Biochemical Pharmacology, Vol. 37, No. 13, pp. 2697–2702, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

TISSUE SPECIFIC REGULATION OF RENAL *N*-NITROSODIMETHYLAMINE-DEMETHYLASE ACTIVITY BY TESTOSTERONE IN BALB/c MICE

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(Received 8 *December* 1986; *accepted* 21 *December* 1987)

Abstract—Nitrosodimethylamine (NDMA), like several other nitrosamines, is activated by the enzymes—mixed-function oxidases—present in the tissue microsomal fractions, producing mutagenic and carcinogenic effects. Previous studies in BALB/c mice have shown an age, sex and androgenic regulation of NDMA-induced mutagenicity. The present study was designed to test the correlation between renal NDMA-demethylase activity and previously published reports on NDMA-induced mutagenicity. Renal and hepatic NDMA-demethylases were determined from the microsomal fractions by quantitating formaldehyde. Renal NDMA-demethylase showed the presence of two isozymes, I and II, with K_m values of 0.6 ± 0.2 and 20.2 ± 6.8 mM respectively. Isozyme I was detected in adult males and first appeared at the onset of puberty; it was absent in adult females and in immature mice. Renal isozyme II was detected in both males and females and was independent of age. Testosterone treatment of adult females resulted in the appearance of renal isozyme I. Castration of adult males caused a dramatic decrease in activity, whereas testesterone administration to such castrates increased activity, of renal isozyme I. Hepatic NDMA-demethylase activities were independent of age, sex or testosterone treatment. In conclusion, these results show an age, sex and tissue specific regulation of renal NDMA activity. Renal and hepatic NDMA-demethylase activities correlated positively with earlier studies on NDMA-induced mutagenesis and carcinogenesis.

N-Nitrosodimethylamine (NDMA‖), a potent carcinogen and mutagen, has been shown to induce tumors in many organs, such as liver, lungs, esophagus and kidney, in several rodent species [1, 2]. Considerable evidence exists which indicates that the tumorigenic [3, 4] and mutagenic [5–9] properties of NDMA and other aliphatic nitrosamines require metabolic activation to proximal carcinogens by the mixed-function oxidase enzyme system present in endoplasmic reticulum of the liver and other organs. These findings demonstrate an association between induction of neoplastic and mutagenic events and enhance the concept that these phenomena are related [5, 6].

Several studies have indicated that species, strain and sex related differences play an important role in the response of individual tissue to the effects of NDMA [6, 9–11]. Further, a correlation exists between the degree of susceptibility to NDMA-induced tumors of certain organs (liver, lung, kidney) in several strains of mice and the differential ability of these organs to activate NDMA to its mutagenic metabolites *in vitro* [6, 7]. Using *in vivo* studies, Noronha [12] has shown that a single dose (10 mg/kg) of NDMA, given intraperitoneally, can induce renal tumors in BALB/c mice. These tumors are morphologically and histologically similar to renal cancer in humans. Further, these NDMA-induced renal tumors were observed in adult male mice only. Even when the dose of NDMA was doubled, females were still unresponsive, and orchiectomy of adult males before NDMA administration totally inhibited renal tumorigenesis in males [12].

These sex differences strongly suggest the role of androgenic hormones in metabolism of NDMA in the renal tissue and help to explain the increased susceptibility of male rodents to renal carcinogenesis. Earlier work [6–8, 10, 11] has indicated that androgens regulate the biotransformation of NDMA to its active mutagenic metabolites in the kidney of BALB/c mice. Renal microsomal fractions from adult males had a higher potential to biotransform NDMA than renal microsomal fractions from adult females, castrated males or immature males and females. Androgen administration can specifically increase the activation potential of renal tissues. Further, even though the liver is more active than the kidney in the activation of NDMA, this androgen effect is observed in the kidney and not in the liver. This androgen regulation in renal tissue is an androgen receptor mediated phenomenon [9]; recent observations also indicate that this effect is observed under conditions when androgen receptors are translocated into the nucleus [13, 14].

The present experiments were designed to determine whether age, sex and tissue specific differences in NDMA activation in the kidney (as evidenced

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‖ Abbreviations: NDMA, *N*-nitrosodimethylamine; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; NADP, nicotinamide adenine dinucleotide phosphate; and testosterone, 1-hydroxy-3-oxo-4-androstene.

by mutagenicity experiments) correlated with the activity of NDMA-demethylase, an enzyme implicated in NDMA metabolism [15–17].

MATERIALS AND METHODS

Animals. BALB/c mice were purchased from the Charles River Laboratory. They were maintained at 25 ± 2° under a 12-hr dark and 12-hr light cycle. Food (Purina Laboratory Chow) and water were provided *ad lib.*

Hormones were administered subcutaneously in 0.1 ml of peanut oil. Animals were bilaterally orchiectomized or sham-operated using light ether anesthesia. Animals were killed by decapitation. Tissues (liver, kidney) were collected and immediately frozen in liquid N_2 and stored at −80° until used (within 4 weeks).

Chemicals. Chemicals and their sources were: NDMA from the Aldrich Chemical Co. (Milwaukee, WI); formaldehyde from Fisher Scientific (Fair Lawn, NJ); and glucose-6-phosphate, glucose-6-phosphate dehydrogenase (Type XXIV), NADP, $MgCl_2$, ammonium acetate, zinc sulfate, barium hydroxide, and testosterone propionate from the Sigma Chemical Co. (St. Louis, MO).

Tissue homogenization and preparation of microsomes. All procedures unless otherwise mentioned were carried out in an ice bath. The frozen tissues were pulverized under liquid N_2 using a Thermovac tissue autopulverizer (Copiague, NY). The pulverized tissues were homogenized, and the microsomal enzyme fractions were obtained as described by Mazel [18], with few modifications. The Brinkmann polytron homogenizer (PT 10) was used to homogenize tissues instead of the Elvehjem–Potter homogenizer. Briefly, the samples were homogenized at 4° in 7 vol. of 10 mM phosphate buffer, pH 7.4, containing 1.15% KCl, using a polytron PT-10 at 30 V for three 30-sec bursts. Each 30-sec homogenization step was followed by a 60-sec cooling period. The homogenate was centrifuged at 9000 *g* for 10 min, and the post-mitochondrial supernatant fraction (S-9) was centrifuged at 105,000 *g* for 60 min to yield the microsomal pellet. The pellet was suspended in 50 mM phosphate buffer, pH 7.4, to restore the original volume using the polytron at 30 V for 10 sec and was used as a source of microsomal enzyme fraction. Preliminary experiments indicated no significant differences in the specific activities of NDMA-demethylases in the renal or hepatic microsomal fractions prepared by the Brinkmann polytron or the Elvehjem–Potter homogenizer. Also, semicarbazide and nicotinamide were omitted from the incubation mixture, since preliminary data showed no advantage at low concentrations (20 mM or below) but dose-dependent inhibitions of NDMA-demethylase activity at higher concentrations were observed.

Measurement of NDMA metabolism by formaldehyde formation. The enzymatic demethylation of NDMA leads to the formation of formaldehyde. Formaldehyde was quantitated using Nash's reagent [19]. Kidneys from eight to ten mice were pooled for each assay. Each experiment was repeated at least three times.

Incubation procedures. Each assay was done in triplicate. The following were added to the incubation tubes at 4°: 0.5 ml of NDMA to the experimental tubes or 0.5 ml of $MgCl_2$ (25 mM) to the control tubes (tissue blanks), 1 ml of cofactors containing 10 mM G6P, 1 mM NADP and 0.5 unit of G6PD in phosphate buffer and a stirring bar in each incubation tube [18]. The tubes were preincubated for 5 min in a 37° water bath with constant stirring. The reaction was initiated by addition of 1.0 ml of microsomal suspension. All tubes were incubated at 37° with constant stirring for 15 min (linear phase of time curve). The reaction was terminated by the addition of 1.0 ml of 15% $ZnSO_4$ followed by 1.0 ml of saturated $Ba(OH)_2$. Ten minutes after the addition of $Ba(OH)_2$, all tubes were centrifuged at 5000 *g* for 10 min at 4° in a Beckman J2-21 centrifuge using Beckman's Series No. 382 rotor. A 2.5-ml sample of the supernatant fraction was assayed for formaldehyde using Nash's reagent [19]. Results were expressed as nanomoles of formaldehyde per milligram of microsomal protein. Protein content of the microsomal fractions was determined according to the method of Lowry *et al.* [20].

Statistical analysis of the data. One-way analysis of variance was performed on the data containing three or more groups. Whenever the analysis of variance showed a significant difference between the means, the Duncan Multiple Range test was performed to determine which group means differed at the 0.01 probability level of significance. Student's *t*-test (two-tail) was used to demonstrate the difference between the means of two groups (i.e. control vs testosterone-treated or males vs females).

The K_m and V_{max} values were calculated from direct linear plots according to Cornish-Bowden [21].

RESULTS

NDMA-demethylase modulation by age and sex. The metabolism of NDMA by microsomal fractions of renal tissue was investigated in 22-day-old (immature), 35-day-old (undergoing puberty), and 60-day-old (sexually mature adult) mice of both sexes. The results indicate that the enzyme activity was not detected in the renal microsomal preparations from immature male or female mice at NDMA concentrations of 1 and 10 mM but only at 100 mM (Table 1). In contrast, the renal microsomal fractions from 60-day-old male mice had significantly higher ($P < 0.01$) enzyme activity than females at 10 and 100 mM NDMA; enzyme activity was undetected at 1.0 mM NDMA in adult females. NDMA-demethylase activity was also undetected at NDMA concentrations of 1 and 10 mM in 35-day-old females (Table 1).

The microsomal enzyme fractions prepared from hepatic tissue showed 3- to 5-fold higher NDMA-demethylase activity compared to renal microsomal enzyme activity (data not shown). However, no difference in enzyme activity was observed between hepatic tissues of males and females or between mature and immature animals. For example, hepatic NDMA demethylase (at 10 mM NDMA) values in immature males and females and mature males and females were 57.5 ± 10.2, 59.2 ± 11.3, 59.3 ± 8.5

Table 1. Effects of age, sex and NDMA concentration on renal NDMA-demethylase activity

Mice (age)	NDMA (mM)	Renal NDMA-demethylase activity (nmol HCHO/mg protein/15 min) Males	Females
22 days	1	0	0
	10	0	0
	100	8.2 ± 1.0	5.2 ± 0.7*
35 days	1	4.1 ± 1.0	0
	10	7.3 ± 1.2	0
	100	17.0 ± 0.6	8.5 ± 0.8*
60 days	1	4.7 ± 0.6	0
	10	8.7 ± 0.4	2.6 ± 1.0*
	100	16.3 ± 0.8	10.9 ± 1.0*

Renal microsomal fractions from 22-, 35- and 60-day-old male and female mice were used. NDMA-demethylase activity was determined at optimal concentrations of cofactors and various concentrations of NDMA. The reaction was terminated after 15 min (linear phase of the time curve), and the amount of formaldehyde was quantitated. Values are means ± SE from nine determinations.

* $P < 0.01$, compared to males of the same age.

and 57.1 ± 12.0 nmol/mg protein/15 min respectively.

Effects of testosterone on NDMA-demethylase activity in the kidney. The metabolism of NDMA by renal microsomal fractions from mice treated with testosterone was investigated in 35- and 60-day-old mice of both sexes. The results indicate that testosterone treatment (1.5 mg/day for 10 days) produced a 2-fold ($P < 0.01$) stimulation in renal NDMA-demethylase activity in both 35- and 60-day-old male mice compared to the oil-treated controls (Table 2). In females, testosterone treatment also induced a 3- to 8-fold increase ($P < 0.01$) in the NDMA-demethylase activity (Table 2). Further, renal NDMA-demethylase activities in testosterone-treated females were quantitatively similar to those of testosterone-treated males.

The data on the enzyme kinetics of renal NDMA-demethylase showed two isozymes (referred to as I and II) in adult males. The K_m and V_{max} values of these isozymes are shown in Table 3. The testosterone-induced increase in the renal NDMA-demethylase activity in the males was due to an increase in the V_{max} of isozyme I with no apparent change in the K_m of isozyme I (Table 3). In females, testosterone treatment resulted in the appearance of isozyme I in the kidney; both the K_m and the V_{max} values of isozyme I were similar to those observed in testosterone-treated males. The K_m and V_{max} values were calculated from direct linear plots according to Cornish–Bowden [21].

Testosterone treatment caused no significant change in the enzyme activity from liver microsomal fractions in either 35- or 60-day-old males and females (data not shown).

Effects of bilateral orchiectomy on renal NDMA-

Table 2. Effect of testosterone treatment on renal NDMA-demethylase activity

Mice age	NDMA (mM)	Renal NDMA-demethylase activity (nmol HCHO/mg protein/15 min) Males Control	Males Testosterone	Females Control	Females Testosterone
35 days	1	4.1 ± 1.0	8.5 ± 0.8*	0	11.7 ± 0.5
	10	7.3 ± 1.2	17.8 ± 1.8*	0	18.1 ± 1.4
	100	17.0 ± 0.6	36.0 ± 1.2*	8.5 ± 0.8†	29.8 ± 0.9*
60 days	1	4.7 ± 0.6	9.2 ± 0.9*	0	10.9 ± 0.5
	10	8.7 ± 0.4	17.4 ± 0.6*	2.6 ± 1.0†	16.7 ± 0.8*
	100	16.3 ± 0.8	30.0 ± 1.7*	10.9 ± 1.0†	31.6 ± 2.0*

Testosterone was administered subcutaneously (1.5 mg/day for 10 days) in 0.1 ml of peanut oil; control animals received 0.1 ml of peanut oil. Renal NDMA-demethylase activity was determined at optimal concentrations of cofactors and various concentrations of NDMA. The reaction was terminated after 15 min (linear phase of the time curve), and the amount of formaldehyde was quantitated. Values are means ± SE from nine determinations.

* $P < 0.01$, compared to controls of the same sex.

† $P < 0.01$, compared to male controls.

Table 3. Renal NDMA-demethylase: kinetic constants

Mice	Kinetic constants*			
	Control		Testosterone-treated	
	K_m (mM)	V_{max} (nmol HCHO/mg protein/15 min)	K_m (mM)	V_{max} (nmol HCHO/mg protein/15 min)
Males				
Isozyme I	0.6 ± 0.2	8.5 ± 0.4	0.4 ± 0.1	14.3 ± 0.8
Isozyme II	20.2 ± 6.8	48.1 ± 7.3	6.6 ± 1.8	34.3 ± 7.1
Females				
Isozyme I	ND†		0.3 ± 0.2	18.8 ± 3.1
Isozyme II	27.8 ± 6.9	32.2 ± 2.8	3.6 ± 1.6	27.9 ± 5.1

* K_m and V_{max} values were calculated from direct linear plots according to Cornish-Bowden [21]. Values are geometric means ± SE from ten determinations.
† Not detectable.

demethylase activity. Sexually mature adult males were bilaterally orchiectomized to determine the effect of testosterone withdrawal on renal and hepatic NDMA-demethylases. Control animals were sham-operated. Animals were killed 3 and 10 days after orchiectomy. The results indicated a 55, 60 and 45% decline within 3 days in renal NDMA-demethylase activity at NDMA concentrations of 1, 10 and 100 mM, respectively, as compared to the sham-operated controls (Table 4). By day 10 after orchiectomy, inhibition of 93 and 78% in renal NDMA-demethylase activities was observed at NDMA concentrations of 1 and 10 mM respectively.

Testosterone treatment was initiated 10 days after castration, and the animals were killed 1, 3 and 7 days after the treatment. The results show a gradual increase in the renal NDMA-demethylase activity from day 1 (3-fold) to day 7 (7-fold increase) as compared to oil-injected castrated controls (Table 5). NDMA-demethylase activity of testosterone-treated castrated mice was not significantly different from testosterone-treated intact mice by day 7. The hepatic enzyme activity was not affected by either castration or testosterone treatment of such castrated mice (data not shown).

DISCUSSION

The present study provides strong evidence of tissue specific modulation of renal NDMA-demethylase activity by testosterone. Two K_m values for renal NDMA-demethylase were observed, indicating the presence of two isozymes. A marked sexual dimorphism in the ability of renal microsomal fractions to metabolize NDMA was observed. The renal microsomal fractions of adult males showed the presence of the low K_m isozyme I which was first evident in the 35-day-old males, when the testicular biosynthesis of testosterone starts to increase [6, 9, 10]. Isozyme I was absent in immature animals and in

Table 4. Effect of bilateral orchiectomy on renal NDMA-demethylase activity

Group	Duration of treatment	Renal NDMA-demethylase activity (nmol HCHO/mg protein/15 min)		
		NDMA concentration 1 mM	10 mM	100 mM
Sham-operated				
	3 days	5.1 ± 0.8	10.1 ± 1.2	18.2 ± 1.7
	10 days	6.8 ± 1.0	9.2 ± 2.0	16.5 ± 0.9
Bilateral orchiectomy				
	3 days	2.4 ± 0.6*	4.2 ± 0.5*	10.4 ± 0.5*
	10 days	0.5 ± 0.2†	2.1 ± 0.4†	7.0 ± 0.5†

Bilateral orchiectomy was performed on 60-day-old male mice; control mice were sham-operated. Animals were killed 3 and 10 days after the surgery. Renal NDMA-demethylase was determined (by quantitating formaldehyde) at optimal concentrations of cofactors and at NDMA concentrations of 1, 10 and 100 mM. All incubations were terminated after 15 min (linear phase of the reaction). Values are means ± SE from nine determinations.
* $P < 0.01$, compared to day 3 sham-operated control.
† $P < 0.01$, compared to day 10 sham-operated control.

Table 5. Effect of bilateral orchiectomy and testosterone treatment on renal NDMA-demethylase activity

Treatment	Renal NDMA-demethylase activity (nmol HCHO/mg protein/15 min)		
	Duration of treatment (days)		
	1	3	7
Castrate (oil-injected)	2.5 ± 0.2	3.0 ± 1.0	2.6 ± 0.2
Castrate (testosterone)	7.3 ± 0.8*	17.7 ± 1.3*	18.2 ± 0.1*
Sham-operated (oil-injected)	10.2 ± 0.3	ND†	9.9 ± 1.3
Sham-operated (testosterone)	14.5 ± 0.9‡	ND	18.4 ± 0.3‡

Bilateral orchiectomy was performed on 60-day-old male mice; control mice were sham-operated. Testosterone (1.5 mg/day for 10 days) was administered 10 days after surgery, and the animals were killed 1, 3 and 7 days after the initiation of testosterone treatment. Renal NDMA-demethylase activity was determined at optimal concentrations of cofactors and 10 mM NDMA for 15 min.

Values are means ± SE from nine determinations.

* $P < 0.01$, compared to oil-injected castrates.

† Not done.

‡ $P < 0.01$, compared to oil-injected sham-operated controls.

adult females but was induced by the administration of testosterone. The high K_m isozyme II was observed in renal microsomal preparations of all age groups in both males and females; however, adult males showed higher values than adult females or immature animals. Testosterone treatment decreased the K_m of renal isozyme II in both males and females. In contrast, NDMA-demethylase activities in the livers of these animals showed no age or sexual dimorphism. Even though hepatic NDMA-demethylase showed a 3- to 5-fold higher value as compared to the renal enzymes, they were independent of testosterone modulation.

Androgenic dependence of renal NDMA-demethylase was further supported by the castration studies. The activity of renal isozyme I declined rapidly after castration and reappeared upon stimulation of such castrates by testosterone. The levels of renal isozyme I reached the same levels as seen in immature animals. Several other renal cytoplasmic enzymes such as β-glucoronidase [22] and ornithine decarboxylase [23] have been shown to be androgen responsive; their activities are stimulated by testosterone and inhibited by orchiectomy. However, the present study demonstrates induction of a specific microsomal mixed-function oxidase.

Epidemiological and clinical observations have led to the hypothesis that human renal cancer may be a hormone-dependent cancer. This hypothesis is based on the following observations: (a) renal carcinoma is twice as common in men as in women: (b) administration of progestins causes a greater regression of metastatic renal cancer in men than it does in women; and (c) androgens promote the growth of renal carcinoma [24–26].

The results presented in this paper provide unequivocal evidence of a correlation between androgenic regulation of renal NDMA-demethylase activity and earlier published results on NDMA-induced mutagenicity and a possible explanation of increased susceptibility of renal carcinogenesis in males.

This androgen regulation of NDMA-induced mutagenicity or stimulation of NDMA-demethylases in the kidney appears to be an androgen receptor mediated phenomenon. For example, earlier studies [6, 7, 9] have shown that testosterone regulation of NDMA-induced mutagenicity in the kidney was not observed in Tfm/y mice, a mutant strain with defective androgen receptors [27]. Earlier studies from this laboratory have also shown that, although cytosolic androgen receptors in the kidney are quantitatively similar in males and females and are independent of age, nuclear androgen receptors were absent in immature mice and were several fold higher in adult males than in adult females [13]. These data on androgen receptors have also been confirmed [14].

NDMA-demethylases are pharmacologically relevant; isozyme I has been implicated in the metabolism of NDMA *in vivo* [17]. Purified preparations of isozyme I have also been shown to cause NDMA-induced mutagenicity in hamster lung cells [28]. Isozyme II is unlikely to play any role *in vivo* because its K_m (25–120 mM) is so much greater than the plasma concentrations (0.6 mM) produced by the median lethal dose [17].

In conclusion, the results clearly indicate a tissue specific stimulation of renal (but not hepatic) NDMA-demethylase in BALB/c mice. The appearance of the low K_m isozyme I in the kidney of males at the onset of puberty, its rapid decline after castration, and its restimulation after testosterone also provide strong support for its functional relevance. Our results have also clearly demonstrated a correlation between NDMA metabolism in the kidney (NDMA-demethylases) and earlier published studies on NDMA-induced mutagenic

responses in the kidney. This correlation may also explain the increased susceptibility of renal tumorigenesis in the males to nitrosamines. Experiments are also underway to determine the optimal time and dose of this testosterone regulation.

Acknowledgements—Supported by NIH RR08016 (F.R.A., S.M. and S.A.), US EPA R810451 (S.M.) and NCI 5P30 CA 14718 (S.M.). The secretarial assistance of Ms. D. Oliver is gratefully acknowledged. This manuscript is taken in part from the dissertation of S. Ahir, submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy from the Department of Pharmacology, Howard University College of Medicine, Washington, DC 20060, U.S.A.

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