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THIYL RADICALS - FORMATION DURING PEROXIDASE-CATALYZED METABOLISM OF ACETAMINOPHEN IN THE PRESENCE OF THIOLS

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SUMMARY: We confirm using EPR spectroscopy in conjunction with the spin probe 2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine (OXANOH) that horseradish peroxidase catalyzed metabolism of the analgesic acetaminophen occurs via a one electron mechanism. When either glutathione cysteine or N-acetylcysteine were included in the reaction the thiols reduced the acetaminophen-derived radicals to generate thiyl radicals which were trapped with the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and observed using EPR spectroscopy. Similarly, DMPO-thiyl radical adducts were observed during prostaglandin synthase catalyzed oxidation of acetaminophen in the presence of either glutathione or N-acetylcysteine. This is a mechanism of removal of reactive xenobiotic free radicals generated in metabolic systems but whether it represents a true detoxification reaction depends on the subsequent fate of the thiyl radicals generated.

Reactive metabolites generated during peroxidase-catalyzed metabolism generally include substrate free radicals. The detection of these compounds using EPR spectroscopy may be difficult due to their instability or reactivity and spin-trapping agents - compounds which will interact with free radicals to form more stable adducts (1) or spin probes - compounds which can be oxidized by free radicals to

Abbreviations:
OXANOH - 2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine.
OXANO$^{\cdot}$ - 2-ethyl-2,5,5,-trimethyl-3-oxazolidinoxyl.
DMPO - 5,5-dimethyl-1-pyrroline-N-oxide. HRP - horseradish peroxidase.
PGS - prostaglandin synthase. RSVM - ram seminal vesicle microsomes.

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generate the stable radical of the spin probe itself (2) are often used to facilitate the detection of radical species.

One of the cells protective mechanisms against reactive endo- and xeno-biotics involves the ubiquitous thiol glutathione. This tripeptide is known to detoxify reactive electrophilic species by the formation of glutathione conjugates which are subsequently excreted from the cell (3). There are few data however concerning the interaction of glutathione with free radicals generated during the metabolism of drugs and toxicants.

The metabolism of acetaminophen in the kidney by peroxidases such as prostaglandin synthase (PGS, EC 1.14.99.1) has been implicated as a possible determinant of the analgesic nephropathy associated with chronic acetaminophen abuse (4-6). We present EPR data obtained using the spin probe OXANOH demonstrating the generation of substrate-derived free radicals during metabolism of acetaminophen by a model peroxidase; horseradish peroxidase (HRP, EC 1.11.1.7). Furthermore, utilizing the spin trap DMPO to detect thiyl radicals we show that the inclusion of either glutathione, cysteine or N-acetylcysteine during the HRP- or PGS-catalyzed metabolism of acetaminophen leads to the generation of the appropriate thiyl radical.

MATERIALS AND METHODS

L-Cysteine, glutathione, N-acetylcysteine, acetaminophen, arachidonic acid, HRP, superoxide dismutase, and DMPO were obtained from Sigma Chemical Co (St. Louis, MO). OXANO$^{\bullet}$ was a gift from Dr L.I. Olsson, Pharmacia Ltd, Uppsala, Sweden and its reduced form OXANOH was prepared as described previously in the presence of DETAPAC (7). Ram seminal vesicle microsomes (RSVM) were used as a source of PGS activity and were prepared as described previously (8).

EPR spectra were recorded on a Varian E9 spectrometer, fitted with a TM110 cavity. Incubations were performed at room temperature in 100 mM phosphate buffer (pH=8) containing 1 mM EDTA.

RESULTS

The oxidation of acetaminophen by HRP was investigated. Paramagnetic species could not be observed using EPR spectroscopy during hydrogen peroxide/HRP catalyzed oxidation of acetaminophen in

the presence or absence of the spin trap DMPO (100 mM). The inclusion of the spin probe OXANOH (1 mM) however resulted in the generation of an EPR signal characteristic of the nitroxide OXANO$^{\bullet}$ (a^N = 16 G). The generation of this signal was enzyme-, substrate- and hydrogen peroxide-dependent and the intensity of the signal was not decreased by the inclusion of superoxide dismutase (80 µg/ml) or sodium benzoate (10-100 mM) in the mixture indicating that $O_2^{\bullet-}$ or OH$^{\bullet}$ were not the oxidizing species. When glutathione was mixed with acetaminophen, HRP and hydrogen peroxide no EPR signal could be detected. The inclusion of the thiol in a reaction containing substrate, HRP, the spin probe OXANOH and an excess of peroxide caused the generation of an EPR signal indicative of OXANO$^{\bullet}$ production as previously but its intensity compared to that observed in the absence of thiol was decreased As we observed in control experiments that glutathione did not reduce the OXANO$^{\bullet}$ signal directly this suggested that glutathione may be competing with OXANOH for the acetaminophen-derived radical generated during hydrogen peroxide/HRP catalyzed oxidation of acetaminophen. Such an interaction between glutathione and an acetaminophen radical would cause the generation of a glutathione thiyl radical. That such a radical was produced was confirmed by the use of the spin trap DMPO which has been used to trap thiyl radicals produced during cysteine oxidation (9,10). When acetaminophen, HRP, hydrogen peroxide and glutathione were mixed in the presence of DMPO the EPR signal shown in Fig. 1B (a^N = 15.0 G, a^H = 16.3 G) was obtained. When cysteine was used in place of glutathione the spectrum in Fig. 1C (a^N = 15.2 G, a^H = 17.0 G) was observed which is almost identical to that proposed for the DMPO-cysteinyl radical adduct in (10). N-acetylcysteine (Fig. 1D) used in a similar manner, also gave rise to a spectrum indicative of a DMPO-thiyl radical adduct (a^N = 15.0 G, a^H = 16.8 G). Such spectra were not obtained in the absence of acetaminophen, enzyme (not shown) or thiol (Fig. 2A). Thus on the basis of the previously

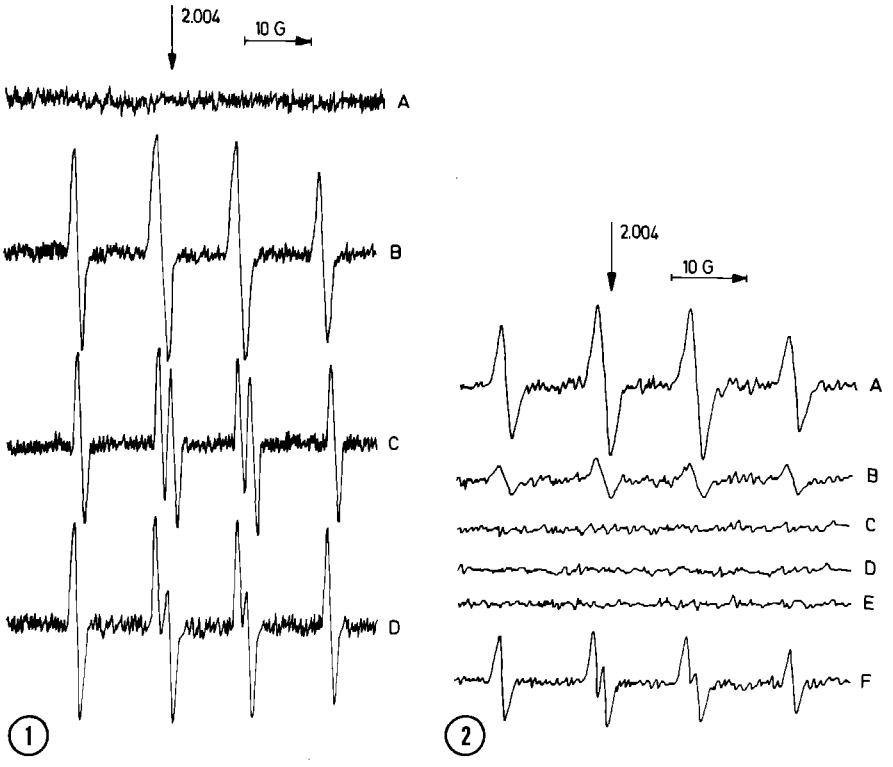

Fig. 1. DMPO-thiyl radical adducts generated during HRP-catalyzed oxidation of acetaminophen in the presence of thiols and DMPO. A - Reaction containing acetaminophen (1 mM), HRP (0.2 µg/ml), hydrogen peroxide (0.25 mM) and DMPO (100 mM); B - as A but in the presence of glutathione (5 mM); C - as A but in the presence of cysteine (5 mM); D - as A but containing N-acetylcysteine (5 mM). Instrument settings were microware power 10 mW modulation amplitude, 0.2 G: receiver gain, 2×10^4; time constant, 0.3 s; scan time, 2 minutes. Reactions were started by addition of peroxide and spectra were recorded 1 minute later.

Fig. 2. DMPO-thiyl radical adducts generated during RSVM-catalyzed oxidation of acetaminophen in the presence of thiols and DMPO. A - Reaction containing acetaminophen (1 mM), RSVM (1 mg/ml), glutathione (5 mM), arachidonic acid (0.1 mM) and DMPO (100 mM); B - as A but without acetaminophen; C - as A but without arachidonic acid; D -as A but with boiled RSVM; E - as A but in the presence of Indomethacin (0.1 mM); F - as A but with N-acetylcysteine in place of glutathione. Instrument settings as above but receiver gain, 10^4; modulation amplitude, 2 G; scan time, 4 minutes. Reactions were started by the addition of arachidonic acid and recorded 1 minute later.

characterized DMPO-cysteinyl radical adduct (9,10) we assign the EPR signals in Figs. 1B-D to DMPO-glutathionyl, DMPO-cysteinyl and DMPO-N-acetylcysteinyl radical adducts respectively. All of the DMPO-thiyl radical adducts were unstable species and significant loss in signal intensity was observed in each case during successive scans.

When PGS, contained in RSVM, was used in place of HRP a similar spectrum to that shown in Fig. 1B was observed in the presence of acetaminophen, arachidonic acid, glutathione and DMPO (Fig. 2A). This spectrum was not observed when arachidonic acid was omitted from the reaction (Fig. 2C), when boiled RSVM were used (Fig. 2D) or when Indomethacin an inhibitor of PGS was pre-incubated with RSVM (Fig. 2E). An EPR signal consistent with the DMPO-glutathionyl radical adduct was observed however when acetaminophen was omitted from the reaction (Fig. 2B) but its intensity was less than 25 % of that observed in the presence of acetaminophen. This suggests that glutathione itself at high (5 mM), but physiological, concentrations may act as a co-substrate for PGS. When N-acetylcysteine was included in the complete reaction mix instead of glutathione the spectrum shown in Fig. 2F, typical of the DMPO - N-acetylcysteinyl radical adduct (cf, Fig. 1D) was observed.

DISCUSSION

Peroxidase-catalyzed metabolism of acetaminophen in the kidney has been implicated as a cause of the nephrotoxicity associated with chronic acetaminophen abuse (5-7) and the metabolism of acetaminophen by a model peroxidase - HRP - has been suggested to occur via a free radical mechanism (11). We could not observe acetaminophen radicals during HRP-catalyzed oxidation of acetaminophen either directly or in conjunction with the spin trap DMPO using conventional EPR techniques. The use of the spin probe OXANOH however indicated that substrate-derived free radicals were produced during this reaction As this technique is based on the one-electron oxidation of the spin probe itself to generate the stable OXANO$^{\cdot}$ radical this method demonstrates only that free radicals are produced in a reaction and imparts no information as to the identity of the radical species. In this system the enzyme-, peroxide- and acetaminophen-dependence of the paramagnetic signal confirmed that the radical species responsible for the

oxidation of OXANOH to OXANO$^{\bullet}$ was a product of the peroxidase-catalyzed oxidation of acetaminophen.

The inclusion of thiols during HRP- and PGS-catalyzed oxidation of acetaminophen led to the production of thiyl radicals which were detected as DMPO-thiyl radical adducts using EPR spectroscopy. Glutathione, cysteine and N-acetylcysteine were shown to reduce the acetaminophen derived radical to produce the appropriate thiyl radicals. The generation of thiyl radicals during HRP- and PGS-catalyzed oxidation of acetaminophen in the presence of thiols demonstrates that both of these oxidations occur via the production of acetaminophen derived free radicals.

Thiyl radicals are known to dimerize to form oxidized glutathione and to interact with oxygen (12). We and other workers have previously used these two factors and inhibition of substrate removal in the presence of thiol as indirect indices of thiyl radical production in metabolic systems (13-16) including peroxidase catalyzed oxidation of acetaminophen (17-19). Cysteinyl radicals have been detected previously using DMPO as a spin-trap during the autooxidation of cysteine (9) and during metabolism of relatively high concentrations of cysteine by HRP (10). This report, utilizing direct methods, shows for the first time that thiols such as glutathione can interact with metabolically-generated xenobiotic radicals in a redox process which generates thiyl radicals.

The removal of the acetaminophen radical by interaction with thiols is of toxicological relevance in the case of glutathione which is present in the body in millimolar concentrations and N-acetylcysteine which is used as a treatment for acute acetaminophen overdosage (19). The removal of the acetaminophen radical by such thiols presumably represents a mechanism whereby reactive xenobiotic-derived free radicals can be removed from a metabolic system. Thiyl radicals however have been proposed as mediators of the mutagenic effects of

glutathione and cysteine observed in bacterial testing systems (20). We are currently investigating the fate of thiyl radicals in metabolic systems, but until their reactions have been fully characterized whether their generation from the interaction of metabolically-derived xenobiotic radicals with thiols represents a true detoxification reaction must remain an open question.

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