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Effect of Starvation and Chlormethiazole on Cytochrome P450s of Rat Nasal Mucosa

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ABSTRACT. Cytochrome P450 (CYP) enzymes of nasal tissue are relatively resistant to induction by classical inducers. In the present study, the effects of starvation on the expression of CYP1A, 2A, 2B, 2C, 2E, 2G, and 3A subfamilies in the nasal mucosa of rat were studied. Fasting for 72 hr caused an increase in 2E1-dependent *p*-nitrophenol hydroxylase and 1A-dependent ethoxy- (or methoxy) resorufin dealkylase activities, but did not affect either 2A-linked coumarin hydroxylase or the testosterone hydroxylase activity, the latter reaction being a marker of several CYPs including 2G1. Whereas increases in 2E1- and 1A- associated catalytic activities were accompanied by a concomitant increase in the corresponding apoproteins as determined by immunoblotting, immunoactive protein bands reactive with antibodies raised against rat 1A1, 2B1, 2C11, 3A1 or rabbit nasal 2A10/11 and 2G1 were not altered. Fasting also increased CYP2E1 and CYP1A2 on the mRNA level, but did not alter CYP1A1 mRNA as determined by hybridization with cDNA probes selective for these cytochromes. A reiterative administration of chlormethiazole, a specific inhibitor of 2E1 in liver, strongly inhibited many CYPs, including 2E1, 1A2, 2G1, and 2A in the nasal mucosa, but did not influence expression of 2B or 3A as determined by immunoblotting or catalytic activities. The chlormethiazole-mediated inhibition of 1A1 and 2E1 was demonstrated to be at the mRNA level. These results suggest that fasting induces the gene expression of 2E1 and 1A2 and that the mechanisms involved in the regulation of CYPs in the nasal mucosa are tissue-specific. The inducibility of the above-mentioned isoforms may have a significant role in the clearance of drugs and bioactivation of inhaled compounds. BIOCHEM PHARMACOL **59**;11:1425–1432, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. nasal mucosa P450s; fasting; chlormethiazole inhibition

The nasal mucosa of many mammalian species exhibits high levels of CYP§ and high activities in the biotransformation of many xenobiotic compounds. A number of CYP isoenzymes have been identified to be constitutively expressed in the nasal epithelia, including members of the CYP1A, 2A, 2B, 2C, 2E, 3A and 4B subfamilies as well as CYP2G1, the latter expressed specifically in the olfactory tissue [1]. Although the primary function of these enzymes in the odorant, pheromone, and toxicant clearance mechanisms has attracted increasing attention, few studies have been carried out regarding their regulation by xenobiotics and other factors in the nasal mucosa. Studies published to date indicate that the nasal mucosa CYP forms are not inducible to the same extent as the hepatic forms, as shown by a resistance to induction by classical CYP inducers, including phenobarbital, 3-methylcholanthrene, benzo(*a*)pyrene, and Aroclor [1,2]. Only CYP2E1 has been demonstrated to be clearly inducible in the nasal tissues by various compounds including ethanol, acetone, and pyrazole [2–4], whereas opposite results have been reported for CYP2A3 [5,6] and CYP1A1/2 [7,8].

It is known that fasting modulates hepatic CYPs in rodents [9–11]. For example, rat CYP2E1, 3A2, 2B1, and 4A are induced by fasting periods of 48 or 72 hr, whereas the expression of CYP2C11 is decreased and CYP1A1, 1A2, and 2A2 do not change [10]. In rat kidney microsomes, fasting induces CYP 2E1, 2B1, and 3A [12]. Recently, it has also been reported that fasting causes the induction of CYP2E1 in fresh lymphocytes of rat [13], but no data are available on the effect of pathophysiological conditions such as diabetes, obesity, or fasting on the CYPs of nasal mucosa of any mammals. Starvation potentiates the hepatotoxicity of many chemicals such as chlorinated hydrocarbons and nitrosamines generally by induction of CYP2E1 [14]. Thus, the modulation in the nasal tissue of CYP2E1 along with other CYPs by starvation may be of considerable importance for the bioactivation *in situ* of inhaled toxic or procar-

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§ *Abbreviations:* CYP, cytochrome P450; CYP isoforms, forms of P450 not intended to catalyze the same reaction but belonging to the P450 superfamily; CMZ, chlormethiazole; CoH, coumarin 7-hydroxylase, pNPH, *p*-nitrophenol hydroxylase; EROD, ethoxyresorufin O-deethylase; MEROD, methoxyresorufin O-demethylase; β-NF, β-naphthoflavone; and Ah, aromatic hydrocarbon.

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cinogenic compounds. Furthermore, the study of CYP expression under starvation conditions may contribute to an understanding of the regulatory mechanism of the constitutive CYPs in different tissues.

In this study, we examined the effects of fasting on a number of CYPs in male rat nasal mucosa. In addition, we investigated the effect of CMZ, an effective and specific inhibitor of hepatic CYP2E1 [15], on the expression in the nasal tissue of this isoform along with the other ones.

MATERIALS AND METHODS

Materials

Ethoxyresorufin and methoxyresorufin were synthesized from resorufin by ethylation and methylation with ethyl and methyl iodide, respectively [16]. Testosterone and its metabolites were obtained as previously reported [17]. Enzymes and coenzymes were obtained from Boehringer Mannheim. CMZ was obtained from Astra Arcus, AB. Rabbit polyclonal antibodies against rat CYP2B1, 1A1, 2E1, and purified rat CYP2E1 were obtained in our laboratory as previously described [18]. Purified rat CYP1A2 was purchased from Gentest. Sheep polyclonal antibodies against rabbit CYP2G1 and rabbit polyclonal antibodies against rabbit CYP2A10/11 were donated by Dr. X. Ding (Albany, NY, U.S.A.). Goat polyclonal antibodies against rat CYP2C11 were supplied by Dr. J.B. Schenkman (Farmington, U.S.A.). Rabbit polyclonal antibodies against rat CYP3A1 and the enhanced chemiluminescence kit were purchased from Amersham. Reagents for development by the alkaline phosphatase method were obtained from ICN. Nylon membranes were purchased from Sartorius. All other chemicals and solvents were of the highest grade available and obtained from common commercial sources.

Animal Treatment and Preparation of Microsomes

The study was performed with male Sprague–Dawley rats (200–250 g, Charles River). In total, three different groups of animals were used. One group of rats received food and water (control); a second group was starved for 72 hr (fasting), but received water *ad lib*; and a third group was treated five times with CMZ every 12 hr at 75 mg/kg i.p. The animals were killed 12 hr after the last CMZ injection. This group received food and water *ad lib*. Microsomes were prepared from rat nasal mucosa and liver as described previously [17].

Immunoblot Analysis

Microsomes were subjected to SDS–PAGE using the method of Laemmli [19] in a Bio-Rad Mini Protean II apparatus. Immunoblot analysis was performed according to Towbin *et al.* [20] using 5% (w/v) milk as a blocking agent. The staining of antigen–antibody complex was carried out either with the enhanced chemiluminescence reagents from Amersham according to the manufacturer's instructions or by the alkaline phosphatase method. The bands on the nitrocellulose membranes were quantified by a laser densitometer (Ultrascan 2201, LKB). Some dilutions (about 3–4) of the microsomal samples were performed to determine the intensity of the protein bands within the linear range of the densitometry response.

Northern Blot Analysis

Total RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction [21]. The RNA was size-fractionated by electrophoresis in 1.25% agarose–formaldehyde gels and then transferred to nylon membrane as described by Sambrook *et al.* [22]. The nylon membranes were hybridized with ^{32}P-labeled CYP2E1 rat cDNA obtained from Dr. F. Gonzalez (NIH, Bethesda, MD, U.S.A.) and with ^{32}P-labeled CYP1A1 and 1A2 rat cDNA fragments. These fragments correspond to nucleotides 1062 to 1362 in the CYP1A1 and to nucleotides 970 to 1252 in the CYP1A2 cDNA. ^{32}P-Labeled β-actin cDNA from mouse was used as a standard during Northern blot analysis. The bands on the autoradiographs were quantified by densitometry.

Histopathological Studies

Fragments of nasal mucosa of control and CMZ-treated rats were fixed in formalin and embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin.

Enzyme Assays

CoH activity was assayed by the fluorimetric determination of 7-hydroxycoumarin [23], pNPH was determined according to Reinke and Moyer [24], and EROD and MEROD were measured by the formation of the resorufin in a Perkin Elmer spectrofluorimeter [25]. Testosterone hydroxylase was determined as reported previously [17].

RESULTS

Effects of Fasting and CMZ on the Nasal Mucosa Content of Different CYP Isoforms

To assess changes in individual nasal mucosa CYPs, we first performed an immunoblot analysis by using polyclonal antibodies prepared against rat liver 1A1, 2B1, 2E1, 2C11, and 3A1 and rabbit nasal 2G1, 2A10/11. Figure 1 shows the results of immunoblots of CYP2B, 3A, 2C, 2A, and 2G1 isoforms in microsomal samples from control rats, rats starved for 72 hr, and rats treated with CMZ. Figure 2 shows the level of CYP apoproteins as determined by densitometry analysis of immunoblots. In regard to the 2G1, 2A10/11, and 2C isoforms, it was observed that starvation did not cause any alterations, whereas CMZ reduced the amount of immunodetectable 2G1, 2A10/11, and 2C to 15, 70, and 5% of their control values, respectively. Neither fasting nor CMZ treatment affected nasal CYP2B and CYP3A. By

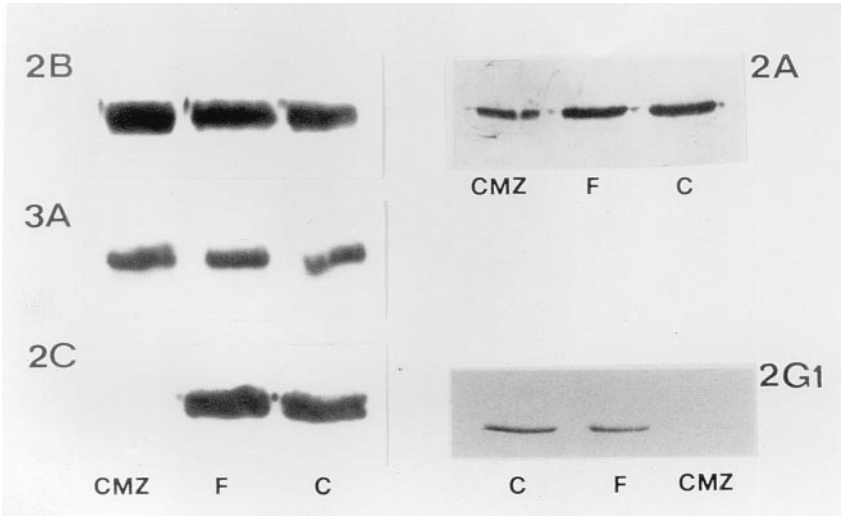

FIG. 1. Immunoblot analysis of CYP2B, 3A, 2C, 2A, and 2G1 in nasal mucosa microsomes from control (C), fasted (F), and CMZ-treated (CMZ) rats. Microsomes, loaded at 30 μg protein/well, were subjected to SDS–PAGE on 7.5% acrylamide gels, transferred to nitrocellulose, and probed with anti-rat liver 2B1, 3A1, and 2C11 and anti-rabbit nasal 2G1 and 2A10/11 immunoglobulin G. The staining of antigen–antibody complex was carried out by enhanced chemiluminescent detection for the 2B, 3A, and 2C and by the alkaline phosphatase method for the 2A and 2G1.

contrast, we did not find any effect of fasting and CMZ treatment on the liver CYP isoforms reactive with anti-rabbit olfactory CYP2A10/11 and anti-rat CYP2C11 (data not shown). Figure 3 illustrates the immunoblots of CYP 2E1 and 1A in nasal microsomal samples from control rats, rat starved for 24, 48, and 72 hr, and rats treated with CMZ. Both anti-CYP2E1 and anti-CYP1A1 recognized a single band which migrated to the same position as purified rat liver 2E1 and 1A2, respectively. That the protein band recognized by anti-rat 1A1 could be CYP1A2 was also supported by the fact that it co-migrated with the lower band ($M_r \cong 52$) in liver microsomes from β-NF-treated rats (blotting not shown). Compared to control microsomes, both the 2E1 and 1A apoprotein contents, as determined by densitometry (Fig. 4), increased to about 260% and 200% after the 48-hr fasting period, respectively. A 72-hr fasting period did not further enhance either the 2E1 or 1A content, whereas the treatment with CMZ significantly decreased both these isozymes below their control levels (Figs. 3 and 4).

Histological Effects of CMZ Administration

To verify whether the CYP alterations following the CMZ treatment shown in Figs. 1 and 3 could depend on the nasal toxicity of this compound, a histological analysis was performed. No cytotoxicity was observed in the nasal mucosa of the CMZ-treated rats (Fig. 5).

Effects of 72-hr Fasting and CMZ Treatment on Some Nasal Monooxygenase Activities

To investigate whether the observed alterations in the microsomal immunoreactive content of CYP isoforms were accompanied by a change in their catalytic activities, coumarin hydroxylase (a marker for 2A3 [26]), pNPH (a marker of 2E1 [24]), ethoxyresorufin and methoxyresorufin dealkylase (markers of 1A1 and 1A2 [27,28]), and testosterone hydroxylase (an index for many CYP isoforms including 2G1, which hydroxylates the testosterone in the 16α-position [29]) were determined in microsomes from control, 72-hr-fasted, and CMZ-treated rats. As shown in Table 1, pNPH, EROD, and MEROD activities were induced about 2-fold by fasting, and CMZ caused a drastic decrease in the rates of both the CYP2E1- and CYP1A-dependent reactions in agreement with the immunoblotting data.

Since *p*-nitrophenol is known to be substrate for 2A3 [26] in addition to 2E1, we inhibited the pNPH activity with anti-2E1 antibodies to evaluate the contribution of

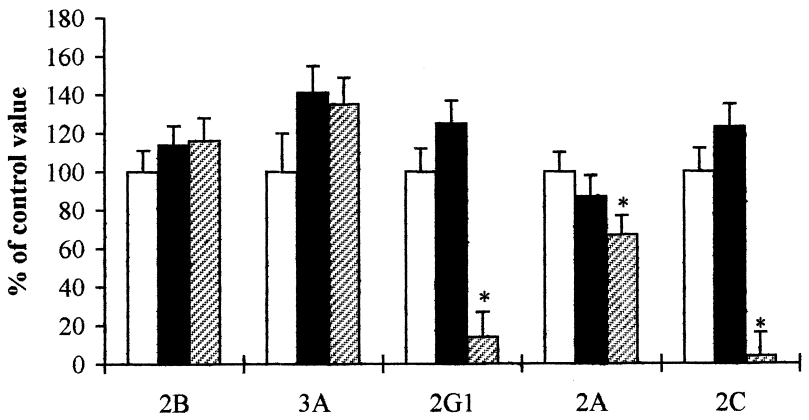

FIG. 2. Immunoblot analysis of the levels of CYP2B, 3A, 2G1, 2A, and 2C in rat nasal mucosa microsomes. The data represent means ± SD (bars) of arbitrary densitometric units from three experiments, expressed as percent of control. Each experiment was carried out with microsomes (30 μg protein for each sample) pooled from four to five control (□), 72-hr-fasted (■), and CMZ-treated (dashed bars) rats. *Values significantly different from control, $P < 0.01$.

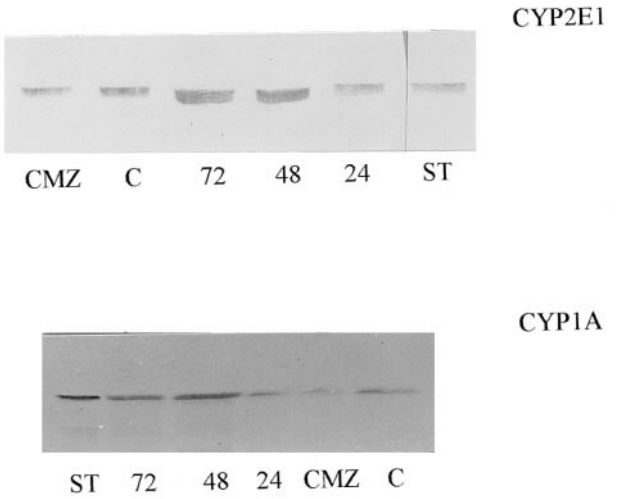

FIG. 3. Immunoblot analysis of CYP2E1 and 1A in nasal mucosa microsomes from control (C), 24-, 48-, and 72-hr fasted rats, and CMZ-treated (CMZ) animals. Microsomes loaded at 25 μg protein/well were subjected to SDS–PAGE on 7.5% acrylamide gels, transferred to nitrocellulose, and probed with anti-rat liver 2E1 and 1A1. The ST lanes contained, as standards for the 2E1 and 1A immunoblots, 0.5 pmol of purified rat liver CYP2E1 and CYP1A2, respectively.

this isoform. The anti-rat CYP2E1 immunoglobulin G (IgG), at a concentration of 8 mg IgG/nmol CYP, inhibited the pNPH activity in the nasal microsomes from control and fasted rats by 23 and 45%, respectively. This finding suggests that 2E1 other than 2A3 is involved in the hydroxylation of *p*-nitrophenol. Since the CYP2A-linked CoH activity did not change with fasting (Table 1), 2E1 appears to account for the increase in pNPH activity observed in the microsomes from fasted rats.

In the nasal microsomes from starved rats, neither the

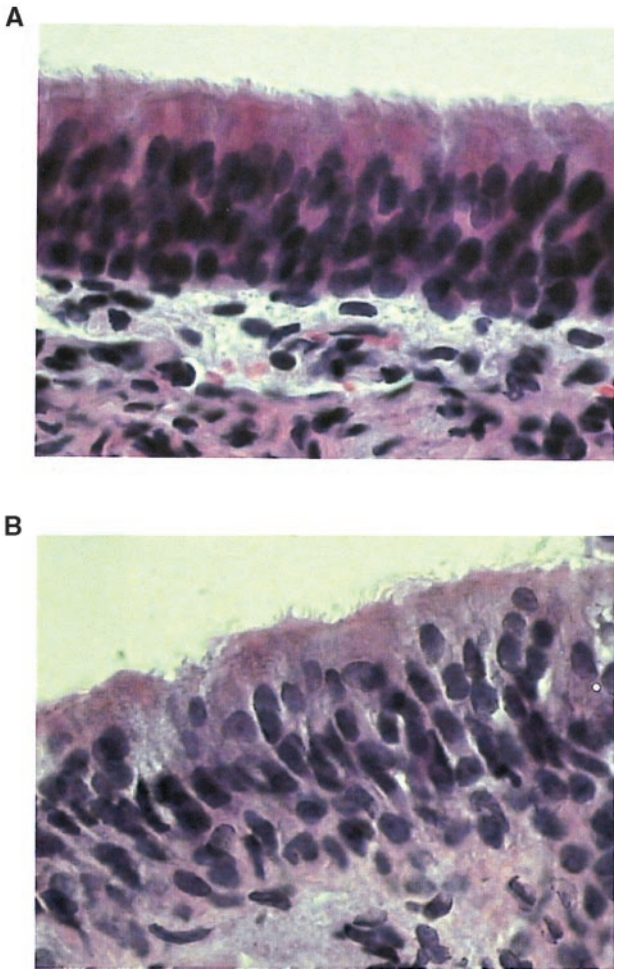

FIG. 5. Effects of CMZ administration in nasal mucosa of rats (A) nasal mucosa of untreated rats. (B) nasal mucosa of CMZ-treated rats (every 12 hr at 75 mg/kg i.p.; 5 times). (hematoxylin and eosin; magnification was 40 ×).

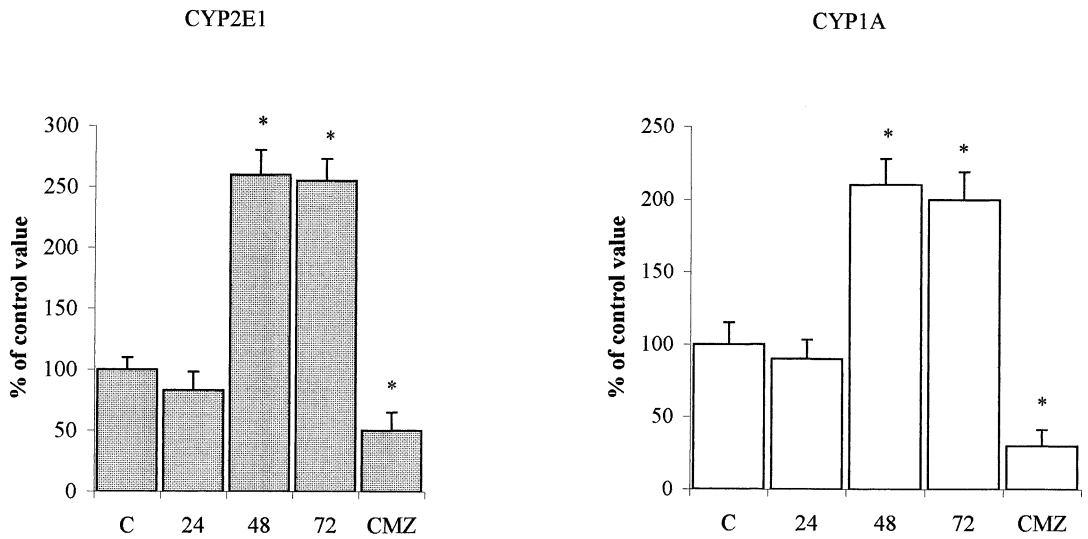

FIG. 4. Immunoblot analysis of the levels of CYP2E1 and CYP1A in rat nasal mucosa microsomes. The data represent means ± SD (bars) of arbitrary densitometric units from three experiments, expressed as percent of control. Each experiment was carried out with microsomes (25 μg protein for each sample) pooled from four to five control rats (C), rats fasted for 24, 48, and 72 hr, and CMZ-treated (CMZ) animals. *Values significantly different from control, $P < 0.01$.

TABLE 1. Coumarin hydroxylase, *p*-nitrophenol hydroxylase, ethoxyresorufin deethylase, and methoxyresorufin demethylase activities in the nasal mucosa microsomes from control, 72-hr-fasted, and CMZ-treated rats

Treatment	CoH	EROD	MEROD	pNPH
Control	0.20 ± 0.03	0.12 ± 0.02	0.016 ± 0.004	0.9 ± 0.2
Fasting	0.22 ± 0.04	0.27 ± 0.03*	0.030 ± 0.004*	1.8 ± 0.3*
CMZ	0.03 ± 0.01*	0.02 ± 0.01*	0.005 ± 0.003*	0.06 ± 0.02*

The activities are expressed in nmol/min/mg microsomal protein. The data represent means ± SD of three different experiments. Each experiment was carried out with microsomes pooled from four to five rats. The substrate concentration in the incubation mixture (final volume 1 mL) was 0.3 μM for MEROD, 0.3 μM for EROD, 0.1 mM for pNPH, and 1 mM for CoH. The incubation time and the protein concentration were 15 min and 1 mg/mL for all the activities, respectively.

*Significantly different from control, $P < 0.01$ by Student's *t*-test.

profile nor the amount of testosterone metabolites was changed, whereas in the microsomes from CMZ-treated rats, all the testosterone metabolites were significantly reduced with the exception of 6β- and 16β-hydroxytestosterone (Table 2). By contrast, the CMZ treatment did not modify the testosterone metabolism in the liver microsomes (data not shown).

Analysis of mRNA of CYP2E1, 1A1, and 1A2

In order to explore the mechanism of CYP2E1 and 1A2 or 1A1 induction in nasal mucosa during fasting, total RNA was isolated and the mRNA of the above-mentioned isoforms was quantified using ^{32}P-labeled cDNA probes for CYP2E1, 1A2, and 1A1. Quantification of the mRNA transcripts revealed significant 2.4- and 2- fold increases in fasted rats for CYP2E1 and 1A2, respectively (Figs. 6 and 7), increases similar in magnitude to those in the corresponding proteins. The CMZ treatment produced a marked decrease in the CYP2E1 and 1A2 mRNA levels below their control values. Fasting or CMZ treatment did not affect the CYP1A1 mRNA level, which remained undetectable as in the control rats.

DISCUSSION

In the present study, we have characterized the changes in nasal mucosa CYPs that occur as a result of fasting. We have shown that the concentration of 2E1 and 1A2 increased significantly, in the nasal microsomes of 48- and 72-hr-fasted rats, whereas 2B, 3A, 2C, 2G1, and 2A were not affected. The induction of nasal 2E1 and 1A2 was manifested by a) immunoblot analysis, b) an increase in CYP1A2-mediated MEROD (or EROD) and CYP2E1-dependent pNPH activities, and c) an enhancement of 2E1 and 1A2 mRNA levels. It is well established that prolonged starvation modulates hepatic and renal CYP enzymes [10, 12] and in particular induces hepatic 2E1 by transcriptional activation [14]. The moderate elevation of the CYP2E1 mRNA level observed in the nasal mucosa of fasted rat, comparable to that found in liver [30], is probably mediated by transcriptional activation, although mRNA stabilization may also occur. As different regulation of 2E1 in the liver and rat nasal mucosa has been reported [2], additional studies will be required to investigate the exact mechanisms responsible for the induction of this isoenzyme by fasting. In considering the 2E1 induction in the rat nasal mucosa, it should be noted that fasting may be able to enhance this isoform as much as the chemical inducers of 2E1 such as ethanol, acetone, and pyrazole [2–4]. It also possible that the 2E1 of nasal mucosa may be modulated by the same agents (for example, diabetes and obesity) found effective in liver. Regarding 1A2, it was unexpectedly found to be induced by fasting in the nasal microsomes. In rat liver, CYP2E1, 3A2, 2B1, and 4A, but not members of the CYP1A subfamily, are inducible by starvation [10]. By using cDNA probes specific for CYP1A1 and CYP1A2, we showed by Northern blot analysis that the rat nasal mucosa

TABLE 2. Oxidation of testosterone in the nasal mucosa microsomes from control, 72-hr-fasted, and CMZ-treated rats

Position of testosterone oxidation	Activity (nmol product/min/mg of protein)		
	Control	Fasting	CMZ
15β	0.263 ± 0.03	0.227 ± 0.03	0.036 ± 0.02*
15α	0.518 ± 0.05	0.430 ± 0.04	0.040 ± 0.02*
6β	0.024 ± 0.01	0.032 ± 0.01	0.032 ± 0.01
16α	0.074 ± 0.02	0.081 ± 0.02	0.019 ± 0.01*
16β	0.163 ± 0.03	0.147 ± 0.02	0.140 ± 0.02
2β	0.269 ± 0.04	0.205 ± 0.03	0.020 ± 0.01*
17	0.204 ± 0.02	0.243 ± 0.03	0.090 ± 0.02*

The data represent means ± SD of three different experiments. Each experiment was carried out with microsomes pooled from four to five rats. The incubations in a 1-mL volume were carried out for 20 min with 1 mM substrate concentration and 1 mg of protein.

*Values significantly different from control, $P < 0.01$ by Student's *t*-test.

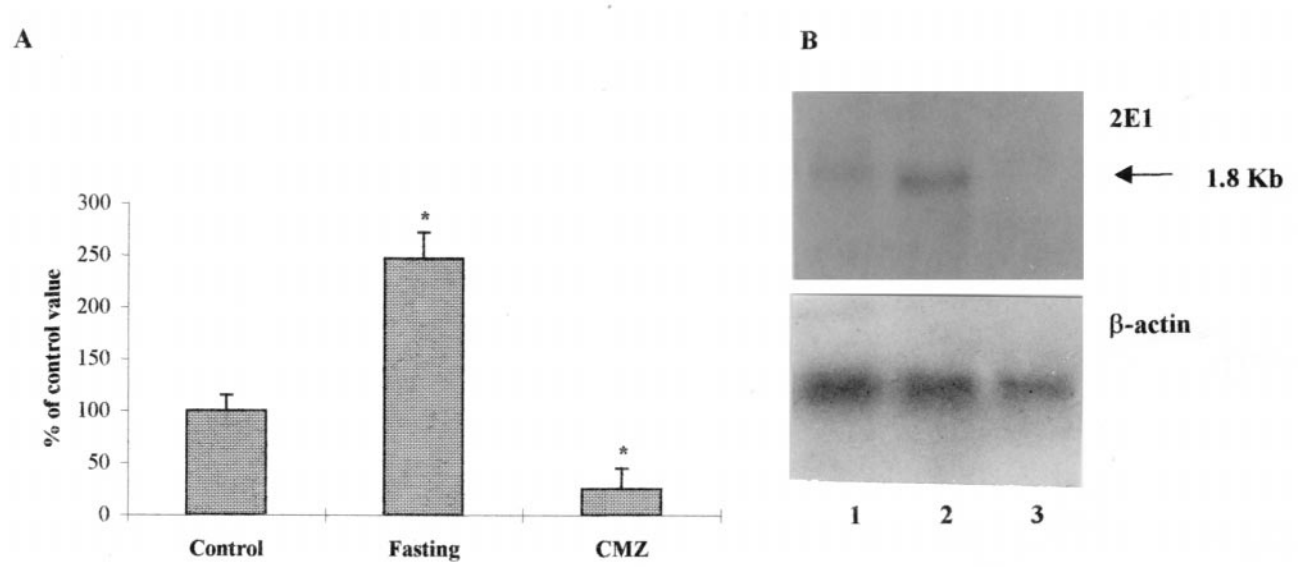

FIG. 6. (A) Analysis of the amount of nasal CYP2E1 mRNA from control, fasted, and CMZ-treated rats. Total RNA was isolated from nasal tissue and subjected to electrophoresis, blotting, and hybridization as described in Materials and Methods. The relative signals were normalized with respect to the intensities obtained using β-actin cDNA. Values are means ± SD of three determinations on pooled samples of total RNA (three rats for each determination). *Values are significantly different from control ($P < 0.01$). (B) Northern blot analysis. Lane 1, control; lane 2, fasting; lane 3, CMZ.

constitutively expresses 1A2 mRNA, but not 1A1 mRNA, in agreement with data by Gillner *et al.* [7] but in contrast to Voigt *et al.* [8]. Ding and Coon also observed that 1A2, but not 1A1, is constitutively expressed in the olfactory epithelium of rabbit [31].

The present finding demonstrates that starvation induces nasal CYP1A2, but not 1A1, at the catalytic level in a more evident fashion (the EROD or MEROD activities in the fasted rats were double those in the control rats) than previously reported after induction by β-NF [7]. In the liver and in various extrahepatic tissues including lung and intestine, many PAHs (polycyclic aromatic hydrocarbons) including β-NF co-induce both CYP1A2 and 1A1 via the Ah receptor [32]. In the nasal mucosa, the increased expression of 1A2, but not 1A1, by starvation or β-NF suggests the presence of an induction mechanism indepen-

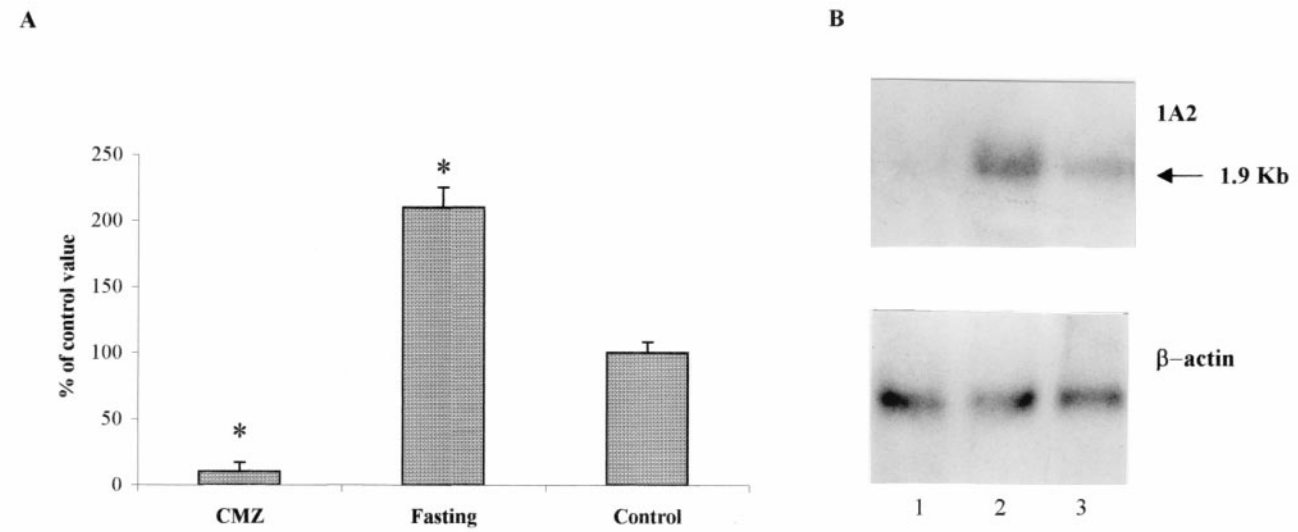

FIG. 7. Analysis of the amount of nasal CYP1A2 mRNA from control, fasted, and CMZ-treated rats. Total RNA was isolated from nasal tissue and subjected to electrophoresis, blotting, and hybridization as described in Materials and Methods. The relative signals were normalized with respect to the intensities obtained using β-actin cDNA. Values are means ± SD of three determinations on pooled samples of total RNA (three rats for each determination). *Values significantly different from control ($P < 0.01$). (B) Northern blot analysis. Lane 1, CMZ; lane 2, fasting; lane 3, control.

dent of the Ah receptor or an Ah receptor-dependent mechanism modulated by tissue-specific proteins. Indeed, an induction of CYP1A2 in mouse liver by tricyclic aromatic hydrocarbons via a mechanism not involving the Ah receptor has recently been reported [33]. In addition to this mechanism, a stabilization of CYP1A2 mRNA might occur in the nose as reported in the liver [34].

It should be noted that in the rat nasal mucosa, 1A1, not constitutively expressed, was found to be inducible by cigarette smoke, as assessed by immunological techniques [28]. This would indicate the presence of a CYP1A induction via the Ah receptor in this tissue as well. Thus, the mechanism by which starvation or other agents induce 1A2 in the nasal tissue is an open question for further study. Stress hormones such as glucocorticoids and thyroxine, both known to be affected by starvation, might have a role in the increase in nasal 2E1 and 1A2 expression.

However, it is quite clear that the effect of starvation on the nasal tissue is different from that on liver: in particular, the strong induction of 3A in liver by fasting [10] was not observed in the nasal microsomes as assayed by the immunoblot and 3A-linked [35] 6β-testosterone hydroxylase activity. A further confirmation of the different regulation of CYP isozymes in the hepatic and nasal tissue derives from the inhibitory experiment with CMZ. This compound, which has been reported to be an effective and specific inhibitor of hepatic CYP2E1 at the transcriptional level [15], inhibited the constitutive expression of many nasal CYPs including 2E1, 1A2, 2G1, and 2A, but not 2B and 3A, as determined by immunoblot analysis and related monooxygenase activities. As shown with the histological analysis, the inhibition did not appear to be due to an intrinsic toxicity of the CMZ treatment. The inhibition of 1A2 and 2E1 expression was also demonstrated at the mRNA level, suggesting that CMZ may interact with transcriptional factors common to the regulation of both genes. This indicates a tissue-specific action of CMZ influencing specific signal transduction chains expressed in the nasal mucosa but not in liver.

In conclusion, our data show: i) a marked induction of nasal 1A2 and 2E1 by fasting and, ii) a different regulation of various nasal CYPs compared to the hepatic CYPs in response to fasting and CMZ. The inducibility of these enzymes may be important, as it may amplify the clearance of hormones, drugs, or inhaled odorants, alter the sense of smell, and change the susceptibility of nose to cancer and toxicity by environmental compound substrates of 1A2 and 2E1 such as polycyclic aromatic hydrocarbons or nitrosamines contained in cigarette smoke and common volatile solvents.

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