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Increased Urinary Excretion of Tryptophan Metabolites in Rats Exposed to Nitrogen Dioxide

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Nitrogen dioxide (NO_2) is widely distributed in nature, and human exposure to it is extensive. It is abundant in all burnt organic materials, including automobile exhaust and cigarette smoke. In the lung of animals exposed to NO_2, some subtle changes will occur and they include inflammation (Gardner et al. 1969), emphysema (Freeman and Haydon 1964) and reduced phagocytic or bactericidal activity of the alveolar macrophages (Suzuki et al.1986). In epidemiological study, Yanagisawa et al. (1986) reported the increased urinary hydroxyproline excretion of residents living in areas of high NO_2 levels. Moreover, in experimental study using animals, exposure to NO_2 induced the alteration of collagen synthesis and/or degradation in lung (Kleinerman 1979) and accelerated collagen degradation in lung by NO_2 exposure was responsible for the increase in the excretion of urinary hydroxyproline (Kosmider et al. 1973). From these findings on the increased excretion of urinary hydroxyproline by NO_2, Yanagisawa et al. (1986) suggest that urinary hydroxyproline could be used as a personal biochemical indicator of the effects of NO_2 on human health prior to manifestation of respiratory symptoms. Moreover, Ripperton and Johnston (1959) reported that urinary content of aspartic acid increased in rats exposed to NO_2. Exposure to NO_2 may influence the metabolism of amino acids to induce their excessive urinary excretion.

Since the maximum permissible limit for NO_2 in working environments in Japan is 5 ppm, in this work, we have studied the changes of urinary excretion of tryptophan metabolites in rats exposed to 5 ppm NO_2 in order to find a new urinary biochemical indicator of the effects of NO_2 exposure on human health.

MATERIALS AND METHODS

Five-week-old, specific-pathogen-free, male Fischer 344

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rats (Japan Charles River Co, Atsugi, Japan) were exposed to 5 ppm NO_2 for 2 and 4 weeks. Eight animals per group exposed to NO_2 or clean air were used. Exposure of animals to NO_2 was conducted by the method previously described by Suzuki et al. (1986).

After exposure to NO_2 or clean air, each rat was placed into a glass metabolism cage (Sugiyama-Gen Environmental Science Co, Tokyo, Japan), and was given pelleted diet ad libitum, and urine was collected for 24 hours. The collected urine was centrifuged at 300 x g for 5 min to remove an extremely small amount of diet mingled into urine, and it was stored at -20°C until analyzed. The contents of xanthurenic acid and kynurenic acid in tryptophan metabolites in urine were determined by the method of Satoh and Price (1958), and the content of each tryptophan metabolite in urine was expressed in μmoles per kg of body weight per day. The contents of xanthurenic acid and kynurenic acid in diet were also checked by the above method.

Rats were sacrificed after collection of urine. A part of the liver was homogenized with 9 volumes of cold 0.25 M sucrose solution containing 0.2 M potassium chloride (pH 7.4), centrifuged at 3,300 x g for 10 min at 4°C, and then the supernatant was re-centrifuged at 15,000 x g for 60 min at 4°C. The final supernatant was dialyzed against 0.2 mM tris buffer (pH 8.0) for 24 hrs at 4°C. The dialyzed sample was stored at -20°C until analyzed. The activity of kynureninase was determined with and without an addition of pyridoxal phosphate (PALP) by the method of Knox (1953). Kynureninase activity was expressed in μmoles of kynurenine utilized per hr per g of tissue.

The pyridoxal phosphate contents in liver were determined by the method of Morita and Mizuno (1984) as modified in our laboratory. One gram samples of liver were homogenized with 9 ml of cold 1 N perchloric acid and the homogenate was centrifuged at 1,500 x g for 30 min at 4°C. The residue was resuspended in 3 ml of cold 0.2 N perchloric acid and re-centrifuged at 1,500 x g for 30 min at 4°C. The two supernatants were combined. The combined mixture was adjusted to pH 2-3 with 5 N potassium hydroxide and centrifuged at 35,000 x g for 30 min at 4°C to remove the resulting fine precipitate of potassium perchlorate. A sample (100 μl) of the final supernatant was analyzed by the high-performance liquid chromatography (HPLC) method. Analytical HPLC was carried out with Shimadzu LC-2 (Shimadzu Co, Kyoto, Japan) equipped with a fluorometric detector (Shimadzu RF-500) at ambient temperature. The fluorescence intensity was measured at 296 nm of excitation and 391 nm of emission. A 0.46 x 25 cm Finepak SIL $C_{18}T$ (Nihon Bunko Co, Tokyo, Japan) stainless-steel analytical column attached to a

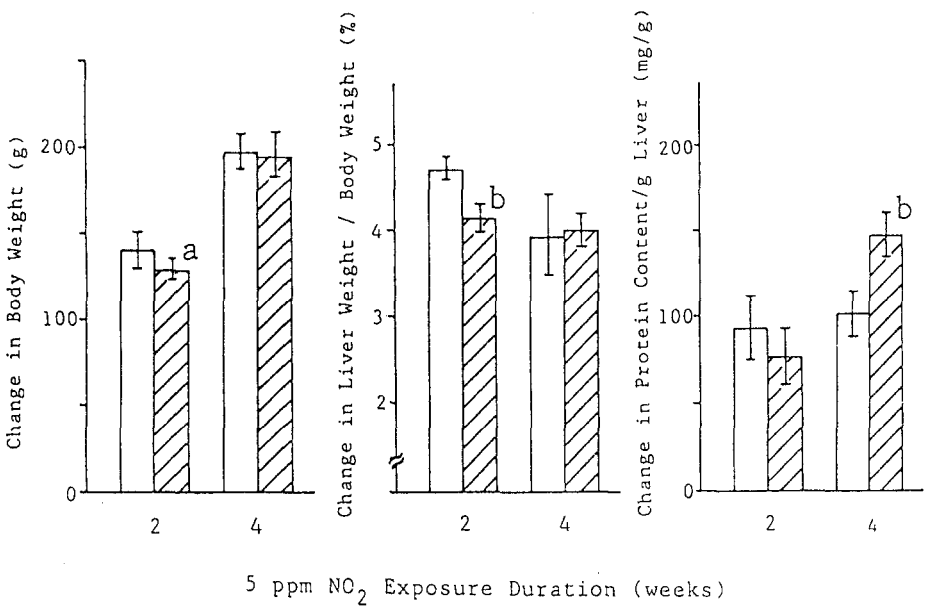

Figure 1. Changes of body weight, liver weight and protein content on liver of rats exposed to 5 ppm NO_2 or air. Data are expressed as the mean ± S.D.(n=8). Symbols indicate the significant difference from control by the Student t test (a; $P<0.05$, $P<0.001$)
□ : Control, ▨ : NO_2

0.46 x 5 cm guard column packed with Finepak SIL C_{18}TP (Nihon Bunko Co) was used. The mobile phase was a mixture of 0.2% monobasic sodium phosphate and 0.085% phosphoric acid (10:11, v/v, pH 2.5). The flow rate was 2 ml/min. The concentration of PALP is calculated from the peak height, based on the calibration chromatogram obtained with a standard solution (5-100 nM/ml) of PALP, which had been treated with perchloric acid and potassium hydroxide before injection to the HPLC, as in the case for liver. The content of PALP in liver was expressed in nanomoles per whole tissue.

RESULTS AND DISCUSSION

As shown in Figure 1, in the rats exposed to NO_2, both the body weight and the liver to body weight ratio decreased on week 2, and they returned to the control levels at week 4. On the other hand, the content of protein per gram of liver did not change up to week 2, but, it increased on week 4. The contents of both xanthurenic and kynurenic acids excreted into the urine of rats exposed to NO_2 increased on week 2, and they returned to the control levels at week 4 (Figure 2). As shown in Figure 3, in the rats exposed to NO_2 for 2 weeks, kynureninase activity in liver decreased without an addition of PALP and with an addition of 20 μg of

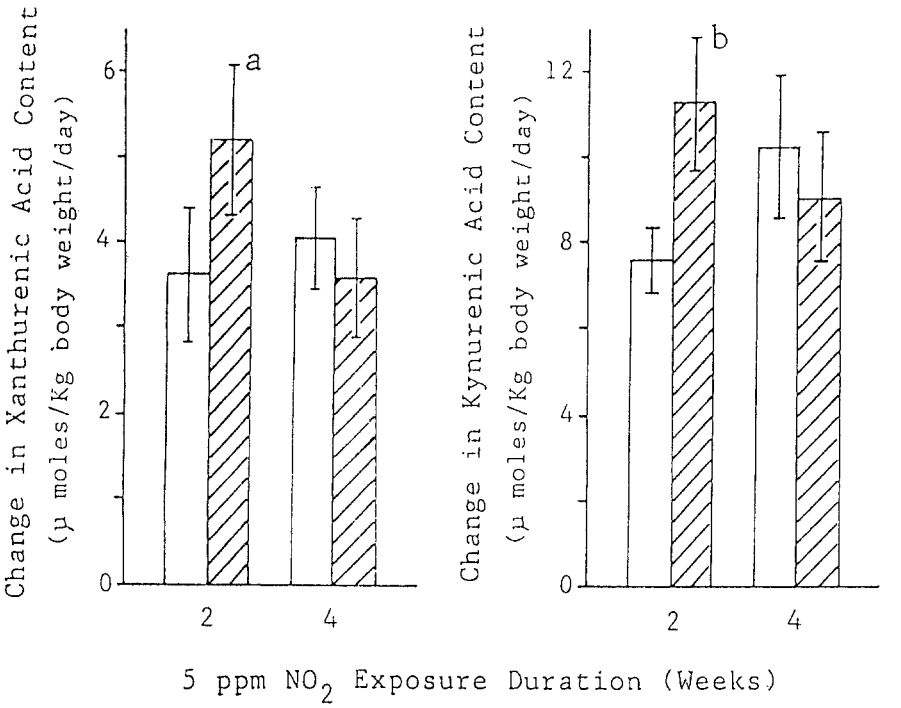

Figure 2. Changes in the contents of xanthurenic acid and kynurenic acid in urine of rats exposed to 5 ppm NO_2 or air. a; $P < 0.005$, $P < 0.001$

PALP, but it returned to the control level with an addition of 100 μg of PALP. On the other hand, the kynureninase activity of rats exposed to NO_2 for 4 weeks remained unchanged with and without an addition of PALP. During the NO_2 exposure period, no significant changes were observed in the contents of PALP in the liver of treated rats (Figure 4). The amounts of xanthurenic acid and kynurenic acid in diet mingled into urine were barely detectable.

It has been well known that a disturbance in tryptophan metabolism consisting of an excessive urinary excretion of xanthurenic acid, 3-hydroxykynurenine, kynurenine and kynurenic acid takes place in a kind of vitamin B_6 dependency (Knapp 1960; Tada et al. 1968). The urinary excretion of these metabolites was decreased, although temporarily, to the normal level by administration of large doses of vitamin B_6 (Knapp 1960). Tada et al. (1968) reported that the level of PALP, which is vitamin B_6 analog showing the same activity as vitamin B_6, in sera from patients with dependent xanthurenic aciduria was found to be within normal limits and the activity of kynureninase in the liver of patients was markedly low. Knapp (1960) assumed the particular disorder in tryptophan

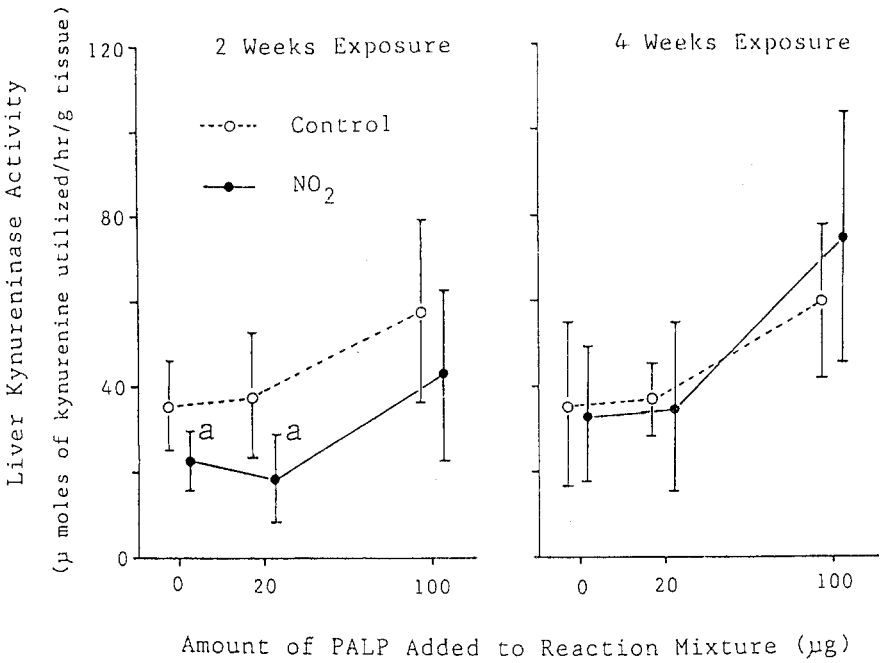

Figure 3. Changes in liver kynureninase activities under various amounts of pyridoxal phosphate (PALP) added to the reaction mixture in rats exposed to 5 ppm NO_2 or air. a; $P<0.05$

metabolism to be a genetically conditional disturbance of vitamin B_6 metabolism. On the other hand, Tada et al. (1968) suggested that the basic defect in the patients is the inability of kynureninase (apoenzyme) to combine normally with the PALP (coenzyme).

The increase of xanthurenic acid and kynurenic acid in the urine of the rats exposed to 5 ppm NO_2 for 2 weeks in our experiment may be due to the inhibition of kynureninase activity in the liver, because in mammals tryptophan is mainly metabolized in the liver (Knox 1953) and kynureninase is a key enzyme for tryptophan metabolism (see Figure 5). As Tada et al. (1968) suggested that the inhibition of binding between kynureninase and PALP may be responsible for the decrease of kynureninase activity in the liver. In othor words, exposure to NO_2 induces an increase in the level of lipid peroxides in the liver (Ichikawa and Yokoyama 1981) and kynureninase is a sulfhydryl enzyme (Moriguchi et al. 1973), which may be inhibit by lipid peroxides (Will 1961). In our experiment, the decreased kynureninase activity returned to the control level with the addition of a large amount of PALP. This suggest that the kynureninase activity was decreased by a defective

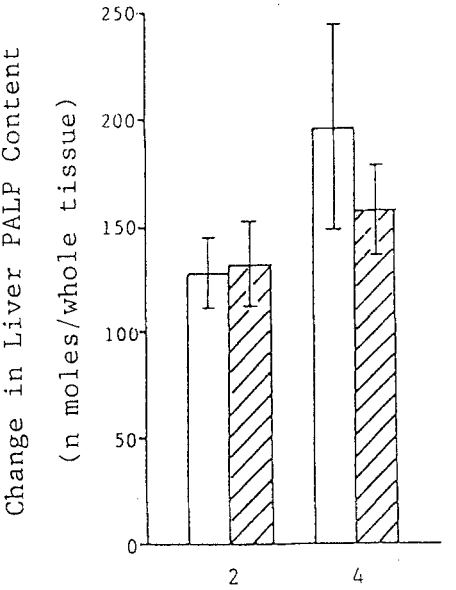

Figure 4. Changes in pyridoxal phosphate (PALP) contents in liver of rats exposed to NO_2 or air.

binding between kynureninase and PALP, not by inactivation of kynureninase itself.

In the rats exposed to NO_2 for 4 weeks, the increases of urinary excretion of xanthurenic acid and kynurenic acid, and the decrease of kynureninase activity in the liver returned to the control levels. There was no significant difference in either the body weight or liver weight between the rats exposed to NO_2 for 4 weeks and the control rats, but increased protein synthesis was observed in the liver of the former rats. These findings suggest that the levels of xanthurenic and kynurenic acids which increased after 2 weeks of NO_2 exposure returned to the control levels on week 4 probably because the synthesis of kynureninase was enhanced as a result of the increase in protein synthesis in the liver.

Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which are produced by the kynurenine pathway of tryptophan metabolisms as shown in Figure 5, are coenzymes indispensable to the metabolisms of many substances, such as amino acids, nucleotides, glucoses, lipids and hormones. Therefore, the disturbance of tryptophan metabolism by NO_2 exposure may induce many damages in human subjects. Moreover, although the contents of PALP in the liver of rats exposed to NO_2 remained unchanged in this experiment, a prolonged

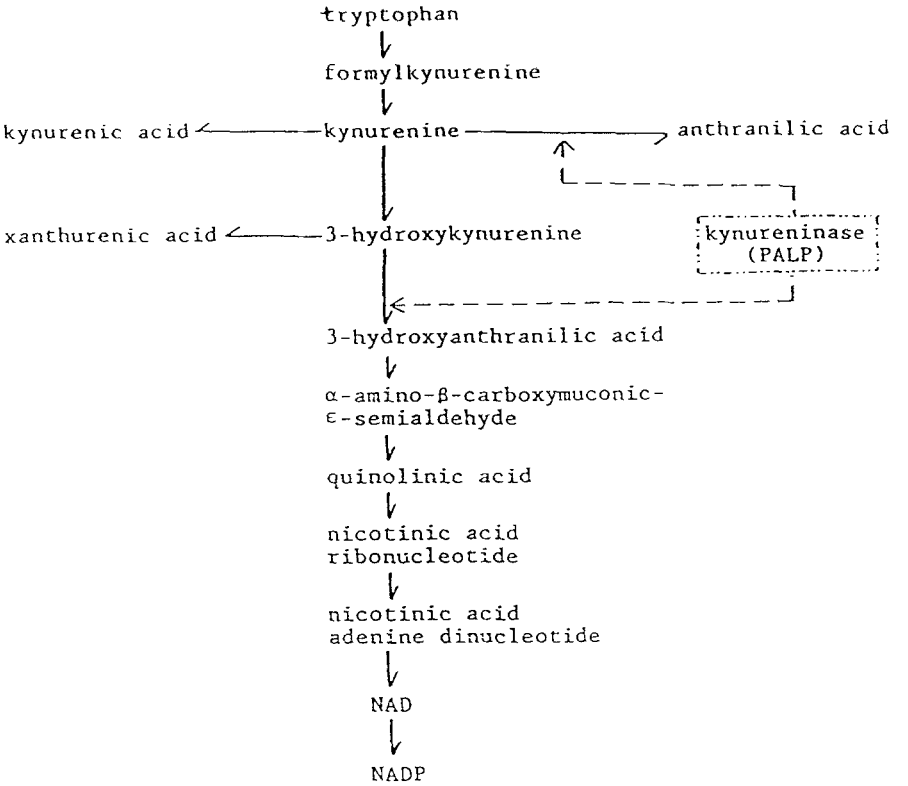

Figure 5. Main pathway of tryptophan degradation.
NAD; nicotinamide adenine dinucleotide
NADP; nicotinamide adenine dinucleotide phosphate

exposure to NO_2 may induce a decrease in PALP content, because Nizhegorodov and Markhotskii (1971) reported decreased contents of vitamin B_6 in various tissues of rats exposed to a mixture of 1.1 ppm NO_2, 18.3 ppm carbon monoxide and 23 ppm ammonia for 139-149 days.

We think that the values of urinary xanthurenic acid and kynurenic acid can be used as convenient biochemical indicators of the effects of NO_2 exposure on human health. In our further study, we will make measurements of prolonged exposure to various doses of NO_2, as a field survey on human subjects.

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