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N-OXIDATION OF *N*,*N*-DIMETHYLANILINE IN THE RABBIT AND RAT LUNG

YOSHIO OHMIYA* and HARIHARA M. MEHENDALE†
Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS 39216, U.S.A.

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Abstract—We have reported previously that chlorpromazine (CPZ) and imipramine (IMP) are metabolized via *N*-oxidation by the rat lung, while they are not appreciably metabolized by the rabbit lung. Indeed, marked species differences exist in the pulmonary *N*-oxidation of these pneumophilic drugs. In the present studies, the isolated, ventilated and perfused lung (IPL) preparations as well as *in vitro* preparations of the rabbit and rat lungs were used to examine the pulmonary disposition of [^{14}C]-*N*,*N*-dimethylaniline (DMA) which has been used frequently as a substrate for *N*-oxidation. Although the IPLs of both species were active in DMA *N*-oxidation, the rabbit lung was more active in DMA *N*-oxidation than the rat lung on the basis of per g lung. The gradual decline in radiolabel concentration in the perfusate was more marked in the rat than in the rabbit IPL. This decline was not due to the drug accumulation in the lung, but to its volatility. There was no dose dependency in the tissue/medium DMA concentration ratios (approximately 1.60), indicating uptake by simple diffusion and low affinity for the lung tissue. *In vitro* lung preparations showed higher DMA *N*-oxidase activity in the rabbit than in the rat, regardless of whether whole homogenate, post-mitochondrial supernatant fraction or microsomal fraction was used, or how the activities were expressed (per mg protein or per g tissue). These results suggest that, although DMA is not highly concentrated in the lung, it is *N*-oxidized by the lung and that DMA *N*-oxidase is different from CPZ or IMP *N*-oxidase reported previously.

A variety of chemicals, which preferentially concentrate in the lung, include antihistamines, antidepressants, β-adrenergic antagonists and morphine-like and synthetic analgesics [1]. In view of their common structural and chemical characteristics with pK_a values greater than 8, these pneumophilic drugs are referred to as basic amines [2]. We have reported previously [3–5] that, while a phenothiazine drug, chlorpromazine (CPZ), and a tricyclic antidepressant, imipramine (IMP), were not metabolized by the isolated, ventilated, perfused rabbit lungs (IPLs), they were metabolized by the perfused rat lungs via *N*-oxidation from whence their *N*-oxides were released into the circulation because of low affinity for the lung tissue. A species comparison using isolated lung microsomes revealed relatively high *N*-oxidase activity for CPZ and IMP in rat and pig lung while little or no activity in goat or rabbit lung [6, 7].

In contrast to our findings, Breyer [8] reported that another phenothiazine drug, perazine, was metabolized by rabbit lung microsomes to perazine *N*-oxide, whereas rat and pig lung microsomes *N*-oxidized perazine at a much slower rate. Devereux and Fouts [9] reported that rabbit lung microsomal *N*-oxidase activity for *N*,*N*-dimethylaniline (DMA) was about three times higher than liver *N*-oxidase at the pH optimum of 8.9, whereas these activities were nearly identical at physiological ranges of pH. These findings can be reconciled on the basis of substrate specificity for pulmonary drug *N*-oxidase activity. The present investigations were undertaken with two objectives: first, to examine the uptake and accumulation of DMA utilizing IPLs; and second, to determine which species, rabbit or rat, is more active in pulmonary DMA *N*-oxidation, using the perfused as well as *in vitro* lung preparations in order to test the above proposal.

MATERIALS AND METHODS

Chemicals. [^{14}C]DMA (5 Ci/mole) was purchased from the California Bionuclear Corp. (Sun Valley, CA) and diluted with unlabeled DMA (Aldrich Chemical Co., Milwaukee, WI) to obtain the desired concentrations. Purity of the labeled drug was 98%, as assessed by T.L.C. Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), and G-6-P dehydrogenase (torula yeast) were obtained from the Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were standard laboratory reagents of analytical grade.

Animals. Male New Zealand white rabbits weighing 2–3 kg (local commercial source) and male Sprague–Dawley rats weighing 350–450 g (Charles River Breeding Laboratories, Wilmington, MA) were maintained in our Central Animal Facilities away from any known inducers, under a 12-hr photoperiod. The animals had access to unlimited water and standard laboratory chow (Ralston Purina Co., St. Louis, MO).

Perfusion procedure. The animals were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.) and heparinized (sodium heparin,

* Visiting Assistant Professor from the Akita University School of Medicine, Japan.
† Send all correspondence to: Dr. Harihara M. Mehendale, Department of Pharmacology and Toxicology, University of Mississippi Medical Center, 2500 North State St., Jackson, MS 39216, U.S.A.

2000 I.U./kg, i.v.) to prevent blood coagulation in the lung. The lungs were surgically removed and prepared for perfusion as described previously [3–5]. The composition of perfusion medium was (mM): NaCl, 118; KCl, 4.75; $CaCl_2$, 2.54; KH_2PO_4, 1.19; $MgSO_4$, 1.19; $NaHCO_3$, 25; glucose, 5; and bovine serum albumin 4.5% (w/v). The pH of the medium, which had been bubbled previously with CO_2/O_2 (5:95, v/v), was adjusted to 7.4 by the addition of 1 N NaOH initially, and during perfusion by controlling the CO_2/O_2 supply. Perfusion apparatus for the rat lungs was a scaled-down version of the apparatus for the rabbit lungs, and the lungs were perfused via the pulmonary artery with the recirculating perfusate and ventilated artificially in a similar manner [3–5]. The volume of the perfusate was 100 ml for the rabbit lung and 33 ml for the rat lung. The perfusate flow rate was controlled at approximately 18 ml per min per g lung. After [^{14}C]DMA (0.1 to 100 μmoles, 0.39 μCi for the rabbit; and 0.1 to 33 μmoles, 0.31 μCi for the rat lung) was added to the perfusate as a bolus at the start of the experiment, perfusate samples (1.5 ml for the rabbit and 0.7 ml for the rat lung) were withdrawn from the circulation at predetermined time points. At the end of the perfusion, the lungs were weighed, dissected free of extrapulmonary tissues, minced, and homogenized in 5 vol. of ice-cold saline. When the gain in lung weight was more than 10% of the lung weight as determined by weighing before and after perfusion, the experiment was discarded.

Tissue preparations. The lungs were homogenized in 5 vol. of ice-cold 1.15% KCl and centrifuged at 10,000 *g* for 30 min; the resulting supernatant fraction was recentrifuged at 105,000 *g* for 60 min to isolate microsomes. All tissue preparation procedures were carried out at 0–4°. Postmitochondrial (10,000 *g*) supernatant and microsomal fractions were resuspended in 1.15% KCl to correspond to a dilution of 0.166 g lung/ml. Protein content in each fraction was determined by the method of Lowry *et al.* [10].

In vitro *incubations.* The following mixture was used: the enzyme preparation corresponding to 0.166 g lung in 1 ml of 1.15% KCl; 1 μmole NADP, 10 μmoles G-6-P, 12.5 μmoles, $MgCl_2$ and 25 μmoles nicotinamide in 0.5 ml; [^{14}C]DMA (0.01 to 1 μmole, 0.02 μCi) in 0.1 ml; G-6-P dehydrogenase 1 unit in 0.1 ml; 0.2 M phosphate buffer, 1.3 ml; made up to a total volume of 3 ml with a final pH of 7.4. The reactions were started by addition of the enzyme preparation, incubated for 10 min at 37° under air in a metabolic shaker, and stopped by addition of 0.5 ml of 1 N NaOH.

Radioassay and analytical procedures. Radioactivity in the perfusate, lung homogenate and incubation mixture was determined by the addition of Universal liquid scintillation mixture (Baker Chemical Co., Phillipsburg, NJ) (10 ml) to samples (0.2 to 0.5 ml) in counting vials and counting in a liquid scintillation spectrometer (Mark II, Searle Analytical Inst., Des Plaines, IL). The radioactivity recoveries were calculated by the difference between known amounts added and the summation of radioactivities in lung plus perfusate after a 60-min perfusion. *N*-Oxidation of DMA was assayed according to the method of Ziegler and Pettit [11] with one modification. The radioactivity remaining in the aqueous phase after extraction with the organic solvent was counted. Our radioactive method and the colorimetric method of Ziegler and Pettit [11] yielded identical results for DMA-NO determination.

RESULTS

Recovery of radioactivity in the isolated perfused lung (IPL) preparations. The percentages of radioactivity recovered from the perfusion system after 60 min of perfusion are shown together with the lung weight and DMA dose in Table 1. The recoveries of radioactivity decreased in a dose-dependent manner except at the two low concentrations used in the IPL experiments of both species. Much better recoveries were consistently obtained in the rabbit IPL than in the rat IPL.

Time–course of total DMA equivalents and DMA-NO in the perfusate. Figures 1 and 2 show the time–course of total radiolabel (DMA equivalents) and DMA-NO in the perfusate of the rabbit and rat IPL respectively. In the rabbit IPL, total DMA equivalents in the perfusate decreased to approximately 85% of dose as early as 1 min, and then an apparent steady-state equilibrium was reached around 10 min at the concentrations of between 1 and 100 μM (Fig. 1, upper panel). At the highest concentration (1000 μM), total DMA equivalents

Table 1. Recovery of [^{14}C]DMA-derived radioactivity after 60 min of perfusion*

Species	Lung weight (g)	DMA dose		Recovery of radioactivity (%)
		μM	μmoles/g lung	
Rabbit	11.9 ± 0.6	1	0.0085 ± 0.0005	90.7 ± 0.6
	11.9 ± 1.0	10	0.086 ± 0.007	91.6 ± 1.2
	11.4 ± 1.2	100	0.92 ± 0.10	83.2 ± 0.9
	10.4 ± 0.7	1000	9.8 ± 0.6	54.0 ± 2.1
Rat	1.8 ± 0.1	3	0.056 ± 0.004	60.2 ± 1.4
	1.9 ± 0.1	30	0.55 ± 0.44	63.4 ± 2.9
	1.7 ± 0.2	300	6.1 ± 0.6	50.6 ± 1.7
	1.7 ± 0.2	1000	19.1 ± 1.0	39.7 ± 0.6

* Rabbit and rat lungs were perfused, respectively with 100 and 33 ml of the perfusate, containing various concentrations of [^{14}C]DMA for 60 min at 37°. Results are the means ± S.E. of four experiments.

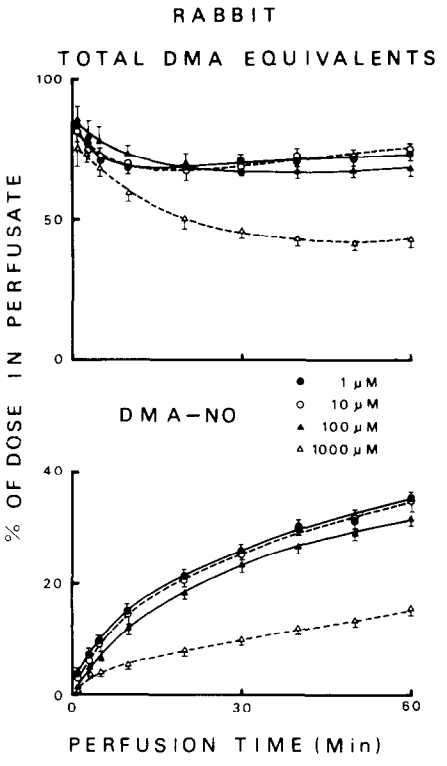

Fig. 1. Time-course of total DMA equivalents and DMA-NO in the perfusate of the rabbit IPL. Each point is the mean ± S.E. for four experiments, and the results are expressed as percentages of the dose in the entire perfusate as a function of time.

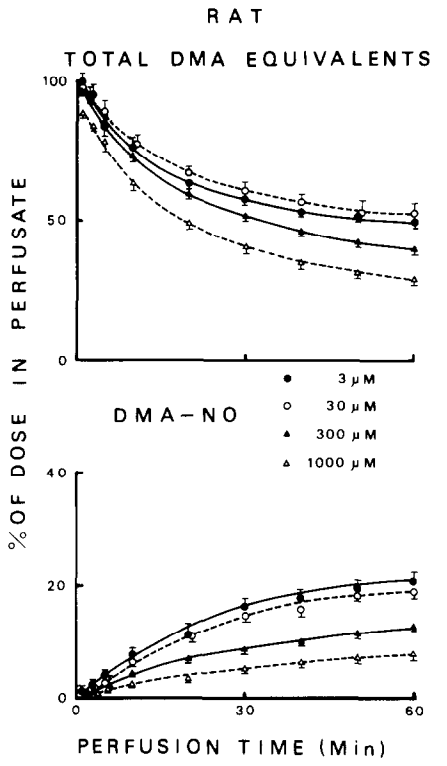

Fig. 2. Time-course of total DMA equivalents and DMA-NO in the perfusate of the rat IPL. Each point is the mean ± S.E. for four experiments, and the results are expressed as percentages of the dose in the entire perfusate as a function of time.

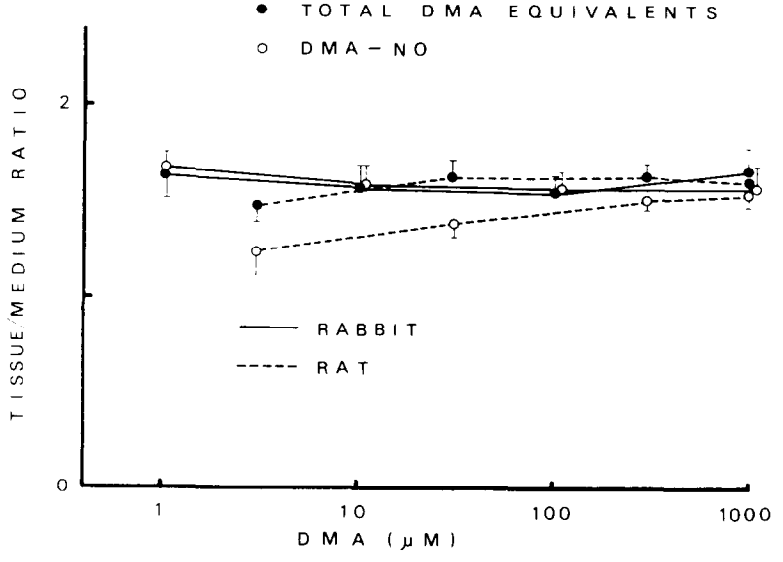

Fig. 3. Accumulation of total DMA equivalents and DMA-NO in the IPL. Each point is the mean ± S.E. for four experiments, and the results are expressed as lung tissue/perfusion medium concentration ratio at the end of 60 min as a function of DMA concentration used.

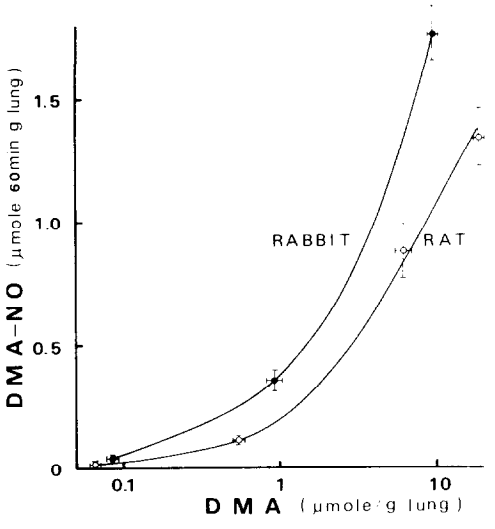

Fig. 4. *N*-Oxidation of DMA by the IPL. Each point is the mean ± S.E. for four experiments, and the results are expressed as amounts of DMA-NO produced during 60 min of perfusion as a function of DMA dose on the basis of per g lung.

decreased gradually up to 60 min. DMA-NO in the perfusate increased parabolically throughout the perfusion (Fig. 1, lower panel). A dose dependency in this increase was evident above 10 μM, and the rate of DMA *N*-oxidation was reduced significantly at 1000 μM. In contrast to the rabbit IPL, total DMA equivalents in the perfusate of the rat IPL continued to decrease steadily throughout the 60-min perfusion at any concentration used (Fig. 2, upper panel). The rate of DMA *N*-oxidation in the rat IPL was much slower than in the rabbit IPL (Fig. 2, lower panel). A dose dependency in the DMA *N*-oxidation was observed except at the lowest concentration used in analogy to the observation with the rabbit IPL.

Accumulation of total DMA equivalents and DMA-NO in the IPL. More than 95% of radioactive materials extracted from the perfusate or lung of both species with organic solvent were identified as unchanged DMA by T.L.C. This suggests small contribution to the metabolism via such pathways as *N*-demethylation, in IPL experiments. Pulmonary accumulations of total DMA equivalents and DMA-NO are expressed as lung tissue/perfusion medium concentration ratios at the end of a 60-min perfusion in Fig. 3. There was no significant difference in the ratios between the rabbit and rat over a wide range of concentrations except for DMA-NO at 3 μM in rat IPL, indicating absence of any saturable uptake phenomena. In general, these ratios were approximately 1.6, indicating relatively low affinity of both parent as well as the metabolite of DMA for the lung tissue.

N-Oxidation of DMA by the IPL. To compare DMA *N*-oxidation activities, total amounts (perfusate + lung) of DMA-NO produced during 60 min of perfusion were plotted against DMA on the basis of per g lung, as shown in Fig. 4. It was apparent that the rabbit IPL was more active in DMA *N*-oxidation than the rat IPL, and this parity widened at higher substrate concentrations.

N-Oxidation of DMA by in vitro *lung preparations.* The results of the *in vitro* metabolism experiments with whole homogenate, postmitochondrial (10,000 *g*) supernatant fraction and microsomal fraction are summarized as kinetic parameters of V_{max} in Table 2. Irrespective of which subcellular fraction was used, it was apparent that the rabbit lung was more active in DMA *N*-oxidation than the rat lung, as indicated by appreciably higher values for V_{max}. Since there was no significant difference in protein content in each fraction between both species, DMA *N*-oxidation activity per g lung was similarly higher in the rabbit than in the rat lung.

DISCUSSION

Our preliminary observations indicate that DMA is volatile at 37°, whereas DMA-NO is not volatile at all. This unavoidable physical loss of DMA has been reviewed by Ziegler [12]. Accordingly, it can be assumed that the larger recoveries of radioactivity in the IPL experiments reflect the larger DMA *N*-oxidation, suggesting a relatively higher activity of the rabbit lung for DMA *N*-oxidation than the rat lung (Table 1). In the case of the non-volatile pneumophilic drugs such as chlorphentermine [13], CPZ [4] and IMP [5], the time–course of their pulmonary uptake and accumulation can be estimated by the decline in radiolabel concentration in the perfusate with time. However, this is not the case with the volatile drug DMA. An apparent equilibrium which was reached in the rabbit IPL around 10 min except at the highest concentration of 1000 μM probably

Table 2. Kinetic parameters of DMA *N*-oxidase*

Subcellular fraction	Rabbit		Rat	
	K_m (10^{-5} M)	V_{max} (nmoles/min/mg protein)	K_m (10^{-5} M)	V_{max} (nmoles/min/mg protein)
Whole homogenate	71.28	3.67	13.84	1.25
Postmitochondrial supernatant	49.50	5.14	9.77	0.76
Microsomal	42.48	12.59	9.91	2.93

* Various concentrations of [^{14}C]DMA were incubated with the subcellular fractions corresponding to 0.166 g lung tissue for 10 min at 37° under air. *N*-Oxidation of DMA was assayed according to the method of Ziegler and Pettit [11]. The values for K_m and V_{max} were calculated from the regression lines of double-reciprocal plots obtained from the results of sixteen incubations. The correlation coefficient was 0.993 or better in each case.

resulted from the balance between DMA *N*-oxidation and volatilization (Fig. 1). On the contrary, the steady decline in radiolabel concentration in the perfusate of the rat IPL resulted from the predominance of volatilization over *N*-oxidation (Fig. 2). This difference in the recoveries of radioactivity provides additional credence to the conclusion of the present IPL experiments that the rabbit IPL is more active in DMA *N*-oxidation than the rat IPL (Fig. 4).

In vivo distribution studies have revealed that many pneumophilic drugs show high lung tissue/blood concentration ratios of over 100 and even 400 in some cases [2]. For instance, accumulation of CPZ by the IPL was against a concentration gradient, reaching a lung tissue/perfusion medium ratio of 180 in both rabbit [3] and rat [4]. Compared to these high ratios, the tissue/medium concentration ratios of total DMA equivalents and DMA-NO was only approximately 1.6, suggesting low affinity for the lung tissue (Fig. 3). Assuming that the difference between amount of total DMA equivalents and DMA-NO is amount of unchanged DMA, the ratio of DMA will be nearly the same, indicating that DMA is not concentrated in the lung tissue. Little or no dose dependency in the tissue/medium ratios suggests that pulmonary uptake of DMA is not mediated by any saturable phenomena, and the uptake appears to be by simple diffusion.

In vitro metabolism experiments with various centrifugation fractions confirmed that the rabbit lung is more active in DMA *N*-oxidation than the rat lung (Table 2). Irrespective of which subcellular fraction was used, or how the activities were expressed (per mg protein or per g lung), the qualitative inferences were unaffected. Although the microsomes have been established as a cleaner drug-metabolizing enzyme preparation with high specific activity, the possibility still remains that species or sex differences observed in studies with microsomal incubation might arise as a result of differences in cell fragmentation or sedimentation. Hence, the microsomal fraction may not be an accurate indicator of mouse hepatic DMA *N*-oxidation activity, and conclusions based only on activity measurements with isolated microsomes can be misleading as reported by Duffel *et al.* [14]. Therefore, we compared DMA *N*-oxidase activity of whole homogenate, postmitochondrial (10,000 *g*) supernatant fraction and microsomal fraction of rabbit and rat lung. A relatively higher DMA *N*-oxidase activity in the rabbit than in the rat lung has been puzzling in view of our previous findings that CPZ and IMP are *N*-oxidized by the rat lung, but not by the rabbit lung [3–6]. Indeed, the identical subcellular fractions of rabbit lung exhibited no CPZ and IMP *N*-oxidase activity in spite of an appreciable DMA *N*-oxidase activity under the same incubation conditions [15]. This apparent discrepancy can be explained on the assumption that rabbit lung DMA *N*-oxidase and rat lung CPZ or IMP *N*-oxidase are separate enzymes.

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