Acknowledgements—This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

Department of Hygienic Chemistry Pharmaceutical Institute, Tohoku University, Sendai 980, Japan

MASAKUNI DEGAWA
AKIO OHTA
MASAYUKI NAMIKI
TAKASHI MASUKO
YOSHIYUKI HASHIMOTO*

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* To whom all correspondence should be addressed.

Biochemical Pharmacology, Vol. 36, No. 19, pp. 3317–3318, 1987.
Printed in Great Britain.

0006-2952/87 $3.00+0.00
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In vitro conversion of pyrazinamide into 5-hydroxypyrazinamide and that of pyrazinoic acid into 5-hydroxypyrazinoic acid by xanthine oxidase from human liver

(*Received* 1 *October* 1986; *accepted* 31 *March* 1987)

An unknown, called compound II, found in human urine containing pyrazinamide and its metabolites, was identified recently by the authors as 5-hydroxypyrazinamide [1], thus establishing that the pathway described by Weiner and Tinker [2] (conversion of pyrazinamide into Compound II) is the same as that described by Pitre *et al.* [3] (conversion of pyrazinamide into 5-hydroxypyrazinamide). However, few *in vitro* studies on the oxidation of pyrazinamide or pyrazinoic acid in human liver have been reported. Considering this need for further study, the investigation of the oxidation of pyrazinamide and pyrazinoic acid by xanthine oxidase found in human liver was undertaken and is herein described.

Materials and methods

A normal human liver was obtained by operative resection following traumatic injury. It was immediately frozen to −80° and stored until used. The homogenates were subjected to differential centrifugation to obtain the cytosol fraction according to the method of Wilgram and Kennedy [4]. The cytosol fraction, precipitated with 30–50% saturated ammonium sulfate, was dialyzed against 10 mM potassium buffer (pH 7.4) containing 1 mM EDTA and 1 mM GSH. Next, using the batch method described previously [5], the dialyzed fraction was treated with hydroxyapatite. After concentrating the post-treated dialyzed supernatant 2-fold, it was incubated by the method of Hande *et al.* [6] except for the use of 1.5 mM dithiothreitol. This dialyzed supernatant fraction was used as the partially purified xanthine oxidase fraction (XO fraction). The specific activity of xanthine oxidase in this fraction was 12.7 nmol uric acid formed · min^{-1} · (mg protein)$^{-1}$, having increased 32.4-fold compared with that in the cytosol fraction. Each reaction mixture consisting of 200 μl of the XO fraction and 100 μl of 50 mM potassium phosphate buffer (pH 7.4) containing the substrate (pyrazinamide or pyrazinoic acid) was incubated at 37° after 10 min of preincubation without substrate. After incubation, extraction was performed by the modified method of Yamamoto *et al.* [7]. The measurements of 5-hydroxypyrazinamide and pyrazinoic acid using HPLC were performed by the method of Yamamoto *et al.* [7] except that the detection of fluorescence was 410/310 nm, the pH of the mobile phase was 2.5, and the flow rate was 1.6 ml/min. Polyacrylamide disc gel electrophoresis was performed by the method of Davis [8] and staining was by the method of Holmes *et al.* [9] using 1 mM hypoxanthine, 80 mM pyrazinamide, 50 mM pyrazinoic acid, 0.5 mM allopurinol, 0.5 mM oxypurinol, 20 mM benzaldehyde or 1.5 mM *N'*-methylnicotinamide (NMN) as the substrate. Xanthine oxidase was measured as described previously [10]. Protein was determined by the method of Lowry *et al.* [11].

Results and discussion

Xanthine oxidase was stained most markedly by hypoxanthine after 60 min of staining. It was also stained by allopurinol, which has been shown to be a powerful and specific inhibitor of xanthine oxidase [12]. It was not stained at all, however, by oxypurinol. The resulting intensity of staining of xanthine oxidase with 1 mM hypoxanthine was inhibited considerably by 0.5 mM oxypurinol over a 10-min staining period, performed after disc gels had been preincubated in the solution with 0.5 mM oxypurinol for 30 min. Furthermore, xanthine oxidase could be stained by benzaldehyde but not by *N'*-methylnicotinamide. It was also stained by pyrazinamide and pyrazinoic acid, though faintly. The addition of 0.5 mM NAD proved to have no effect on the staining patterns of the disc gels. As conversion of pyrazinamide into 5-hydroxypyrazinamide and that of pyrazinoic acid into 5-hydroxypyrazinoic acid, respectively, increased linearly over a 60-min incubation period in the XO fraction, the K_m value of xanthine oxidase for pyrazinamide or pyrazinoic acid was determined after the reaction mixtures were incubated for 40 min. The K_m value for pyrazinamide was 2.4 mM and that for pyrazinoic acid was 0.7 mM. The double-reciprocal plots from experiments on the XO fraction were linear for both pyrazinamide and pyrazinoic acid [Y (1/μmol 5-hydroxypyrazinamide formed per h per mg protein) =0.017 + 0.0412 X (1/mM pyrazinamide), r = 0.99, and Y (1/μmol 5-hydroxypyrazinoic acid

per h per mg protein) =0.0091 + 0.0064 X (1/mM pyrazinoic acid), $r = 0.99$], indicating the presence of only one enzyme in the XO fraction for both pyrazinamide and pyrazinoic acid. The conversion of pyrazinamide into 5-hydroxypyrazinamide or that of pyrazinoic acid into 5-hydroxypyrazinoic acid increased 1.30- and 1.37-fold, respectively, in the XO fraction when 0.7 mM NAD was added to the reaction mixture containing 10 mM pyrazinamide or 3 mM pyrazinoic acid respectively. It was thus demonstrated that some dehydrogenase(s) that metabolized both pyrazinamide and pyrazinoic acid existed in the XO fraction. As the staining pattern of the disc gels was not changed by the addition of NAD, this may suggest that the dehydrogenase(s) in this case was identical to xanthine dehydrogenase. The inhibitory studies with oxypurinol were done over a single period of 40 min, indicating that the greater the quantity of oxypurinol used, the more marked was the inhibition of conversion of either pyrazinamide or pyrazinoic acid in the XO fraction (data not shown).

As for pyrazinamide, however, there is evidence that xanthine oxidase may not be the main catalyst responsible for its oxidation. In one case, Weiner and Tinker found Compound II in the urine of a human subject pretreated with allopurinol; they did not find 5-hydroxypyrazinoic acid in this subject [2]. In another case, Auscher *et al.* [13] found Compound II in the urine of a xanthinuric subject who was supposedly without xanthine oxidase, though they did not find 5-hydroxypyrazinoic acid in this subject. These findings indicate that further study is warranted to identify in humans a pyrazinamide-oxidizing enzyme other than xanthine oxidase.

In summary, using disc gel electrophoresis and high performance liquid chromatography, it was shown that both pyrazinamide and pyrazinoic acid were oxidized to their 5-hydroxy forms by xanthine oxidase from human liver (K_m values of xanthine oxidase with pyrazinamide and pyrazinoic acid were 2.4 and 0.7 mM respectively).

*Address all correspondence to: Dr Tetsuya Yamamoto, The Third Department of Internal Medicine, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya Hyogo, 663 Japan.

The Third Department of Internal Medicine
Hyogo College of Medicine
Hyogo, 663 Japan

TETSUYA YAMAMOTO*
YUJI MORIWAKI
SUMIO TAKAHASHI
TOSHIKAZU HADA
KAZUYA HIGASHINO

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Biochemical Pharmacology, Vol. 36, No. 19, pp. 3318–3320, 1987.
Printed in Great Britain.

0006-2952/87 $3.00+0.00
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Mouse brain ATPase activities after chronic nicotine infusion

(*Received* 3 *November* 1986; *accepted* 3 *April* 1987)

Tolerance to the effects of nicotine develops with chronic exposure to the drug [1–5], and a portion of this tolerance may be related to the observation that chronic treatment with nicotine results in an increase in the number of putative nicotinic receptors [4–6]. The changes in receptor number do not seem to explain all of the tolerance, however [5].

Nicotinic receptors from *Torpedo* and *Electrophorus* contain ion channels that are activated after interaction with agonists [7]. The normal ionic conductance observed after the activation of these channels is the inward flux of Na^+ down its electrochemical gradient. Therefore, it may be that a persistent activation of nicotinic receptors arising from the constant presence of the nicotinic receptor agonist, nicotine, may increase intracellular Na^+ and result in a chronic depolarization of the nerve membrane.

Chronic membrane depolarization may indirectly affect the activity of the enzyme responsible for maintaining the membrane potential, Na^+, K^+-ATPase [8], by increasing the concentration of its intracellular ion substrate, Na^+. It has been reported that rats chronically exposed to nicotine in their drinking water appeared to have elevated levels of total Na^+, K^+-ATPase activity [9]. Na^+, K^+-ATPase in the brain is represented by two isozymes with different molecular weights [10], and different affinities for the cardiac glycosides [10, 11]. Both of these isozymes appear to function in ion transport [12]. The present study was undertaken to determine whether the two ouabain-sensitive Na^+, K^+-ATPase activities are equally affected by chronic nicotine treatment in four strains of mice that differ in their abilities to develop tolerance to the effects of nicotine [5].

Methods

Materials. ATP, phosphoenolpyruvate (PEP), ouabain octahydrate and pyruvate kinase (PK) were purchased from Boehringer–Mannheim (Indianapolis, IN) and bovine serum albumin, L-nicotine and imidazole were purchased from the Sigma Chemical Co. (St. Louis, MO).

Mice. Females of four inbred mouse strains were used: BALB/cByJ, C57BL/6J/Ibg, DBA/2J/Ibg and C3H/2Ibg. All mice were bred at the Institute for Behavioral Genetics at the University of Colorado, Boulder.

Chronic drug treatment. Mice were chronically treated with nicotine by constant intravenous infusion [4, 5]. The final treatment dose was 3.0 $mg \cdot kg^{-1} \cdot hr^{-1}$, and animals were treated with this dose for 10 days.

Tissue preparation. At the completion of the treatment period, mice were killed by cervical dislocation, and their brains were dissected into six regions: cerebral cortex,