0026-895X/86/020168-05$02.00/0

MOLECULAR PHARMACOLOGY, 29:168–172

Biochemistry of Misonidazole Reduction by NADPH-Cytochrome *c* (P-450) Reductase

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Received March 15, 1985; Accepted November 29, 1985

SUMMARY

The biochemical mechanism for the reduction of misonidazole [1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol] by purified rabbit liver NADPH-cytochrome *c* (P-450) reductase, the primary nitroreductase of liver, has been studied. Neither the anaerobic nor the futile aerobic reduction velocities exhibited signs of Michaelis-Menten saturation at concentrations less than 5 and 10 mM, respectively. The anaerobic reduction of misonidazole resulted in the formation of glyoxal from fragmentation of the imidazole ring in 25% yield. The rate of glyoxal formation was linear with time and paralleled the reduction of misonidazole, suggesting that it was derived from the partitioning of a reactive intermediate between at least two alternative pathways. Negligible amounts of the 2-amino derivative of misonidazole were formed, however, indicating the existence of alternative reduction/fragmentation pathways.

The hypoxic cell compartment of solid tumors contains viable neoplastic stem cells which are capable of reentering the rapidly proliferating oxygenated cellular pool of the tumor (1–4). It is well established that hypoxic cells are relatively resistant to the cytotoxic effects of ionizing radiation and are also presumed to be relatively resistant to most chemotherapeutic agents (1, 2). Thus, the hypoxic cell population has the capacity to limit the curability of solid tumors. For this reason, agents directed specifically against hypoxic cells would appear to have considerable potential in cancer therapy in combination with other drugs or treatment modalities capable of eradicating the oxygenated neoplastic cell component of solid tumors.

Various nitro-containing compounds exhibit significant selective cytotoxicity toward hypoxic cells (5). Among the more thoroughly studied of these agents are misonidazole and metronidazole, which have been tested clinically as radiation sensitizers (6). The radiosensitizing capability and the anaerobic cytotoxicity of these compounds are apparently derived from their high electron affinity. Misonidazole is readily reduced, either by radiation-generated free radicals or by enzymatic systems (7, 8). The first stage in the reduction appears to be the formation of the nitro radical anion, which has been detected in liver microsomes incubated anaerobically with metronidazole or nitrobenzene (9, 10). In the presence of oxygen, the radical anion is rapidly reoxidized by oxygen (9) whereas, in the absence of oxygen, further reduction occurs, presumably via the formation of nitroso and hydroxylamine derivatives, ultimately leading to the attainment of an amino-containing compound and the fragmentation of the imidazole ring (see Fig. 1) (7, 11–14). The selective kill of hypoxic cells by nitroimidazoles presumably results from the fact that the various anaerobic reduction products are more toxic than superoxide.

To guide the development of new antitumor agents containing the nitroimidazole nucleus, an understanding of the biological reduction processes underlying the selective toxicity of these agents to hypoxic cells is essential. In mammalian liver, NADPH-cytochrome *c* reductase (EC 1.6.2.4; FPT) appears to be the primary nitroreductase, although DT-diaphorase, xanthine oxidase, and other enzymes also exhibit this activity (15). Thus, Mason and Holtzman (10) reported that rat liver microsomes exhibited much higher activity in generating the nitrobenzene radical than mitochondrial or cytosolic fractions. Similarly, although both microsomal and cytosolic fractions were reported to be capable of catalyzing the reduction of niridazole, microsomes were 100 times more active at saturating concentrations of the substrate (16, 17). It was suggested that NADPH-cytochrome *c* reductase was responsible for this activity in the microsomes, and that xanthine oxidase was the active enzyme in the cytosol. Similar results were obtained by Wang *et al.* (18), who demonstrated that nitroreductase activity copurified with xanthine oxidase in cytosol and with partially purified NADPH-cytochrome *c* reductase in microsomes, each enzymatic activity producing a species capable of reacting with protein upon anaerobic reduction of a nitroacetamide derivative. More recently, McManus *et al.* (19) showed that purified

This research was supported by American Cancer Society Grant CH-211 and United States Public Health Service Fellowship CA-07521 to D. C. H.

ABBREVIATIONS: AIM, 1-(2-amino-1-imidazolyl)-3-methoxy-2-propanol; HPLC, high performance liquid chromatography.

NADPH-cytochrome *c* reductase generates a reactive species when incubated anaerobically with misonidazole; similar results were observed by West *et al.* (20) using ronidazole.

The purpose of this report is to clarify the reduction pathways of misonidazole from a biochemical perspective, using purified rabbit liver NADPH-cytochrome *c* (P-450) reductase as the nitroreductase. The results show that (a) the initial velocity of nitroimidazole reduction does not saturate at substrate concentrations below 10 mM, (b) significant amounts of the amino-containing reduction product of misonidazole are not produced by this enzymatic action, and (c) glyoxal formation accounts for approximately 25% of the reduced nitroimidazole, which results from the partitioning of the reduced intermediate between multiple reduction/fragmentation pathways.

Materials and Methods

NADPH-cytochrome *c* reductase was purified from the livers of phenobarbital-treated rabbits by the method of French and Coon (21). The purified enzyme contained 40–50 units/mg of protein. Misonidazole reduction was assayed spectrophotometrically using a deoxygenated solution containing 1.0 unit/ml (0.25 μM) of NADPH-cytochrome *c* reductase, an NADPH regenerating system (5 units/ml of glucose-6-phosphate dehydrogenase, 500 μM glucose-6-phosphate, and 50 μM NADP), an oxygen-scavenging system (10 units/ml of glucose oxidase, 10 units/ml of catalase, and 500 μM glucose), and 30 μg/ml of dilauroylphosphatidyl choline in 100 mM Tris-HCl or 100 mM K_2HPO_4 (pH 7.4) incubated in an anaerobic cuvette at 30°. The reaction was initiated by the addition of an aqueous solution of misonidazole through a rubber septum, and the change in absorbance as a function of time was recorded at 325 nm. Reaction velocities were calculated from the loss of misonidazole absorbance using $E_{325} = 7.8\ mM^{-1}\ cm^{-1}$.

Glyoxal was assayed spectrophotometrically by a modification of the method of Mitchell and Birnboim (22). To each of two polyethylene tubes was added 0.2 ml of the enzyme reduction mixture and 0.2 ml of 0.5 N sodium formate (pH 2.9). To the reference tube was added 0.1 ml of water and to the sample tube 0.1 ml of 100 mM Girard's reagent T (trimethylaminoacetohydrazide chloride). After 15 min at 30°, 0.5 ml of 0.1 N sodium formate (pH 2.9) was added to each tube, the contents were transferred to quartz cuvettes, and the absorbance at 295 nm was recorded. The extinction coefficient of the bis-adduct was 27.3 $mM^{-1}\ cm^{-1}$. The concentration of misonidazole was determined from the reference cuvette. Glyoxal formation was also measured by high performance liquid chromatography (HPLC). To 100 μl of the enzyme reaction mixture was added 2 μl of 45% formic acid and 20 μl of 100 mM Girard's reagent T. After 15 min at 30°, 50 μl of the reaction mixture was injected onto a Whatman Partisil 10 SCX column which was then eluted with 1 N KH_2PO_4 (pH 3.5) at 30°. The adduct (retention time = 22 min, 2 ml/min) was detected at 295 nm on a Varian Varichrome detector.

The aerobic reduction and reoxidation of misonidazole were monitored with a Gilson Oxy-graph equipped with a Clark electrode or by measuring adrenochrome formation from adrenaline (19, 23). Conditions were as described in the spectrophotometric assay, omitting the oxygen-scavenging system.

Measurement of the 2-amino-containing product of misonidazole was performed by a modification of the procedure of Flockhart *et al.* (11). To 1 ml of the reaction mixture was added 1 ml of saturated $NaHCO_3$, 1 ml of ethanol, and 0.1 ml of 2,4-dinitrofluorobenzene. After shaking for 2 hr at 30°, 1.5 ml of water was added and the excess 2,4-dinitrofluorobenzene was extracted with 5 ml of toluene/cyclohexane (1:4, v/v). The dinitrofluorobenzene-amine adduct was extracted with toluene, concentrated under a stream of nitrogen, and assayed by HPLC on a Partisil 10/25 silica column. The mobile phase was 98% $CHCl_3$/2% methanol, and detection was at 400 nm. A pure standard of the derivatized amine was prepared by applying the above procedure to a methanolic solution of Pd/C-reduced misonidazole; after derivatization, the product was purified on a silica gel column, using methanol/chloroform (1:19, v/v) as the elution solvent. After removal of the solvent, a red solid was obtained; recrystallization from chloroform/petroleum ether yielded the chromatographically pure derivatized amine, m.p. 161–163°.

Misonidazole was generously provided by Dr. Sara Rockwell (Department of Therapeutic Radiology, Yale University). All other compounds were from standard suppliers.

Results

Initial studies were performed to determine the relationship between the kinetics of superoxide formation and the aerobic metabolism of misonidazole by NADPH-cytochrome *c* reductase using the adrenochrome assay. These experiments were conducted in an effort to clarify the implications of an earlier report by McManus *et al.* (19) which indicated that misonidazole at a concentration of 0.5 mM did not increase the production of superoxide by microsomes as measured by adrenochrome formation; the reactions involved in the production of superoxide are depicted in Fig. 1. Addition of misonidazole at final concentrations up to 5 mM to a solution containing NADPH and 0.1 unit of NADPH-cytochrome *c* reductase did not result in an increase in the rate of adrenochrome formation, as reported by McManus *et al.* (19) for microsomes at a misonidazole concentration of 0.5 mM. Because adrenaline has been reported to be a substrate for NADPH-cytochrome *c* reductase (23), however, additional aerobic turnover studies were performed using an oxygen electrode.

Studies with a Clark oxygen electrode demonstrated that misonidazole does stimulate oxygen consumption by NADPH-cytochrome *c* reductase, in accord with the reaction scheme presented in Fig. 1. The increase in the rate of oxygen consumption was linear with respect to the concentration of misonidazole added over the range of 0–10 mM misonidazole (Fig. 2). No evidence of Michaelis-Menten saturation of the rate of

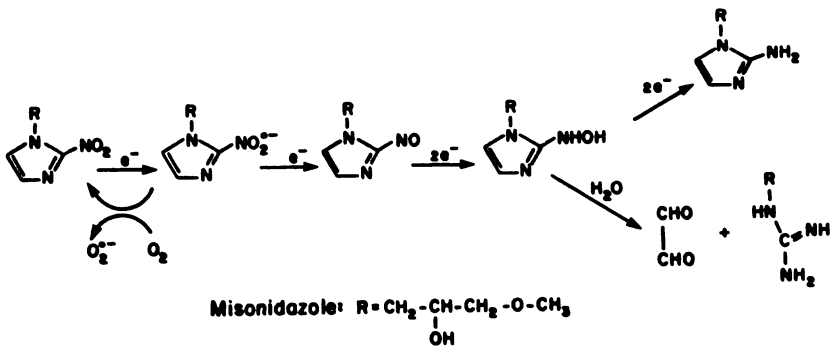

Fig. 1. Schematic diagram of misonidazole reduction. In the presence of O_2, futile cycling between the oxidized and one-electron reduced species produces superoxide and prevents net reduction of the nitroimidazole (25, 26).

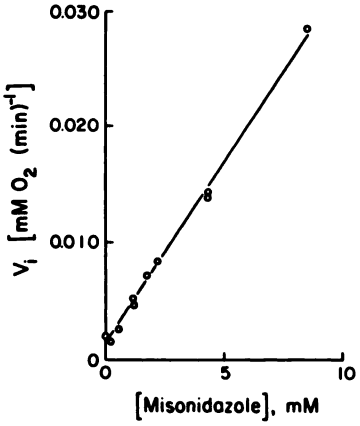

Fig. 2. Aerobic reduction and reoxidation of misonidazole. The misonidazole-stimulated oxygen consumption by NADPH-cytochrome *c* reductase was determined using a Clark-type oxygen electrode in the presence of an NADPH-regenerating system (see Materials and Methods).

oxygen consumption was observed in this concentration range. These findings indicate that the adrenochrome reaction is not the method of choice for monitoring drug-induced stimulation of superoxide formation by microsomes or by purified microsomal enzymes because the reagent itself stimulates the reaction to be monitored, generating a high background rate, and may competitively inhibit the metabolism of the added drug.

The anaerobic reduction of misonidazole by NADPH-cytochrome *c* reductase was monitored spectrophotometrically by the loss of the nitro group absorbance at 325 nm. Over the concentration range investigated (i.e., 0–140 μM), the anaerobic reduction velocity was linear with respect to the concentration of misonidazole and NADPH-cytochrome *c* reductase, yielding a second order rate constant of 0.011 $(\text{min})^{-1}$ $(\mu\text{M})^{-1}$. No spectral changes attributable to misonidazole metabolism were observed under aerobic conditions.

The interaction of misonidazole with NADPH-cytochrome *c* reductase was also studied in the presence of the electron recipient, cytochrome P-450. Addition of purified rabbit liver cytochrome P-450_{LM2} in a 10-fold molar excess over the concentration of NADPH-cytochrome *c* reductase did not affect the rate of misonidazole reduction, consistent with the hypothesis that cytochrome P-450 does not function as a nitroreductase in the presence of NADPH-cytochrome *c* reductase with this substrate (18).

At least two distinct metabolic pathways have been described for the bioreduction of misonidazole; these are illustrated in Fig. 1. The amino-containing product of misonidazole (AIM) has been isolated from cells and animals treated with this agent (7, 11, 24). Similarly, fragmentation of the imidazole ring, proposed to occur by hydrolysis of the hydroxylamine derivative, has been reported to yield glyoxal and a guanidine derivative in both chemical and biochemical reduction systems (25–27). Because glyoxal forms stable covalent adducts with guanosine and has been suggested to be one of the cytotoxic reduction products of misonidazole (25, 26, 28), these two alternative reduction pathways were investigated using purified NADPH-cytochrome *c* reductase as the nitroreductase.

The use of Girard's reagent T allowed the detection of glyoxal formed by enzymatic reduction of misonidazole and permitted the acquisition of detailed kinetic data on this reaction. The Girard T adduct obtained as a product from the enzymatic reduction mixture possessed chromatographic and spectroscopic properties identical to those of the reaction product of Girard's reagent T with authentic glyoxal. Attempts to corroborate the assay with the results of earlier studies using Zn/NH_4Cl-reduced misonidazole and 2,4-dinitrophenylhydrazine as a derivatizing reagent (25) were unsuccessful, because the Zn/NH_4Cl reduction system destroyed glyoxal with a half-life of approximately 20 min at 60°. These results suggest that this latter reduction system may not accurately mimic the biological reduction of the 2-nitroimidazoles because it yields artificially low levels of glyoxal. The Girard's reagent T permitted a measurement of the ratio of glyoxal formed to misonidazole reduced, by simultaneous monitoring of glyoxal formation and misonidazole reduction by NADPH-cytochrome *c* reductase as a function of time under anaerobiosis. These data are presented in Fig. 3. The findings demonstrate that glyoxal accounts for about 25% of the misonidazole reduced. This value is relatively constant with time, suggesting a partitioning of the reduced intermediates between the glyoxal-yielding fragmentation pathway and other as yet undefined metabolic pathways. Under aerobic conditions, or in the absence of NADPH-cytochrome *c* reductase, no glyoxal was observed.

Measurement of the formation of glyoxal allows this assay to be used to obtain anaerobic reduction velocities at misonidazole concentrations that are much higher than those attainable using a direct spectrophotometric assay. The results of these experiments are illustrated in Fig. 4. The anaerobic velocity of glyoxal formation was proportional to the initial concentration of misonidazole, and no saturation of the anaerobic reduction velocity was apparent at concentrations below 5 mM. These findings are consistent with those determined by monitoring oxygen consumption under aerobic conditions, and indicate that the fundamental properties of the interaction between NADPH-cytochrome *c* reductase and misonidazole are unaffected by the presence or absence of oxygen.

The other reduction pathway delineated in Fig. 1 results in the formation of the amino derivative of misonidazole. Isolation of this metabolite from reduction mixtures containing NADPH-cytochrome *c* reductase was attempted by derivatization with 2,4-dinitrofluorobenzene, extraction, and HPLC (11). Under the conditions described, the 2,4-dinitrofluorobenzene adduct had a retention time of 9 min, whereas derivatized Tris and unreacted 2,4-dinitrofluorobenzene eluted at 8.0 and 3.2 min, respectively. The efficiency of extraction, derivatization,

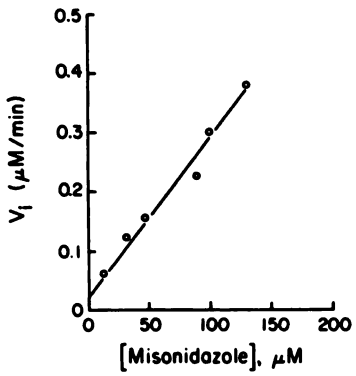

Fig. 3. Anaerobic reduction of misonidazole. The anaerobic reduction of misonidazole by NADPH-cytochrome *c* reductase was determined by monitoring the loss of misonidazole spectrophotometrically at 323 nm (see Materials and Methods).

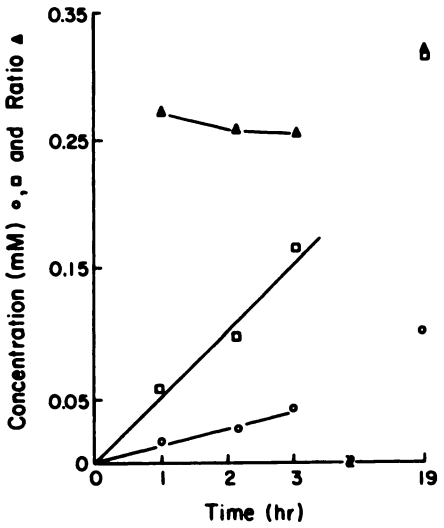

Fig. 4. Fragmentation of misonidazole. The change in concentration of misonidazole and glyoxal in an anaerobic reduction mixture containing NADPH-cytochrome *c* reductase was determined at various times, and the molar ratio of glyoxal formation/misonidazole loss was used to obtain the fragmentation ratio (see Materials and Methods). ○, concentration of glyoxal (mM); □, change in concentration of misonidazole (mM); ▲, ratio, concentration of glyoxal to change in concentration of misonidazole.

and detection of AIM by this procedure was observed to be greater than 30% (data not shown).

The anaerobic reduction of misonidazole by NADPH-cytochrome *c* reductase did not yield significant quantities of AIM. By spectrophotometric determination of the amount of misonidazole reduced (300 μM), the extraction efficiency noted above can be utilized to ascertain that AIM is produced with less than 1% efficiency during the anaerobic reduction of misonidazole by NADPH-cytochrome *c* reductase. Thus, this pathway makes a negligible contribution to the overall metabolism of misonidazole by purified rabbit liver NADPH-cytochrome *c* reductase.

Discussion

Misonidazole is a clinically relevant agent, making an understanding of the biochemistry of the reductive activation of this compound important. The present report represents the first description of detailed kinetic data for the metabolism of misonidazole by a purified mammalian nitroreductase. The absence of Michaelis-Menten saturation during the reaction of misonidazole with NADPH-cytochrome *c* reductase under both aerobic and anaerobic conditions suggests that the interaction of the nitroimidazole with this nitroreductase is unaffected by the presence of oxygen. The fact that the cytotoxicity of this agent is greater toward hypoxic cells than to their oxygenated counterparts appears to be due to the fact that the net reduction process leads to a more toxic product under hypoxia than under aerobic conditions.

The use of Girard's reagent T for the spectrophotometric determination of glyoxal production represents a major improvement over the previously reported assay method of isolating the precipitate resulting from the reaction of 2,4-dinitrophenylhydrazine with glyoxal (25, 26). Formation of a UV-absorbing species with Girard's reagent T requires the presence of an α-dicarbonyl moiety; thus, no reaction was observed with formaldehyde, glyceraldehyde, glyoxylic acid (22), or hydroxyurea (data not shown). Varghese and Whitmore (27, 29) have recently reported the net formation of a glyoxal-guanosine adduct from chemically and biologically reduced misonidazole. They have proposed that the reactive intermediate is not glyoxal itself, but an unstable product of the hydrolysis of the hydroxylamine derivative. Although the properties of the possible reaction product of Girard's reagent T with this intermediate might be identical to the glyoxal-Girard T adduct, it seems unlikely that Girard's reagent T is actually measuring such an intermediate. The proposed hydrolytic intermediate is much less reactive with guanine than with glyoxal itself, and shows no reaction with 2,4-dinitrophenylhydrazine (25), which would be expected to have a reactivity similar to that of Girard's reagent T. The kinetics of the reaction of Girard's reagent T with enzymatically reduced misonidazole and authentic glyoxal were indistinguishable, and the reaction was complete in 15 min at 30°, in contrast to the greater than 3 hr at 60° required for the reaction with guanosine (25). It seems likely, therefore, that Girard's reagent T is in fact reacting with free glyoxal, which must ultimately be derived from fragmentation of the imidazole ring after bioreduction, possibly via the scheme proposed by Varghese and Whitmore (27, 29).

The relatively constant value for the fragmentation ratio as a function of time suggests that the rate of glyoxal production is comparable to the reduction rate and is not the result of a slow hydrolysis reaction. Therefore, the glyoxal assay provides a sensitive and specific assay for a reduction product of misonidazole, allowing the acquisition of anaerobic reduction kinetics at concentrations beyond those which can be obtained spectrophotometrically (Fig. 5). The observed lack of saturation of the anaerobic reduction rate is consistent with the oxygen

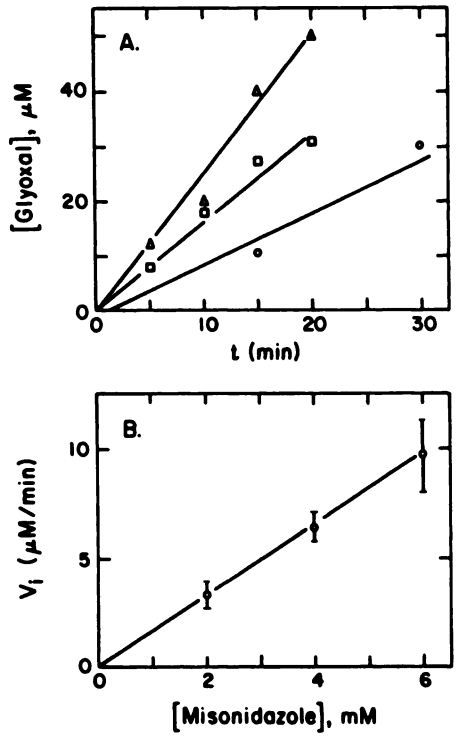

Fig. 5. Anaerobic reduction of misonidazole. The time-dependent formation of glyoxal at various concentrations of misonidazole (○, 2 mM; □, 4 mM; △ 6 mM) shown in panel A was used to obtain initial velocities of misonidazole reduction at high concentrations of the 2-nitroimidazole. Panel B represents a replot of the average velocities of misonidazole reduction at each concentration, assuming a constant ratio of fragmentation = 0.25. Error bars in panel B represent the standard deviation.

consumption data and the high misonidazole concentrations required to produce cytotoxicity (30), but is inconsistent with the reported K_m of 740 μM for the microsomal catalyzed reduction of misonidazole (19). NADPH-cytochrome *c* reductase is the primary reductase in microsomes (16–18), but the participation of other enzymes, different lipid concentrations and composition, and/or the use of a different assay system may be responsible for the observed difference between previous data and the findings presented in this report.

The lack of production of AIM by the purified reductase is consistent with the $4e^-$ reduction stoichiometry obtained in electrochemical reduction studies of misonidazole (13). Nevertheless, AIM has been shown to be produced in biological systems (7, 11, 24), pointing out the existence of other pathways of metabolism. The lack of significant AIM production leaves approximately 75% of the reduced misonidazole unaccounted for, although chemical model studies suggest the formation of azo and hydrazo dimerization products, and alternative fragmentation pathways are likely, even using the purified nitroreductase (13). Thus, the scheme depicted in Fig. 1 is clearly an oversimplified one. The studies presented in this report have documented a major bioreductive fragmentation pathway; this mechanism is currently being exploited in our laboratory to generate new bioreductive alkylating agents with increased selective toxicity to hypoxic tumor cells.

Acknowledgments

We wish to thank Drs. K. Shyam, S. R. Keyes, S. Rockwell, and R. T. Hrubiec for helpful discussions.

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