hypoxic conditions (data not shown).

Discussion

Our results show that hypoxic conditions accelerated the reduction and covalent binding of misonidazole in perfused rat livers and, therefore, are in accord with those from misonidazole binding studies using cultured cells [4–7]. Perfusion of the livers under aerobic conditions diminished, but did not prevent, misonidazole reduction and binding. These occurrences may have resulted from localized areas of hypoxia, as the livers were perfused with a cell-free medium.

Misonidazole reduction was determined by measuring the formation of misonidazole-amine, the terminal product. We do not believe that misonidazole-amine was the reactive product generated by reductive metabolism, since this derivative is a stable compound [10]. Rather, it is more likely that the reactive product was a precursor to the amine, such as the nitroso- or hydroxylamine analog of misonidazole, as suggested by others, or further metabolies of these products. GSH appears to have played an intervening role, since tissue GSH was consumed under conditions where covalent binding to tissue protein by misonidazole products was extensive. In addition, depletion of hepatic GSH with DEM significantly enhanced the covelant binding to protein ($P < 0.025$). This finding is in contrast to results from studies using cultured Chinese hamster ovary cells where GSH depletion with DEM did not increase the extent of covalent binding [6, 7]. We have isolated a biliary metabolite that appears to be a GSH conjugate of a misonidazole metabolite, and we are currently confirming the structural identity of this material.

The reason for the strikingly greater binding to RNA than to DNA is not clear. Possibly, activation of misonidazole took place in the cytoplasm or in cytoplasmic organelles, and nuclear material was thus less exposed. Miller *et al.* [18] also reported greater binding of misonidazole to RNA compared to DNA in cultured EMT-6 tumor cells. However, their methods for determining "bound" radioactivity did not clearly distinguish metabolites from misonidazole derivatives covalently linked to cellular macromolecules. Therefore, our results and theirs are not easily comparable, as evidenced by their finding that less than 1% of the bound metabolites was associated with protein [18]. In contrast, protein was the major site of macromolecular covalent binding in our study (protein binding accounted for about 75%, and RNA binding about 4%, of the total bound radioactivity).

Application of the concepts summarized by Mitchell *et al.* [19] to our data leads us to suggest that the GSH transferase enzymes (EC 2.5.1.18) are probably not required for the formation of conjugated metabolites. This idea follows from the observation that GSH diminished but did not block misonidazole binding in experiments using low drug concentrations (0.5 mM) in which the hepatic GSH concentration remained in the normal range; if the reaction with GSH were enzyme-catalyzed, the conjugation would be rapid and binding of the metabolite to protein would not be expected to take place until after tissue GSH had been consumed.

Although we have not demonstrated that the observed covalent binding by misonidazole was deleterious to tissue viability, it is conceivable that extensive binding could be toxic. For example, the covalent binding that occurred following a 2.5 mM initial misonidazole concentration was nearly ten times greater than the level of binding required to produce acetaminophen-induced hepatotoxicity in mice [20]. Our results, therefore, support the proposal that misonidazole-dependent cytoxoicity is a consequence of extensive alkylation of essential macromolecules by a

reductively-generated, reactive metabolite. This proposal is also supported by results from other laboratories. For example, alkylation of enzymes could result in their inactivation. Varnes and Giaglow [21] recently reported that glycolytic enzymes in Ehrlich ascites tumor cells and Chinese hamster V79-379A cells were inhibited by exposure to misonidazole under hypoxia, possibly by binding to essential sulfhydryl groups. Such toxicity in hypoxic tumor cells may explain the ability of misonidazole to augment the effectiveness of conventional radiation therapy and chemotherapy. Further investigation may reveal that the peripheral neuropathy that occasionally follows misonidazole therapy [22] is related to reductive activation in isolated hypoxic areas of normal tissue.

In lack of definitive evidence that misonidazole binding is toxic, our results also support an alternative hypothesis that radiation therapy or chemotherapy is enhanced by cellular GSH depletion [7, 23], although a very high concentration of circulating misonidazole was required in our study to accomplish this depletion.

The concept that a nitroaromatic compound can be reductively activated to an alkylating agent may explain the biological properties of other nitro-substituted chemicals, including environmental agents. Misonidazole itself appears to be an excellent model compound for investigations concerning the bioactivation, the toxicity, and the detoxication of nitrocompounds.

In summary, we have demonstrated that misonidazole undergoes reductive, metabolic activation in intact mammalian tissue. Formation of a reactive product was evidenced by extensive covalent binding of misonidazole to tissue protein and RNA, and both covalent binding and reductive metabolism were enhanced by hypoxia. Depletion of tissue GSH stores with DEM increased the covalent binding to protein, and misonidazole alone (2.5 mM) depleted hepatic GSH. These results indicate that GSH plays an intervening role in the binding of misonidazole products to protein.

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