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Possible Role of Free Radical Formation in Clozapine (Clozaril)-Induced Agranulocytosis

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SUMMARY

The use of clozapine, a unique antipsychotic drug, has been restricted due to a 1–2% incidence of drug-induced agranulocytosis. Metabolic activation of clozapine in neutrophils or stem cells could be the molecular mechanism underlying this side effect. Clozapine oxidation by human myeloperoxidase and horseradish peroxidase was evident from the disappearance of the UV absorbance at 290 nm. High performance liquid chromatography analysis revealed the formation of at least four radioactive peaks as a result of clozapine metabolism, including radioactivity coeluting with the protein. The tight association of radioactivity with the enzymatic protein was metabolism-dependent. This protein binding, which correlates with the total metabolism of clozapine, was reduced in the presence of glutathione and was absent in the presence of ascorbate. Similarly, in the presence of both reducing agents, the metabolite peaks in the high performance liquid chromatography radiogram, which are not associated with protein, disappeared. In contrast, in the presence of glutathione, two additional metabolites were found that could be isolated and identified by NMR and mass spectroscopy as clozapine glutathionyl adducts. Evidence for one-electron transfer reactions or the intermediate formation of a clozapine radical during the peroxidase-mediated metabolism of clozapine stems from the observation of thiyl and ascorbyl radicals in the presence of glutathione and ascorbate, respectively. The ascorbyl radical was detected by direct ESR spectroscopy in a peroxidase system. Its steady state concentration was significantly increased in the presence of clozapine. Glutathionyl radical formation was demonstrated by radical trapping with 5,5-dimethyl-1-pyrroline *N*-oxide in a peroxidase system. Again, the radical adduct concentration was significantly increased in the presence of clozapine. Similarly, when oxygen consumption was measured in peroxidase systems in the presence of glutathione or NADPH, the rate of oxygen uptake was markedly enhanced upon addition of clozapine. Thus, the data support the possibility of clozapine activation to free radical metabolites, which may cause oxidative stress or lead to adduct formation. Further, it can be concluded from these data that radical scavengers such as ascorbic acid, when coadministered with clozapine to patients, may reduce oxidative stress and protein adduct formation.

Clozapine (Clozaril) is a unique antipsychotic drug that is superior to standard neuroleptics due to its lack of extrapyramidal side effects and its effectiveness with otherwise "treatment-resistant" patients. Despite its advantages, the use of clozapine has been hampered due to a 1–2% incidence of drug-induced agranulocytosis. The exact mechanism of this adverse reaction is not known, yet clinical observations suggest an immune-mediated hypersensitivity reaction (1). Drug-induced hypersensitivity reactions in general appear to be linked to the metabolic activation of the drug to reactive metabolites. A good correlation exists between the incidence of agranulocytosis formation and drug activation by myeloperoxidase, which is present in both polymorphonucleocytes and bone marrow cells (2).

The major metabolic pathways reported for clozapine in human subjects are *N*-oxide formation and *N*-demethylation (3). Other metabolites are formed by either substitution of the chlorine or addition of a hydroxyl or methylthio functional group to the aromatic moiety (4). The formation of such methylthio conjugates indicates the intermediate formation of strong electrophiles (5), which do not normally appear in urine but react readily with protein thiols, glutathione, or methionine. Drugs with arylamine functional groups, such as clozapine, are known to be activated by peroxidases, forming free radicals and other reactive species that are able to initiate oxidative stress or bind to macromolecules (6, 7). Glutathione either can detoxify such reactive intermediates by conjugate formation, leading to products that are more hydrophilic and easily excreted (5), or can reduce free radical metabolites, resulting in the formation of the unchanged parent molecule and the glutathionyl radical and radicals derived therefrom. Reduction of free radicals by glutathione is known as thiol pumping and is suggested

ABBREVIATIONS: HRP, horseradish peroxidase; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylenetriaminepentaacetic acid; HPLC, high performance liquid chromatography.

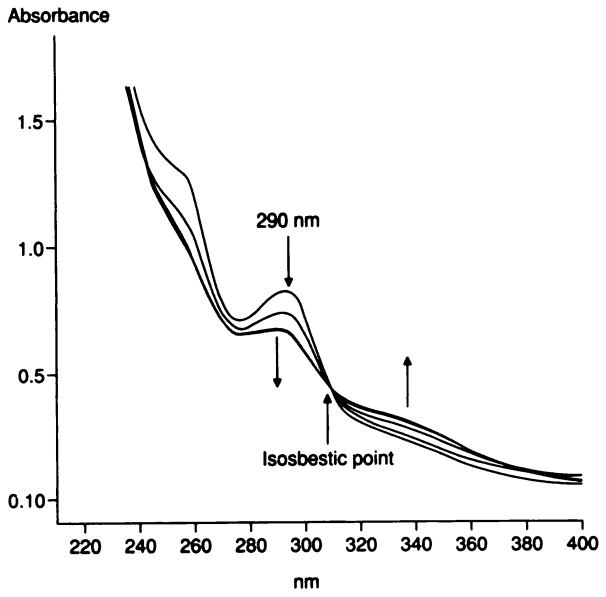

Fig. 1. Oxidation of clozapine by HRP/H_2O_2: optical spectra. The incubation mixture contained 100 μM clozapine, 1.25 μg/ml HRP, and 50 μM H_2O_2, in phosphate buffer, pH 7.4. At zero time, HRP was added and repetitive scanning was started. Scans were taken every 2 min with a scan speed of 250 nm/min.

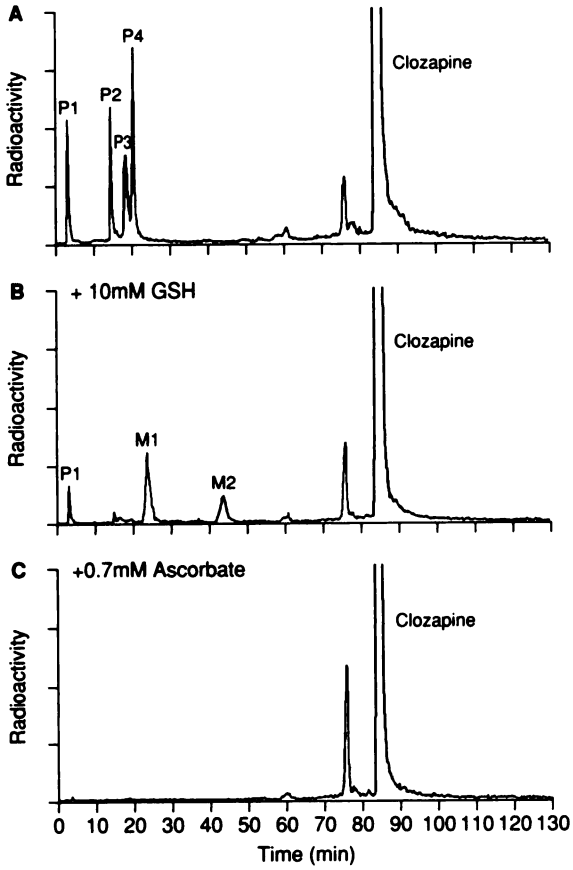

Fig. 2. HPLC separation of clozapine and its metabolites formed by human myeloperoxidase. A, Myeloperoxidase (20 units/ml) was incubated with 100 μM clozapine and 50 μM H_2O_2, in 0.05 M phosphate buffer (pH 7.4), for 4 h. B, Same as in A, but in the presence of 10 mM glutathione. C, Same as in A, but in the presence of 0.7 mM ascorbate. The incubation media were injected directly onto the column.

as a possible mechanism for the detoxification of the free radical metabolites of xenobiotics in biological systems (8–13). In aerobic systems, thiol pumping leads ultimately to superoxide formation, which can be observed by monitoring oxygen consumption (14, 15). Alternatively, free radicals are detoxified by other free radical scavengers, with ascorbic acid being one of the most potent scavengers (8, 13, 16, 17).

In this paper, we provide evidence for the formation of clozapine-derived free radicals in the presence of peroxidases. These radicals are capable of initiating oxidative stress or binding to macromolecules; thus, radical formation may be directly related to the observed agranulocytosis. We also demonstrate that antioxidants such as ascorbate effectively reduce the clozapine-derived free radical concentrations.

Materials and Methods

[^{3}H]Clozapine (8-chloro-11-[4-methyl-1-piperazinyl]-5*H*-dibenzo-[*b*,*e*][1,4]diazepine) was synthesized by Dr. R. Voges of the Synthetic Tracer Laboratories of Sandoz Pharma Ltd. (Basle). The specific activity was 677 μCi/mg, and the purity was >95%. Unlabeled clozapine was obtained from the Preclinical Research Department of Sandoz Pharma Ltd. NADPH, glutathione, 5,5-dimethyl-1-pyrroline *N*-oxide, human myeloperoxidase, HRP type VI (EC 1.11.1.7), catalase, and DTPA were obtained from Sigma. Hydrogen peroxide was purchased from Fluka. Sodium ascorbate was purchased from Calbiochem-Behring.

All enzymatic incubations of HRP or human myeloperoxidase with clozapine were performed in phosphate buffer, pH 7.4, containing 0.5 mM DTPA. Other details, including concentrations for the different reagents, are given in the figure legends. Optical spectra to monitor the enzymatic reaction were recorded on a Philips PU 8700 UV/visible spectrophotometer. For HPLC analysis, the enzymatic reactions were stopped by addition of 1 N hydrochloric acid. The resulting mixture was then directly injected into the HPLC. The system consisted of Kontron 420 pumps, a Kontron 460 autosampler, a Kontron UVIKON 730 S LC UV spectrophotometer, and a Berthold LB 507 A radioactivity monitor. The column was a 250- × 4.6-mm Supelco LC-18-DB, with 5-μm particle size packing (Supelco Inc., Bellefonte, PA). The material was eluted at 45°, using a flow rate of 1 ml/min. The mobile phase consisted of ammonium carbonate (25 mM) with 0.1% triethylamine, adjusted to a final pH of 8.5 (solvent A), and acetonitrile (solvent B). The proportion of solvent B was 0% up to 5 min and was increased linearly to reach 20% at 10 min, 24% at 60 min, and 80% at 100 min. Positive-ion fast atom bombardment mass spectra for the identification of metabolites were taken on a MAT 212 mass spectrometer. Thioglycerol was used as a matrix. NMR spectra of the metabolites were recorded on Bruker AM360 or AM500 superconducting spectrometers. The spectra were obtained at room temperature in methyl alcohol-d_4, using tetramethylsilane as an internal standard.

Oxygen uptake measurements were made using a Gilson model 5/6 oxygraph (Gilson Medical Electronics, Middleton, MI). Experiments were performed at 37° in a 1.8-ml water-jacketed cell. All ESR spectra were recorded at room temperature on a Varian E-109 or a Bruker ER 200 D spectrometer equipped with a TM_{110} microwave cavity and an aqueous flat cell. Detailed experimental conditions are described in the figure legends.

Results

The peroxidase-mediated oxidation of clozapine was studied using HRP and myeloperoxidase/hydrogen peroxide systems. Through the use of UV spectroscopy, the enzymatic oxidation of clozapine was demonstrated (Fig. 1). Decay of the clozapine absorption at 290 nm was accompanied by a simultaneous increase in the absorption around 340 nm, but no distinct peak

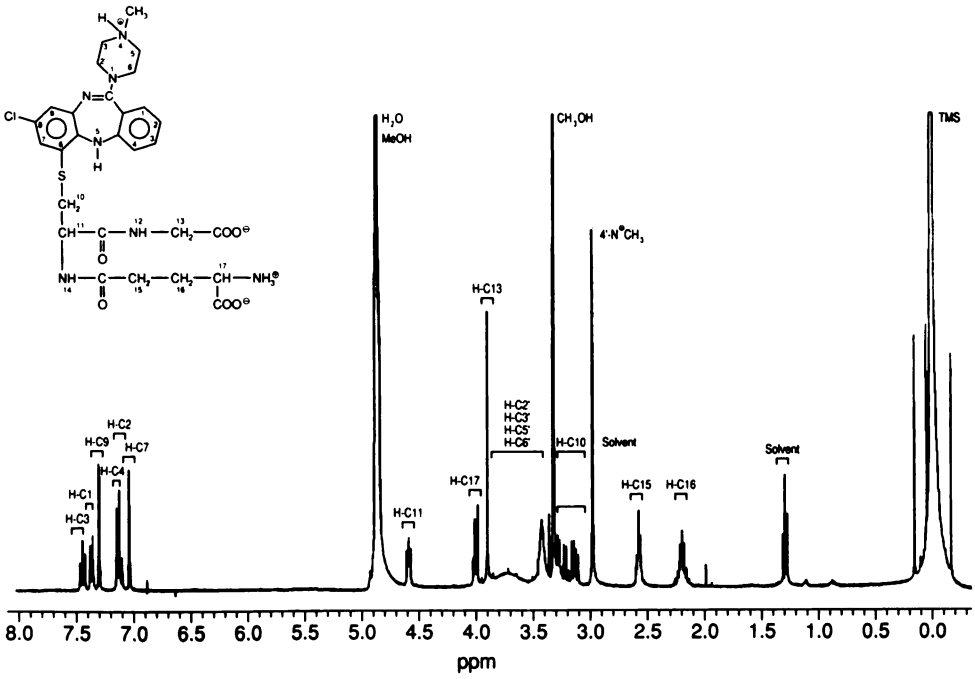

Fig. 3. ^{1}H NMR spectrum (360 MHz) of the glutathione conjugate M2 of clozapine.

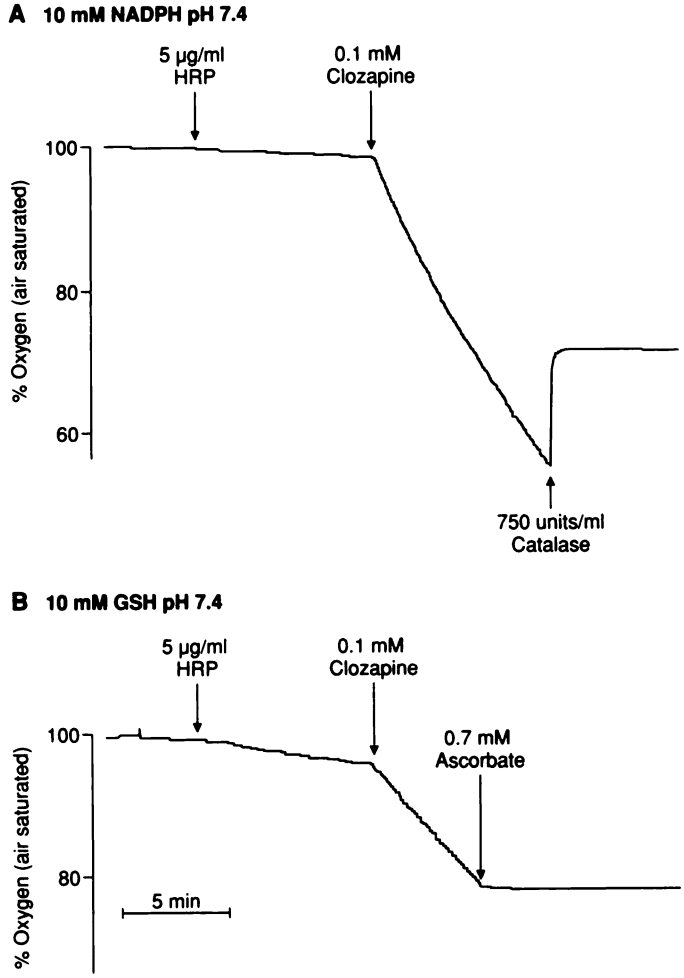

Fig. 4. Oxygen consumption curves of 1.8-ml incubations containing 10 mM NADPH (A) or 10 mM glutathione (B), in 0.05 M phosphate buffer adjusted to pH 7.4. HRP (5 μg/ml) (type VI), 100 μM clozapine, 0.7 mM ascorbate, and 750 units/ml catalase were added at the points indicated.

was apparent. Characteristic of the spectral change is an isosbestic point at 306 nm. The reaction was totally dependent on the presence of both the native enzyme and hydrogen peroxide.

Clozapine metabolism was further evaluated by HPLC analysis of incubations containing radiolabeled clozapine with either HRP or human myeloperoxidase and hydrogen peroxide. The results were qualitatively similar for both enzymes, in that a number of polar metabolites were found. Fig. 2A shows the HPLC separation of clozapine and its metabolites after an incubation with human myeloperoxidase and hydrogen peroxide. The radioactive peak eluting at 76 min was also present in incubations when hydrogen peroxide was replaced with 1500 units/ml catalase and should, therefore, not be due to peroxidative metabolism. All other peaks, except for clozapine itself, were absent in the presence of catalase (data not shown). Peak 1 coeluted with the enzymatic protein at 3.5 min and was not separable by HPLC. Peaks 2–4 eluted at 15, 19, and 21 min, respectively. In the presence of 10 mM glutathione, peak 1, which coeluted with the protein, was somewhat reduced. More noticeable, however, was that the other three metabolite peaks were not detectable; instead, two additional peaks, M1 and M2, eluting at 24 and 44 min, respectively, were found (Fig. 2B). Both radioactive peaks, M1 and M2, were isolated by HPLC from incubations with HRP and were subjected to ^{1}H NMR spectroscopic analysis.

^{1}H NMR data are consistent with clozapine glutathione adducts at the 9-position for metabolite M1 and at the 6-position for metabolite M2 (Fig. 3). The assignment for a substitution at the 6-position is consistent with two doublets for the protons at positions 7 and 9, with a coupling constant of 1–2 Hz. Substitution at positions 1 to 4 can be excluded, because the apparent triplet assigned to position 3, which is due to two neighboring protons, shows an additional splitting of 1–2 Hz. This splitting must be due to a proton in the *meta*-position. The substitution at the 9-position for metabolite M1 was further confirmed by nuclear Overhouser experiments

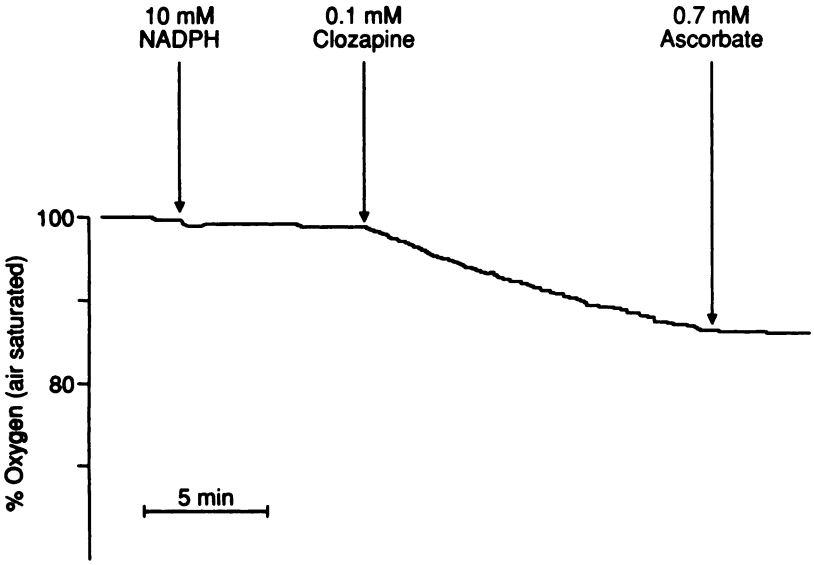

Fig. 5. Oxygen consumption curves of 1.8-ml incubations containing 10 units of human myeloperoxidase, in 0.05 M phosphate buffer adjusted to pH 7.4. NADPH (10 mM) 100 μM clozapine, and 0.7 mM ascorbate were added at the points indicated.

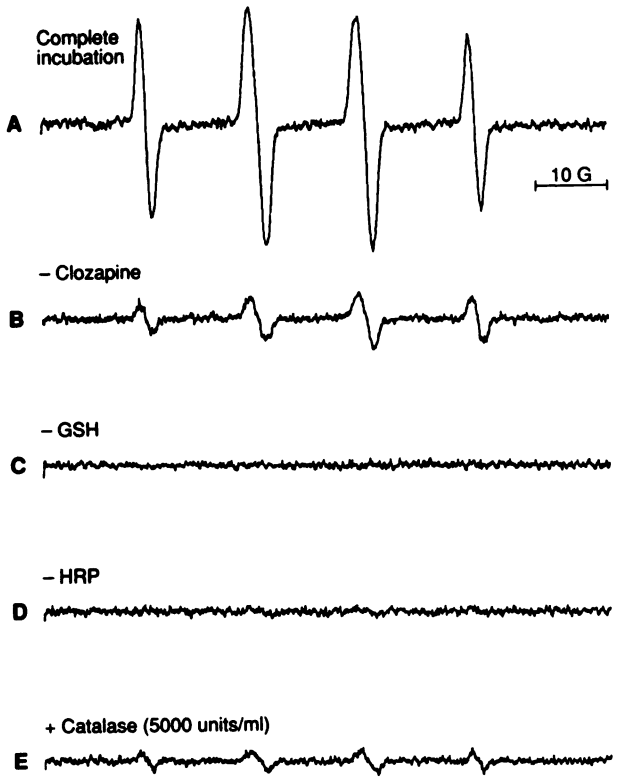

Fig. 6. ESR spectrum of the DMPO/glutathione thiyl radical adduct produced in a system of clozapine and HRP. A, Incubation containing 100 μM clozapine, 10 mM glutathione, 100 mM DMPO, 25 μg/ml HRP, and 0.5 mM DTPA, in 0.05 M phosphate buffer, pH 7.4. B, Same as in A, but without addition of clozapine. C, Same as in A, but without glutathione. D, Same as in A, but without HRP. E, Same as in A, but with the addition of 5000 units/ml catalase. The instrumental conditions were as follows: 20 mW microwave power, 0.6 G modulation amplitude, 0.160 sec time constant, and 40 G/min scan rate.

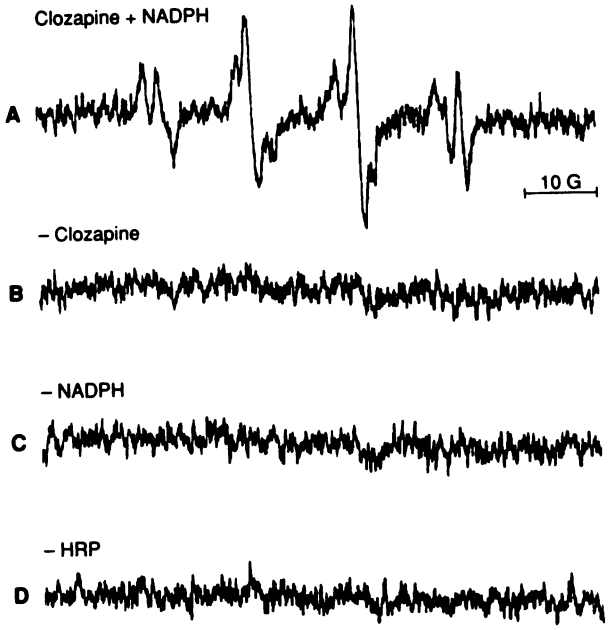

Fig. 7. ESR spectrum of the DMPO/·OOH adduct produced in a system of clozapine and HRP. A, Incubation containing 100 μM clozapine, 10 mM NADPH, 100 mM DMPO, 25 μg/ml HRP, and 0.5 mM DTPA, in 0.05 M phosphate buffer, pH 7.4. B, Same as in A, but without addition of clozapine. C, Same as in A, but without NADPH. D, Same as in A, but without HRP. The instrumental conditions were as follows: 20 mW microwave power, 1 G modulation amplitude, 0.330 sec time constant, and 40 G/min scan rate.

(data not shown). The molecular weight of 631, as determined by fast atom bombardment mass spectroscopy, for both M1 and M2 is also consistent with this assignment. In addition, 20 to 50% less total products were detected in the presence of 10 mM glutathione in incubations containing human myeloperoxidase or HRP, respectively. Upon replacement of 10 mM glutathione with 0.7 mM ascorbate, only parent drug and the peak at 76 min were detected (Fig. 2C). We cannot explain the apparent increase in the formation of the peak at 76 min; however, it is always present and its formation does not correlate with the rate of metabolism. In particular, in incubations containing 5 μg/ml HRP, where a higher rate of metabolism was observed, the formation of this peak did not increase.

The effects of glutathione and ascorbate on clozapine metabolism in peroxidase systems were further investigated by following oxygen uptake, using a Clarke oxygen electrode. Upon the addition of HRP (Fig. 4) to an aqueous solution of 10 mM glutathione or 10 mM NADPH, a small amount of oxygen

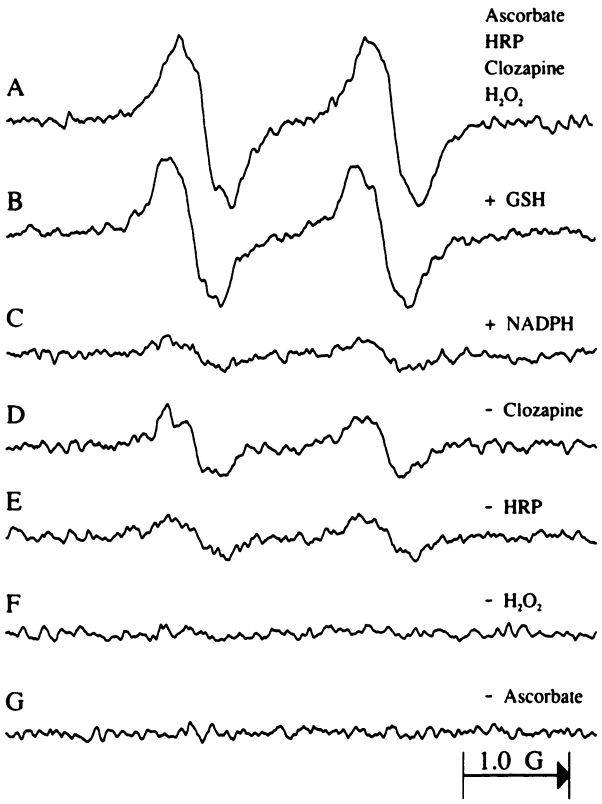

Fig. 8. A, ESR spectrum of the monodehydro-ascorbate radical (A^-) generated in a system of 100 μM clozapine, 50 μM hydrogen peroxide, 100 μM ascorbate (A^-), and 8.3 μg/ml HRP, in 50 mM phosphate buffer, pH 7.4. B, Same as in A, but in the presence of 1 mM glutathione. C, Same as in A, but in the presence of 1 mM NADPH. D, Same as in A, but in the absence of clozapine. E, Same as in A, but in the absence of HRP. F, Same as in A, but in the absence of hydrogen peroxide. G, Same as in A, but in the absence of ascorbate. The instrumental conditions were as follows: 20 mW microwave power, 0.1 G modulation amplitude, 0.128 sec time constant, and 10 G/min scan rate.

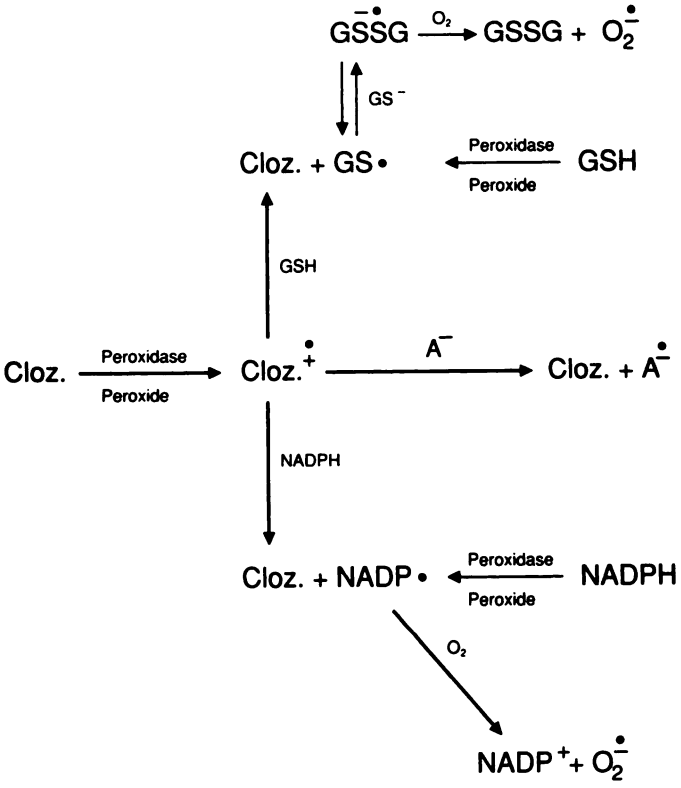

Fig. 9. Scheme for the formation of a clozapine (*Cloz.*)-derived free radical and subsequent reactions following the reduction of the radical by glutathione, ascorbate (A^-), or NADPH.

uptake was observed as a result of direct oxidation of NADPH or glutathione by the enzyme and the reaction of the resulting radicals with molecular oxygen (14, 15). Upon addition of clozapine, oxygen uptake was significantly increased. Addition of hydrogen peroxide was not required, yet when catalase was added an oxygen rebound was observed, indicating an autocatalytic formation of hydrogen peroxide (Fig. 4A). Ascorbate at a concentration of 0.7 mM completely inhibited oxygen consumption (Fig. 4B). Similar results were obtained with human myeloperoxidase (Fig. 5). When NADPH was added, only a little oxygen consumption was observed. However, oxygen consumption increased markedly after addition of clozapine and was inhibited upon addition of ascorbate.

Glutathionyl free radical formation due to thiol pumping was further confirmed by radical trapping with DMPO (Fig. 6). A four-line ESR signal, corresponding to the DMPO/glutathione thiyl radical adduct, was obtained at pH 7.4 in a clozapine/glutathione/HRP/DMPO system. The adduct resulted in a distinctive ESR spectrum (a^N = 15.4 G and a^{H_β} = 16.2 G), with hyperfine splittings similar to those reported by Harman *et al.* (14). Without clozapine, the same signal was observed, but with much smaller intensity, due to the direct oxidation of glutathione by the enzymatic system. No signal was observed in the absence of glutathione or HRP. The addition of catalase (5000 units/ml) decreased the signal amplitude significantly. When glutathione was replaced with NADPH (Fig. 7), a weak ESR signal was observed, which can be attributed to the DMPO adducts of superoxide, DMPO/·OOH (a^N = 14.3 G, a^{H_β} = 11.35 G, and a^{H_γ} = 1.25 G), and hydroxyl radical, DMPO/·OH (a^N = 14.9 G and a^{H_β} = 14.9 G). When clozapine, NADPH, or HRP was omitted from the incubation, no signal was observed. The DMPO/·OH adduct can be formed either by decomposition of DMPO/·OOH or by the trapping of hydroxyl radical from trace transition metal-dependent reactions (18).

In the presence of 100 μM ascorbate and 100 μM clozapine in the HRP/hydrogen peroxide system, the ascorbyl radical was formed (Fig. 8A). The ascorbyl radical concentration was only marginally affected in the presence of 1 mM glutathione (Fig. 8B), but the radical was almost absent in the presence of 1 mM NADPH (Fig. 8C). In the absence of clozapine, much lower concentrations of the ascorbyl radical were detected (Fig. 8, D and E). No ESR signal was observed in the absence of hydrogen peroxide and ascorbate (Fig. 8, F and G).

Discussion

Aromatic amines that cause idiosyncratic hematological disorders in humans are thought to be metabolized to reactive intermediates by monocytes and neutrophils (2). When these cells are activated during phagocytosis, significant quantities of hydrogen peroxide and myeloperoxidase are released (19). Peroxidases generally activate aromatic amines via one-electron transfer reactions, leading to free radical metabolites. In addition, myeloperoxidase converts hydrogen peroxide and chloride ion to hypochlorous acid, which is a powerful oxidant capable of catalyzing two-electron oxidations (19). Evidence

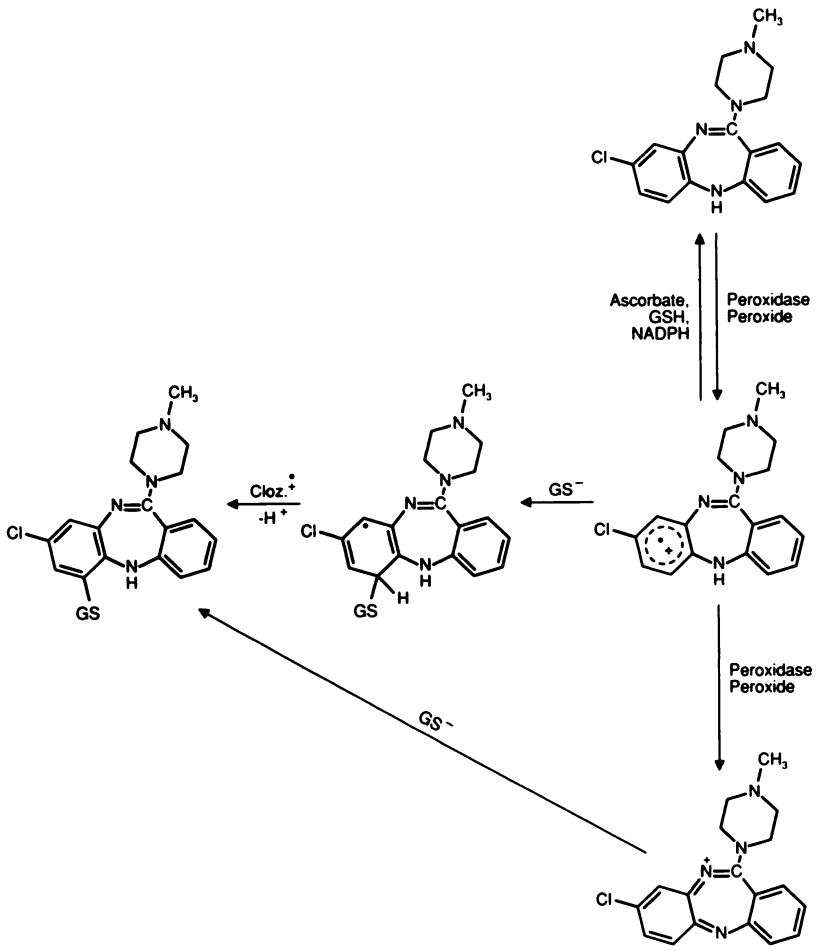

Fig. 10. Proposed clozapine activation by HRP and human myeloperoxidase to reactive intermediates and their reactions with nucleophiles.

that peroxidase systems also activate clozapine to free radical intermediates stems from the detection of glutathionyl and superoxide radicals as reaction products of glutathione, or NADPH, and oxygen with clozapine metabolite(s) (Fig. 9). Additional evidence arises from the >2-fold increase in the steady state concentration of the ascorbyl radical in a peroxidase system when clozapine is present. This indicates one-electron transfer reactions and strongly suggests that clozapine is metabolized to a free radical by both the model peroxidase HRP and human myeloperoxidase. The rate of clozapine oxidation by these peroxidases must be considerably greater than the oxidation of either NADPH, glutathione, or ascorbate, which are known to be weak substrates for peroxidases.

Even though we do not have spectroscopic evidence on the structure of the clozapine radical, the aromatic amine structure of the compound suggests a radical with the spin density delocalized over the aromatic ring and the two adjacent nitrogen atoms. Dreiding models indicate a 100° angle between the two benzene rings, which should prevent the spin density from being delocalized over the second benzene ring. At physiological pH, the radical should exist in its protonated form as a cation radical (Fig. 10), similar to the *p*-phenetidine-derived 4-ethoxyaniline cation radical (20). A clozapine cation radical would be a very electrophilic species, which may react readily with protein thiols or other nucleophiles such as glutathione. The observed adduct formation could proceed through various mechanisms, such as radical-radical coupling or addition of the nucleophilic glutathione anion to the strongly electrophilic clozapine radical cation (Fig. 10). The resulting neutral radical adduct can then be oxidized by a second clozapine cation radical, or by another oxidizing agent, to the stable adduct. Such a mechanism would be analogous to the decay of the chlorpromazine cation free radical, which is second-order in radical concentration (21, 22). Alternatively, second-order radical disproportionation will form the two-electron oxidation product, the di-imine, as proposed in Fig. 10. This is consistent with the shift observed in the UV absorption, from 290 nm to about 340 nm, when clozapine was incubated with peroxide and peroxidases. Conjugate formation would then proceed via addition of the glutathione anion to the di-imine, according to the Michael reaction. The observed conjugate formation at positions 6 and 9, but not at the second benzene ring, is consistent with a radical mechanism and the proposed radical structure. Interestingly, all metabolic changes reported for the aromatic ring system of clozapine (4) occurred at the same ring as found in this study, where glutathione adducts were found in positions 6 and 9.

Some controversy still exists on whether clozapine-induced agranulocytosis is due to a cytotoxic action towards bone marrow cells or whether an immune-mediated mechanism could explain the agranulocytosis formation. The latter mechanism is favored, however, in view of the observation that clozapine suppresses granulocyte division only in extremely high concentrations, 5×10^{-3} M, which are not achieved *in vivo* (1). The

data reported in this study allow for both cytotoxicity and immunogen formation. Tight protein binding of clozapine-derived radioactivity, which is a prerequisite for an immune-mediated mechanism, was also observed in this study. Its inhibition by glutathione and, in particular, by ascorbate indicates that the tight binding is dependent on metabolism and suggests protein adduct formation. *In vivo*, glutathione competes for adduct formation with proteins and, in general, glutathione conjugation is considered to be a detoxification process. However, there is evidence that these conjugates do not necessarily represent a detoxification. Glutathione conjugation of semiquinones or aminophenoxyl free radicals does not necessarily lead to less redox-active species, and these metabolites should be considered among the possible species responsible for toxicity (23–25). The clozapine cation radical itself also behaves as a strong oxidant, as demonstrated by the oxidation of glutathione, ascorbate, or NADPH, which is an important mechanism for the removal of xenobiotic-derived free radical metabolites. Yet, if thiyl radicals are formed in excessive amounts, oxidative stress results, due to the depletion of glutathione pools and the formation of reactive oxygen species. Oxidative stress leads to modifications of protein thiols and is considered to be a major cause of cellular toxicity. Such a mechanism would be of importance if the clozapine-induced agranulocytosis were due to a cytotoxic action towards bone marrow cells.

Ascorbic acid is a reducing agent and radical scavenger that is found in normal human plasma at concentrations of 50–150 μM and in the cytosol of human neutrophils at concentrations of 1.0–1.4 mM (26). Its function has been suggested to be the protection of neutrophils or host tissues by the scavenging of oxygen radicals (27–29) or xenobiotic-derived free radicals (8, 13, 16, 17), at which it is much more efficient than glutathione. Ascorbate at physiological concentrations also reduced the clozapine cation free radical, thereby inhibiting not only oxidative stress but also protein and glutathione adduct formation. These data indicate a protective role of ascorbate against toxicity related to free radicals, such as the clozapine cation free radical, regardless of whether the disorder is caused by a cytotoxic mechanism or an immunological response.

It cannot be decided from this study whether the onset of agranulocytosis due to clozapine treatment in a small group of patients is favored by a genetic predisposition with respect to the patient's immune response, as suggested by Lieberman *et al.* (1, 30), or favored by low levels of ascorbate in schizophrenic patients (31–34). A combination of several factors, including the variability of clozapine metabolism and the natural fluctuation of a person's antioxidant levels, might be responsible for the outbreak of this possibly fatal disease. In any case, the availability of large amounts of antioxidants such as glutathione and, probably more importantly, ascorbate efficiently reduces reactive intermediates *in vitro*. This suggests that the coadministration of clozapine with antioxidants such as ascorbic acid to patients would be beneficial in reducing the risk of agranulocytosis. Ascorbic acid is one of the most important biological antioxidants (35), and its efficacy in reducing the clozapine free radical is more than one order of magnitude greater than that of glutathione. In addition, ascorbic acid exhibits very little toxicity and can safely be used as a dietary supplement.

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