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CARBON-CENTERED FREE RADICAL FORMATION DURING THE METABOLISM OF HYDRAZINE DERIVATIVES BY NEUTROPHILS

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Abstract—The neutrophil-catalyzed metabolism of hydrazine derivatives to carbon-centered radicals was investigated by the spin-trapping technique using α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN). Oxidation of methylhydrazine (MeH), dimethylhydrazine (DMH), phenylethylhydrazine or procarbazine by neutrophils from rat peritoneal exudates led to the formation of alkyl radicals. The monosubstituted hydrazine oxidation by phorbol ester (PMA)- or Zymocel-activated neutrophils generated, on average, 2- to 4-fold more POBN-alkyl adducts than di-substituted hydrazines. Supernatant from sonicated neutrophils generated similar yields of radicals. Azide, an inhibitor of myeloperoxidase, effectively reduced the neutrophil-catalyzed radical yield from the oxidation of MeH but not DMH. On the other hand, superoxide dismutase and catalase effectively inhibited radical formation in DMH metabolism by PMA-activated neutrophils, in contrast to MeH metabolism. Our results show that neutrophils are able to metabolize hydrazine derivatives, the pathway depending on the hydrazine substitution. Alkyl radical production during the oxidation of mono-substituted derivatives, such as MeH, was mediated mainly by myeloperoxidase, and that of di-substituted derivatives, such as DMH, was mediated mainly by active oxygen species.

Neutrophils (PMNs)† are involved in the defense against infectious microorganisms. Their activation by phagocytic or chemotactic stimulus leads to an abrupt increase in oxygen consumption followed by the production of remarkable quantities of superoxide anions (respiratory burst), which leads to the formation of hydrogen peroxide by dismutation [1].

In addition, activated PMNs produce powerful oxidants, through the myeloperoxidase system [2]. Metabolism of pharmacologically active drugs, such as arylamines, hydrazines, hydantoins, and dapsones, by PMN-derived oxidants or myeloperoxidase has been described [3]. In some instances, phagocyte activation can replace the cytochrome P450 mixed-function oxidase system in the oxidation of carcinogens to reactive intermediates [4–6]. This raises the question of whether PMN-induced xenobiotic-derived free radicals could have a role in the induction of the carcinogenic processes observed at inflammatory sites.

Hydrazine derivatives are an interesting class of chemicals since, besides their several industrial uses, many of them have pharmacological activities [7]. 2-Phenylethylhydrazine (PEH) is a monoamine oxidase inhibitor used clinically as an antidepressant; procarbazine [*N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide] (PCZ) is an effective antitumor agent; 1,2-dimethylhydrazine (DMH) is a potent carcinogen and methylhydrazine (MeH) has been used frequently as a model to study the metabolism of these compounds [8]. Most hydrazines are carcinogenic to experimental animals [7]. These derivatives are metabolically activated to reactive intermediates, such as carbon-centered radicals [9, 10], active oxygen species [11, 12] and probably alkyldiazonium ions [13–16]. Formation of carbon-centered radicals has been well established by spin-trapping during the enzymatic oxidation of several mono- and some di-substituted derivatives by hemoproteins, microsomes or hepatocytes [17–23].

PMNs could metabolize hydrazine derivatives to reactive intermediates through the myeloperoxidase oxidation system [3, 4]. The oxidation of phenylhydrazine to phenyl radicals by $HOCl^-$ produced by PMNs has been demonstrated [24]. In the present study, we show that the oxidative metabolism of the hydrazine derivatives MeH, DMH, PEH and PCZ by PMNs from rat peritoneal exudates leads to the formation of carbon-centered radicals and investigate the possible mechanisms using the spin-trapping technique.

MATERIALS AND METHODS

The following reagents were obtained from commercial sources: Hanks' balanced salt solution (HBSS), glycogen (Type II), cytochrome *c*, phorbol-12-myristate-13-acetate (PMA), diethylenetriamine

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† Abbreviations: PMNs, neutrophils; MeH, methylhydrazine; DMH, 1,2-dimethylhydrazine·2HCl; PEH, 2-phenylethylhydrazine·H_2SO_4; PCZ, procarbazine, *N* - isopropyl - α - (2 - methylhydrazino) - *p* - toluamide hydrochloride; DTPA, diethylenetriamine pentaacetic acid; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; Tempol, 4 - hydroxy - 2,2,6,6 - tetramethylpiperidine - *N*-oxyl; HBSS+G, Hanks' balanced salt solution plus 5 mM glucose; SOD, superoxide dismutase; ssPMN, supernatant from sonicated neutrophils; and PMA, phorbol-12-myristate-13-acetate.

pentaacetic acid (DTPA), *o*-dianisidine·2HCl, DMH, PEH, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN), myeloperoxidase and catalase (Sigma Chemical Co.); Heparin (Roche), superoxide dismutase (SOD) (ICI) and Zymocel (Alpha-Beta Technologies); 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempol) and MeH (Aldrich Chemical Co.). MeH was precipitated with H_2SO_4 to form crystalline MeH·H_2SO_4 and recrystallized from methanol. PCZ was supplied by the Drug Synthesis and Chemistry Branch of the National Cancer Institute. Water grade Milli-Q (Millipore) was used throughout. Stock solutions of hydrazine derivatives were prepared immediately before use in HBSS.

Preparation of PMNs. PMNs from female Wistar rat peritoneal exudates were obtained as previously described [25] by injection of 0.8% glycogen dissolved in saline, 12 hr before the rats were killed with diethyl ether. Cells were collected by peritoneal washing with 10% heparin in HBSS. The collected cells were centrifuged and washed twice with HBSS plus 5 mM glucose (HBSS+G) to eliminate most of the heparin from the final cell suspension, which was adjusted to $2–3 \times 10^7$ cells/mL. This procedure regularly produces a population with 98% viability, determined by Trypan blue exclusion; 90% of the cells were PMNs. The cellular suspension was maintained at 0–4° until used in a maximum of 4 hr. Supernatant from sonicated PMNs (ssPMN) was prepared by sonicating a suspension of $2–3 \times 10^7$ cells/mL at 0° for 6 × 3 min on a Branson Sonifier 450 with a duty cycle of 30% and the output control setting at 4. The supernatant was collected after centrifugation. Superoxide production was followed by reduction of cytochrome *c* [26] and myeloperoxidase activity was determined [27] as already described.

Lactate dehydrogenase assay. The activity of lactate dehydrogenase was measured by the consumption of NADH (0.16 mM), followed at 340 nm, by the supernatant of PMNs in the presence of pyruvate (1.25 mM) in 0.05 M Tris buffer, pH 7.4, at 37° as described [28].

Electron paramagnetic resonance (EPR) studies. Spectra were recorded at room temperature on a Bruker ER 200D-SRC spectrometer. Aliquots (100–200 μL) from incubation mixtures were transferred to flat quartz cells. The standard reaction mixtures containing 1×10^7 PMNs/mL (or the sonicated supernatant), 75 mM POBN and 0.1 mM DTPA in HBSS+G were sparged with nitrogen for 5 min after which 2×10^{-7} M PMA or 0.8 mg/mL Zymocl plus 0.1 to 5 mM hydrazine derivative was added and incubated with agitation for 1 hr in a reciprocating water bath at 37°. In some incubation mixtures 20 μg/mL SOD, 5 μg/mL catalase or 1 mM azide was included. Other modifications of the incubation mixtures are described in the text or figure legends. The concentration of POBN radical adducts was calculated by double integration using Tempol as the standard.

RESULTS

Carbon-centered radical formation. Incubation of the hydrazine derivatives, MeH, PEH, DMH or PCZ, with PMNs from rat peritoneal exudates activated by the tumor promoter, PMA, in the presence of POBN, led to the formation of carbon-centered radicals, which could be detected by EPR (Fig. 1). These same adducts were observed whether the hydrazine derivatives were metabolized by Zymocel-activated PMNs or by ssPMN (results not shown). The EPR spectra of the adducts had similar parameters ($a_N = 15.25$, $a_H = 2.75$), indicating trapping of the corresponding carbon-centered radicals [29]. In agreement, radical adducts with smaller hyperfine constants ($a_N = 13.75$, $a_H = 2.00$) were occasionally observed but their appearance could be eliminated by nitrogen sparging. The latter species should correspond to alcoxy radicals formed by the reaction of primary alkyl radicals with oxygen [30, 31]. The fact that it is difficult to distinguish different POBN-alkyl radical adducts by their EPR parameters is emphasized in Fig. 1d. PCZ oxidation

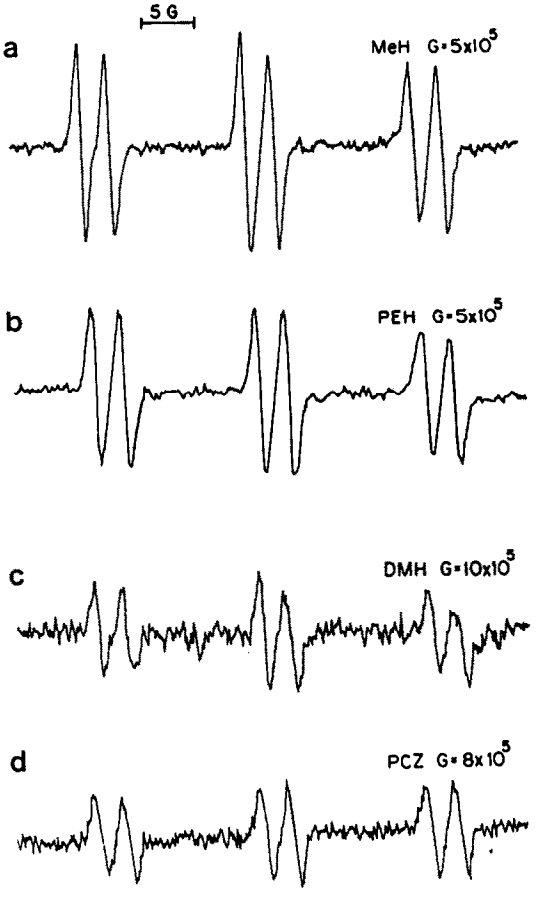

Fig. 1. EPR spectra of POBN-radical adducts from incubations of PMNs (1×10^7/mL) and PMA (2×10^{-7} M) with the following hydrazine derivatives: (a) 1 mM MeH, (b) 2 mM PEH, (c) 5 mM DMH and (d) 2 mM PCZ, in the presence of 75 mM POBN and 0.1 mM DTPA, in HBSS+G as described in Materials and Methods. Spectrometer conditions: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 0.5 sec; and scan rate, 0.2 G.

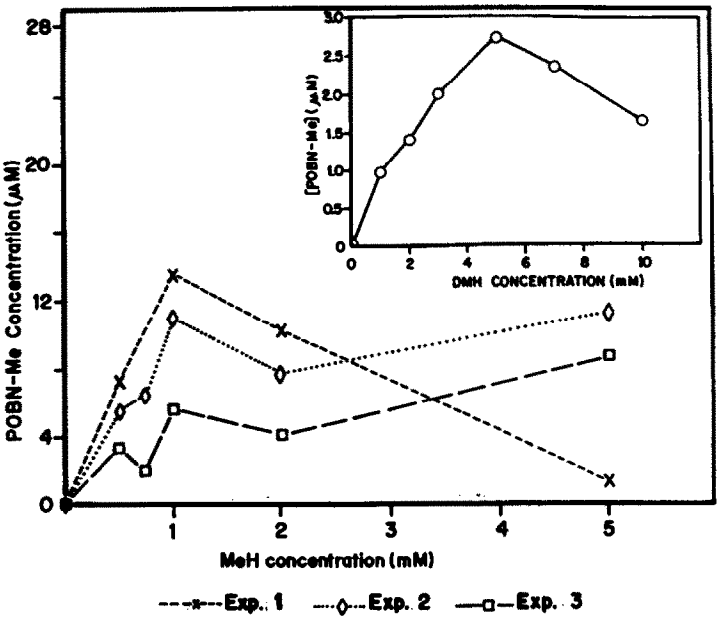

Fig. 2. Dependence of POBN-methyl radical adduct formation on MeH (figure) or DMH (inset) concentration. PMNs (1×10^7/mL) were incubated in the presence of 2×10^{-7} M PMA, 75 mM POBN and 0.1 mM DTPA in HBSS+G in the presence of increasing concentrations of the hydrazine derivative for 1 hr at 37°. Radical concentration was calculated as described in Materials and Methods.

has been shown to generate both methyl and benzyl-type free radicals, but these species cannot be distinguished by the EPR spectrum of the POBN adducts ([21, 29]; Fig. 1d). The use of spin traps that would give a more informative EPR spectrum such as methylnitrosopropane, did not lead to radical adduct detection. In spite of low radical yields, trapping with POBN can be ascribed to its efficiency in reacting with carbon-centered radicals [32].

Due to previous reports on the toxicity of spin traps to cells [33, 34], control experiments were performed to determine the optimum trap concentration. There was a maximum of radical adduct formation in the oxidation of MeH by the PMN/Zymocel system at 75 mM POBN (data not shown); higher concentrations of the spin-trap inhibited radical yield. Myeloperoxidase activity released into neutrophil supernatant upon activation by Zymocel was also inhibited at spin-trap concentrations higher than 75 mM (data not shown). For these reasons, all the experiments were performed with 75 mM POBN. Metal-catalyzed auto-oxidation of the hydrazine derivatives was inhibited by inclusion of the chelator DTPA.

POBN-methyl radical adduct yield increased with MeH concentration up to a maximum of 1 mM MeH during oxidation of the drug by PMA-activated PMN (PMN/PMA) (Fig. 2) or by Zymocel-activated PMN (PMN/Zymocel) (data not shown). At concentrations above 1 mM MeH a decrease in radical adduct yield was observed, which may reflect toxicity of the drug to the PMNs. At a concentration of 5 mM MeH the results were not reproducible. The enhanced release of lactate dehydrogenase into supernatant of PMNs, used as a measure of hydrazine toxicity, demonstrated that, in fact, concentrations of MeH higher than 1 mM are toxic to the cells (Table 1).

Table 1. Lactate dehydrogenase release from PMNs

System	Lactate dehydrogenase activity* (U/L)
PMN	139 ± 9
ssPMN	1207 ± 122
PMN/MeH 1 mM	125 ± 15
PMN/MeH 5 mM	201 ± 64
PMN/DMH 2 mM	157 ± 48
PMN/DMH 5 mM†	227 ± 88
PMN/PEH 2 mM	129 ± 15
PMN/PCZ 2 mM	170 ± 20

* Lactate dehydrogenase activity was determined in the supernatant of PMNs treated with the indicated concentrations of the hydrazine derivatives for 1 hr at 37° as described in Materials and Methods. Values are means ± SD of three independent determinations.

† At this concentration of DMH, inhibition of lactate dehydrogenase activity was observed.

The results in Fig. 2 show that the absolute values obtained for the radical concentrations varied from one experiment to another, but the general trend was maintained. By increasing the number of experiments we observed that the average values and the dispersion were maintained (data not shown). Although we used a pool of PMNs from

three individuals and standardized the experiments in relation to the intensity of the respiratory burst and release of myeloperoxidase into the supernatant, other unidentified variations must occur between pools. Hypochloride has been shown to catalyze the oxidation of phenylhydrazine to phenyl radicals [24], but its formation is expected to be constant since superoxide formation and myeloperoxidase release are being controlled. One possible explanation for the dispersion was given in experiments in which the neutrophils were obtained from a single animal, where there seemed to be two populations of neutrophils, one with high and the other with low oxidation capacity (data not shown). We attribute the observed dispersion to intrinsic properties of the PMN pools; the deviations observed in the assays described below would reflect the variations between these pools and not random errors, maintaining the same tendency in the individual experiments.

In the metabolism of DMH by PMN/PMA, the yield of POBN-methyl radical adduct was also dependent on drug concentration, with a maximum at 5 mM DMH, decreasing at higher concentrations possibly due to its toxicity (inset Fig. 2). Lactate dehydrogenase release into PMN supernatant, also indicated toxicity of the drug to the cells (Table 1).

Mechanism of carbon-centered radical formation. We used different activation systems to investigate the metabolism of the hydrazine derivatives. Activation by PMA leads to the induction of the respiratory burst, but not to an additional release of myeloperoxidase in relation to control PMN [2]. Zymocel, a highly purified β-glucan, substitutes opsonized zymozan in the activation of PMNs [35] stimulating phagocytosis and, consequently, the respiratory burst and myeloperoxidase release. ssPMN contains myeloperoxidase with no production of active oxygen species.

Comparing the methyl radical adduct formation in the oxidation of MeH by the different activation systems, we observed that the ssPMN, which contained four times more myeloperoxidase than that released by Zymocel activation (data not shown), provided the highest yield (Table 2). Purified myeloperoxidase, at a concentration equivalent to that released by Zymocel activation also catalyzed alkyl radical production (Table 2). PMA activation, which produced a more intense respiratory burst, formed fewer methyl radicals than Zymocel activation (Table 2), although alkyl radical production from the reaction of hydrazines with superoxide anions has been described [36]. It is important to keep in mind that the observed deviations were not random errors, but fluctuations between PMN pools. In this sense radical yield was higher with the PMN/Zymocel system then with PMN/PMA in all the experiments performed. Resting PMNs also catalyzed oxidation of MeH to carbon-centered radicals, although in lower yields. Results obtained with PEH were comparable to those obtained with MeH (Table 2); although smaller differences between the different activating systems were observed, mono-substituted hydrazines should be oxidized by the same mechanisms.

We observed that the oxidation of DMH in the different systems had a lower yield of methyl radicals than MeH or PEH (Table 2). In this case, the PMN/PMA system was more efficient than the myeloperoxidase-releasing systems. It seems that active oxygen species are relatively more important in the oxidation of this drug. PCZ seemed to be an intermediate case, since oxidation to alkyl radicals by ssPMN was lower than by the activated PMNs, but oxidation by the PMN/PMA system was not more efficient than that by PMN/Zymocel. In general the di-substituted hydrazine derivatives showed a lower yield of carbon-centered radical formation by activated PMNs than the mono-substituted compounds.

Incubations of MeH with activated neutrophils were performed in the presence of the active oxygen species scavengers, catalase for H_2O_2 and SOD for superoxide anion, or the myeloperoxidase inhibitor, azide, to determine the most important oxidants in the formation of carbon-centered radicals. The inhibitory effect was similar whether activation of PMNs was induced by PMA or Zymocel (Fig. 3 and inset). SOD and catalase inhibited radical formation by 20–40%, while azide inhibition was over 70%, confirming the indication that myeloperoxidase has a major role in MeH metabolism to methyl radicals by activated PMNs. Catalase concentration had to be adjusted since above 5 μg/mL it catalyzed oxidation of hydrazine derivatives to carbon-centered radicals (data not shown).

POBN-methyl radical adduct formation in the oxidation of DMH by PMN/PMA was inhibited between 45 and 70% by catalase and SOD, while azide inhibition was around 20% (Fig. 4), supporting the idea that myeloperoxidase has a minor role in the oxidation of the di-substituted derivatives by activated PMNs, when compared with active oxygen species.

Incubation of the ssPMN with hydrazine derivatives led to the formation of carbon-centered radicals, which in the case of the mono-substituted but not the di-substituted derivatives can be inhibited by azide (Fig. 5). Azide is not a specific inhibitor of myeloperoxidase and would also inhibit the peroxidase activity of catalase. Incubation of MeH with ssPMN in the presence of 3-amino-1,2,4-triazole, a specific inhibitor of catalase [37], did not inhibit the generation of methyl radicals (data not shown), demonstrating that myeloperoxidase and not catalase is responsible for this oxidative process.

DISCUSSION

In the present study, we demonstrated that activated PMNs can metabolize MeH and PEH to the respective methyl and phenylethyl radicals (Figs. 1–3, Table 2). Radical formation was dependent upon cell concentration (data not shown) and upon drug concentration (Fig. 2). The substantial inhibition of POBN-methyl radical adduct formation in the metabolism of MeH by activated PMNs or ssPMN when in the presence of azide indicates a role for myeloperoxidase. On the other hand, the low inhibition observed when reactions were carried out in the presence of SOD or catalase (Fig. 3) suggests that active oxygen species have a minor role in

Table 2. Alkyl radical formation by different systems

System	Radical adduct concentration* (μM)			
	MeH	PEH	DMH	PCZ
PMN/PMA†	8.4 ± 4.2	8.1 ± 0.9	2.7 ± 1.5	4.3 ± 2.8
PMN/Zymocel‡	11.4 ± 8.2	9.0 ± 1.5	1.4 ± 0.4	5.0 ± 3.3
ssPMN§	20.1 ± 0.5	9.3 ± 0.2	1.3 ± 0.3	3.0 ± 2.0
PMN‖	3.7 ± 0.9	2.1 ± 0.3	1.0 ± 0.5	1.6 ± 1.4
Myeloperoxidase¶	3.1 ± 1.5	—	1.2 ± 0.4	—

* Values are means ± SD of three independent determinations. Radical concentrations were calculated as described in Materials and Methods.

† PMNs (1×10^7 cells/mL) and PMA (2×10^{-7} M) were incubated in the presence of 75 mM POBN and 0.1 mM DTPA in HBSS + G for 1 hr at 37° with: 1 mM MeH, 2 mM PEH, 5 mM DMH, or 2 mM PCZ.

‡ Same as † except that Zymocel was substituted for PMA (0.8 mg/mL).

§ Same as † except that ssPMN was substituted for PMN and PMA as described in Materials and Methods.

‖ Same as † in the absence of an activator.

¶ Same as † except that purified myeloperoxidase was substituted for PMN and PMA at a concentration equivalent to that released during Zymocel activation (100 U/mL).

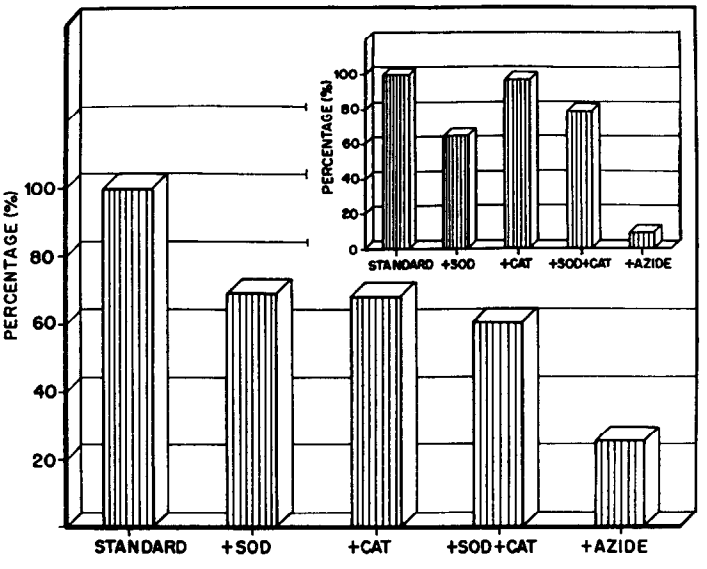

Fig. 3. Effect of scavengers and inhibitors on POBN-methyl radical adduct formation in the oxidation of MeH by PMNs activated by PMA or Zymocel. PMNs (1×10^7/mL) were incubated with 1 mM MeH, 75 mM POBN and 0.1 mM DTPA, in HBSS+G in the presence of an activator, PMA (2×10^{-7} M) (figure) or Zymocel (0.8 mg/mL) (inset), with the following scavengers: 5 μg/mL catalase, 20 μg/mL SOD or 1 mM azide for 1 hr at 37°. The values are from one representative experiment and the standard varied from 2.4 to 12.7 μM for PMA activation and from 4.5 to 22.9 μM for Zymocel activation. Radical concentrations were calculated as described in Materials and Methods.

mono-substituted hydrazine derivative oxidation by PMNs.

Activated PMNs also catalyzed the oxidation of DMH and PCZ to alkyl radicals (Figs. 1, 2 and 4 and Table 2), and radical formation was dependent upon drug concentration in the oxidation of DMH by PMN/PMA (Fig. 2, inset). Radical adduct yield in the oxidation of the di-substituted derivatives was low when compared to the mono-substituted compounds (Fig. 1 and Table 2), which may indicate that the drug specificity is governed by their redox potential. On the other hand, the oxidation processes appear to be different for mono- and di-substituted hydrazines.

In contrast to mono-substituted hydrazine derivatives, DMH and PCZ oxidation to carbon-centered

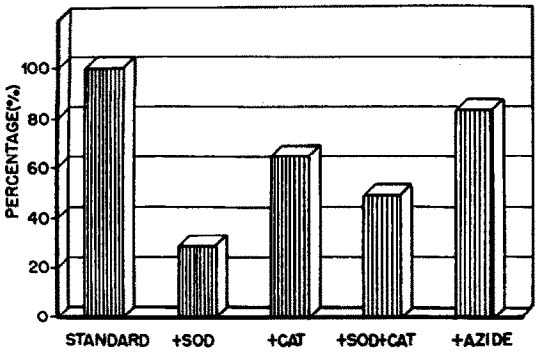

Fig. 4. Effect of scavengers and inhibitors on POBN-methyl radical adduct formation in the oxidation of DMH by PMNs activated by PMA. PMNs (1×10^7/mL) were incubated with 5 mM DMH, 75 mM POBN and 0.1 mM DTPA, in HBSS+G in the presence of the activator PMA (2×10^{-7} M) with the following scavengers: 20 μg/mL SOD, 5 μg/mL catalase, or 1 mM azide for 1 hr at 37°. The values are from one representative experiment and the standard varied from 2.2 to 7.6 μM. Radical concentrations were calculated as described in Materials and Methods.

radicals showed similar yields when catalyzed by activated or resting PMNs or by ssPMN. A minor inhibition was observed in the oxidation of DMH by activated PMNs when incubated in the presence of azide (Fig. 4). Neither DMH nor PCZ oxidation by ssPMN was inhibited significantly by azide (Fig. 5). On the other hand, catalase and SOD significantly inhibited radical formation in DMH oxidation by activated PMNs. These results indicate that myeloperoxidase has a minor role in di-substituted hydrazine derivative oxidation to carbon-centered radicals, when compared to active oxygen species.

When hydrazine derivatives, especially mono-substituted derivatives, enter circulation, they are

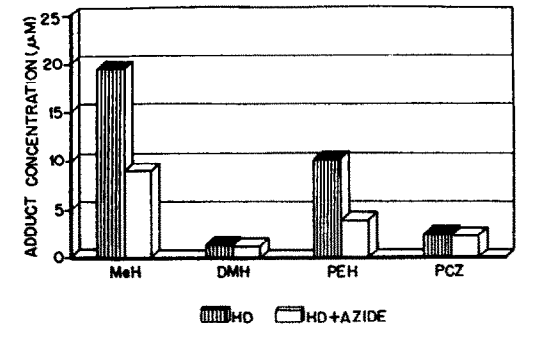

Fig. 5. Effect of azide on the formation of POBN-radical adducts in the oxidation of hydrazine derivatives by ssPMN. ssPMN (from 1×10^7/mL cells) was incubated in the presence of 75 mM POBN and 0.1 mM DTPA in HBSS+G for 1 hr at 37° with: 1 mM MeH, 5 mM DMH, 2 mM PEH or 2 mM PCZ in the presence or absence of 1 mM azide. The values are from one representative experiment. Radical concentrations were calculated as described in Materials and Methods.

metabolized by erythrocytes [22,38,39] and PMNs (our results) before reaching the tissues. This would protect critical sites from being reached by high concentrations of hydrazine derivatives. If an inflammatory process occurs during hydrazine derivative administration, toxic metabolites would be formed at the inflammatory site, which could contribute to future development of a carcinogenic process in the surrounding cells. In fact, carbon-centered radicals and cationic metabolites generated during oxidation of hydrazine derivatives bind covalently to DNA [32, 40–42] and proteins [32]. Alkylation of proteins such as myeloperoxidase is likely to occur and could be responsible for the hypersensitivity reactions of some hydrazine derivatives [3, 8].

In conclusion, PMNs may have a role in hydrazine derivative metabolism and/or in their activation to toxic intermediates during inflammatory processes.

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