Variations in the Level of Urinary Thiobarbituric Acid Reactant in Healthy Humans under Different Physiological Conditions

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The level of urinary thiobarbituric acid (TBA) reactants in healthy human subjects due to malonaldehyde derivatives was measured to assess the lipid peroxidation status of the whole body. For each subject the TBA reactant level over a day varied over a 2—3 fold range while the daily level varied over a 1.5—3 fold range under normal life-style conditions. One of the factors causing an increase in the reactant level within a single day may be the subjects' physical activity, because the reactant level of each subject was higher in the afternoon or in the evening than in the morning. Remaining awake all night or hard exercise caused a dramatic increase in the reactant level over a day and in the daily reactant level. The reactant level within a single day for a subject was increased 5.5 fold and the daily level 3 fold by remaining awake all night, and the level within a day was increased 22 fold by hard exercise while the corresponding daily level was increased 7 fold. It is unlikely that food, alcohol and smoking greatly affect the reactant level. The results suggest that increased physical activity enhances lipid peroxidation in the whole body and thus the increased urinary excretion of malonaldehyde derivatives.

Keywords thiobarbituric acid reactant level; urine; lipid peroxidation; physical activity; stress

Oxidative stress or lipid peroxidation has been implicated in diverse pathological conditions including aging, carcinogenesis, atherosclerosis, inflammation, ischemia and drug toxicity.^{1,2)} The level of lipid peroxidation products in human plasma or serum, as assessed by the thiobarbituric acid (TBA) reactant level, is considered an important index for the lipid peroxidation status of the whole body.³⁻¹⁹⁾ While many workers have demonstrated a relationship between the TBA reactant level in plasma or serum and age, sex, exercise and diseases, others have not. Indeed, in our previous study we were unable to identify the TBA reactants present in plasma or serum²⁰⁾ and there is some doubt whether the TBA reactants in plasma or serum reflect lipid peroxidation status of the body.²¹⁾ The TBA reactants in urine, however, have been characterized adducts of malonaldehyde with amino acids or amines.²²⁻²⁷⁾ To determine the urinary TBA reactant level, it is necessary to measure the reactant level after removing interfering chromogens by high-performance liquid chromatography.²⁷⁻³⁰⁾

In our previous study,²⁷⁾ we found that the daily urinary TBA reactant level in healthy Japanese subjects ranged from 26 to 95 nmol red pigment/kg · d, and the reactant level was not related to age or sex. In the present study, we have investigated the within-day, day-to-day and month-to-month variations in the urinary TBA reactant levels of healthy Japanese, and found some relationship between urinary TBA reactant level and life-style. While the type of food eaten, alcohol intake or stopping smoking (in the case of a smoker) slightly affected the reactant level, physical activity and/or stress had a dramatic effect.

MATERIALS AND METHODS

Reagents TBA and 2,4-dinitrophenylhydrazine (DNPH) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Tetramethoxypropane (TMP) was obtained from Tokyo Kasei Kogyo Company (Tokyo, Japan).

Apparatus High-performance liquid chromatography (HPLC) was carried out using a Hitachi L-600 or L-655-A11 (Tokyo, Japan) liquid chromatograph equipped with a column (4.6 mm i.d. × 250 mm) of YMC A-303 ODS (Yamamura Chemical Laboratories, Kyoto, Japan), and peaks were detected using a Hitachi L-4200 UV-VIS (Tokyo, Japan) or a Shimadzu SPD-6A (Osaka, Japan) detector.

Subjects Six male and four female healthy Japanese aged 21—53 were chosen as subjects for the experiment, and the body weight (kg) of each subject was measured and recorded. Several series of experiments were performed on successive days. Each subject was fed "regular" Japanese food, vegetable-rich food (V) or meat-rich food (M) throughout a day. "Regular" Japanese food was composed of rice, vegetables and meat, V consisted of rice, a large amount of vegetables and a small amount of meat, and M consisted of rice, a large amount of meat and a small amount of vegetables. One subject was given an alcoholic drink (about 500 ml of beer containing 3% alcohol), while another subject who smoked about 20 cigarettes every day was obliged to stop. Another subject was obliged to work without sleeping during the night, or made to perform hard exercise. The experimental conditions, however, did not exceed by much their usual life-style.

Collection of Urine Urine obtained at midnight was discarded. Then urine was collected 5—10 times at arbitrary intervals and finally at midnight. The volume of each urine sample collected and the time of collection were recorded exactly. An aliquot of each urine sample was stored in a 10-ml test tube fitted with a screw-cap, containing a drop of toluene until analyzed within 1 week. The TBA reactant level in the stored urine remained unchanged during the 1-week storage period.

TBA Assay The TBA assay was performed according to the previously reported method.²⁷⁾ To a test tube fitted with a screw-cap, were added in succession 2.0 ml of a solution containing 10 nmol TMP/ml in water or 2.0 ml

of urine, 0.10 ml 0.5% butylated hydroxytoluene solution in glacial acetic acid (the final concentration of acetic acid was 2%), and 3.0 ml aqueous 0.5% TBA solution. The mixture was kept at 5 °C for 60 min and then heated at 100 °C for 20 min. After cooling, the mixture was extracted with 3 ml chloroform and centrifuged at 650g for 10 min. The aqueous phase was subjected to HPLC using a mobile phase composed of 0.04 M acetate buffer (pH 5.5)/methanol (6:4, v/v) at a flow rate of 0.8 ml/min. The peak was detected at 532 nm and a red pigment appeared at a retention time of 8.0 min. The amount of red pigment in 1 ml urine was determined by comparing the peak area of the red pigment with that of the standard TMP solution. The amount of red pigment from the same urine sample was very similar even if the assay was performed on a different day. Hence, reproducible data could be obtained by this procedure.

The amount of red pigment, in nmol/kg, formed by each urine collected was calculated by (red pigment nmol/ml urine) $\times$ (urine volume ml/body weight kg). In order to monitor the change in the urinary TBA reactant level within a single day, the TBA reactant level (red pigment nmol/kg $\cdot$ h) was calculated from the red pigment nmol/kg/interval (h) between two urine collections, and plotted against the mid-time of the urine collection interval. In order to monitor daily changes of in the urinary reactant level, TBA reactivity (red pigment nmol/kg $\cdot$ d) was obtained from the daily sum of the reactant level within a single day and plotted over a period of days.

Determination of Malonaldehyde Derivatives by DNPH Malonaldehyde derivatives were determined as 1-(2,4-dinitrophenyl)pyrazole (DNPP) using DNPH, according to the published method^{31,32)} with slight modifications.²⁷⁾ The reference standard, DNPP, was prepared as described elsewhere.³³⁾ Thus, a mixture of 1.5 ml of urine or an aqueous solution containing 20 nmol TMP/ml, and 1.2 ml 0.25% (w/v) DNPH solution in 1 N HCl was heated at 100 °C for 30 min. The reaction mixture was extracted with chloroform as described³³⁾ and the extract subjected to HPLC using a mobile phase composed of acetonitrile/0.01 N HCl (45:55, v/v) at a flow rate of 1.5 ml/min. The peak due to DNPP appeared at a retention time of 7.0 min when detected at 300 nm. The malonaldehyde content of urine was determined by comparing the peak height of DNPP with that of the TMP standard solution.

The amount of malonaldehyde derivatives excreted (nmol/kg $\cdot$ h) was determined by dividing the amount in urine by the body weight (kg) and by the interval (h) between two urine collections.

Determination of Creatinine Urine creatinine concentrations (mg/l) were determined according to the established method using picric acid. Creatinine excretion (mg/kg $\cdot$ h) was calculated by dividing the amount of creatinine in urine by the body weight (kg) and by the interval (h) between two urine collections.

RESULTS

Urine samples were collected from six male and four female healthy Japanese aged 21–53, and the TBA reactant levels (red pigment nmol/ml) were determined by HPLC. In order to monitor the variation in urinary TBA reactant level within a single day, the TBA reactant level (red pigment nmol/kg $\cdot$ h) was calculated. In order to monitor the daily variation the TBA reactant level (red pigment nmol/kg $\cdot$ d) was calculated.

Subject HE (21-Year-Old Female) During the experimental period for successive 7 d, she was instructed to keep to her own usual life-style. She worked moderately in the daytime and slept well during the night; her diet was changed from “regular” Japanese food to V or M for two successive days. Her urinary TBA reactant level within a day and the daily reactant level during the experimental period are shown in Fig. 1. Every day her reactant level in the afternoon was 2–3 fold higher than in the morning. Her daily reactant level for the 7 successive days exhibited a 2-fold variation. Her creatinine output remained constant throughout the experimental period.

Subject MF (22-Year-Old Female) During the experimental period for 4 successive days, she kept to her own usual life-style consuming a V or M diet. Every day her urinary reactant level in the afternoon was higher than in the morning (Fig. 2). Her daily reactant level exhibited a 2–3 fold variation.

Subject HK (52-Year-Old Female) Two series of experiments for successive periods of 8 and 7 d were carried out in different months. On the first 5 d of the series 1 experiment, she kept to her own usual life-style consuming a M or V diet. Her reactant level in the afternoon were 2–3 fold higher than in the morning (Fig. 3, series 1). On

Fig. 1. Urinary TBA Reactant Levels in Subject HE (21-Year-Old Female)

Every day she worked moderately in the daytime and slept soundly at night. She consumed a V or M diet for the last 4 d. Creatinine output of the subject was constant at 40–50 mg/h and 1–1.2 g/d every day.

the 6th day she remained awake all night and continued working at her desk. The reactant level in the afternoon increased by a factor of 5.5. On the 7th and 8th days she kept to her own usual life-style and the increased level in the afternoon fell. The daily TBA reactivity on the 6th day was three times as high as those seen on the other days of the series 1 experiment.

During the series 2 experiment she kept to her own life-style eating "regular" Japanese food. Every day her reactant levels in the afternoon were higher than in the morning, and her daily reactivity exhibited a 2-fold variation during this period (Fig. 3, series 2).

Subject MM (24-Year-Old Male) Two series of experiments for successive periods of 5 and 3 d were performed. Throughout the series 1 experiment he kept working as usual. On most days, his reactant level in the afternoon or in the evening was higher than in the morning (Fig. 4, series 1). His daily reactant level remained constant at low levels. In the series 2 experiment, one year after the series 1 experiment, he worked as usual in the daytime except that he played some vigorous tennis in the morning of the 2nd day. His reactant level in the afternoon or in

the evening was higher than in the morning throughout the series 2 experiment (Fig. 4, series 2). His reactant level at around noon on the 2nd day exhibited a dramatic 22-fold increase whereas his creatinine level increased slightly at noon, and his daily reactant level on the 2nd day was 7-fold higher.

Subject KH (30-Year-Old Male) Three series of experiments for successive periods of 3, 4 and 2 d were

Fig. 2. Urinary TBA Reactant Levels in Subject MH (22-Year-Old Female)

She kept her own usual life style taking food V or M.

Fig. 4. Urinary TBA Reactant Levels in Subject MM (24-Year-Old Male)

The series 2 experiment was performed one year after the series 1 experiment. He played tennis in the morning of the 2nd day of the series 2 experiment. On the other days he kept to his own normal life-style. In the series 2 experiment his creatinine output was kept at 35–45 mg/h except for the higher level of 50 mg/h seen at around noon on the second day.

Fig. 3. Urinary TBA Reactant Levels in Subject HK (52-Year-Old Female)

On the 6th day of the series 1 experiment she worked all night without sleeping, and on the other days she kept to her own normal life-style. She consumed a V or M diet on the first 6 d of the series 1 experiment.

Fig. 5. Urinary TBA Reactant Levels in Subject KH (30-Year-Old Male)

The series 2 and 3 experiments were performed one year after the series 1 experiment. He kept to his own normal life-style except for taking beer at the indicated times.

Fig. 6. Urinary TBA Reactant Levels in Subject KK (53-Year-Old Male)

The series 2 experiment was performed one year after the series 1 experiment. He was a smoker and kept to his own normal life-style except for taking beer on the 3rd day of the series 1 experiment and stopping smoking on the last 3 d of the series 2 experiment.

TABLE I. Comparison of the Daily Urinary TBA Reactant Levels in 10 Japanese Subjects

Subject	Sex	Age	Number of experiment	The daily TBA reactant level Red pigment, nmol/kg·d			
				Minimum	Maximum	Average $\pm$ S.D.	
HE	Female	21	7	23	44	37.4 ± 7.7	Fig. 1
YI	Female	22	4	42	78	56.5 ± 15.2	
MF	Female	22	4	30	80	42.5 ± 16.5	Fig. 2
HK	Female	52	15	43	145 ^{a)}	86.6 ± 32.0	Fig. 3
NK	Male	23	3	48	125	75.3	
MM	Male	24	8	19	145 ^{b)}	48.6 ± 42.2	Fig. 4
KA	Male	25	2	23	38	30.5	
KH	Male	30	9	31	111	56.0 ± 26.1	Fig. 5
MB	Male	44	4	36	71	51.5 ± 14.5	
KK	Male	53	8	80	162	103.8 ± 26.2	Fig. 6

Minimum and maximum levels under normal life-style conditions except that the maximum obtained after staying awake all night^{a)} and the maximum obtained after vigorous exercise^{b)} are listed.

performed in different months over two years. He kept to his own usual life-style except for taking beer in the evening of the 3rd day of the series 1, the 1st and the 2nd days of

the series 2 and the 1st day of the series 3 experiments. On most days, his reactant level in the afternoon or in the evening was higher than in the morning (Fig. 5). His daily

Fig. 7. Comparison of the Urinary TBA Reactant Levels (Δ) with the Amount of Malonaldehyde Derivatives Excreted into Urine ($\blacktriangle$)

The amounts of TBA red pigment and malonaldehyde derivatives determined by the DNPH method in the urine of subject HK are compared.

reactant level exhibited 3-fold variation. It is unlikely that taking an alcoholic drink affected the reactant level throughout the three series of experiments.

Subject KK (53-Year-Old Male) Two series of experiments for successive periods of 3 and 6 d were performed in different years. This subject had smoked for many years and he kept to his usual life-style except for taking beer in the evening of the 3rd day of the series 1 experiment and stopping smoking during the last 3 d of the series 2 experiment. Every day his reactant level in the afternoon or in the evening was higher than in the morning (Fig. 6). The daily reactant levels exhibited a 2-fold variation.

The daily TBA reactant levels of 10 subjects, including the 6 subjects described above, are listed in Table I. The daily reactant level of each subject maintaining a normal life-style exhibited a 1.5–3-fold variation, while the variation in the subjects staying awake all night was 3-fold and after hard exercise 7-fold.

In order to confirm that the urinary TBA reactant level always reflected the amount of malonaldehyde derivatives in urine, the TBA reactant level and the malonaldehyde level estimated by the DNPH method were compared in urine collected at any time within a day (Fig. 7). It was found that the amount of red pigment, nmol/kg·h, and the amount of malonaldehyde derivatives, nmol/kg·h, in any urine samples collected throughout a day were equal. Hence, the TBA reactant level in urine determined in this way was due exclusively to malonaldehyde derivatives.

DISCUSSION

Measurement of Urinary TBA Reactant Levels as an Index of Lipid Peroxidation in the Whole Body TBA reactant levels in human plasma or serum have been reported to be affected by age,^{3–6} sex,^{5,6} exercise,⁷ pregnancy¹⁰ and disease,^{8,9} such as diabetes, liver disease⁶ and alcoholism.¹¹ The reactant levels in plasma or serum varied from 0.5 to 50 nmol red pigment/ml in

these reports. However, there are three disadvantages associated with the measurement of reactant levels in plasma or serum. The first is that the reactivity is influenced greatly by the type of method employed: nature of acid used in the reaction and the procedure used to determine the red pigment, *i.e.*, direct spectrometry,^{3,12–14} direct fluorometry,^{4,6–11,15} HPLC-absorption spectrometry^{5,16–18} and HPLC-fluorometry.¹⁹ According to the Yagi method involving direct fluorometry, the normal level of red pigment was estimated to be 3–4 nmol/ml.⁹ The second disadvantage is that the TBA reactants are not well characterized^{20,21} while the third is that the TBA reactants which exist in the circulating plasma may be readily excreted into urine.^{22–27} In spite of these disadvantages, the TBA reactant level in plasma or serum has been used for the interpretation of many disorders related to lipid peroxidation.

Few studies on the TBA reactant levels in human urine have been reported. Because human urine yields at least three pigments produced by the TBA reaction, 455 nm yellow, 532 nm red and 518 nm orange pigments, the urinary red pigment should be estimated by HPLC-spectrometry.^{27–30} The formation of the pigment is affected little by the nature of the acid used as long as HPLC-spectrometry is employed.²⁷ Furthermore, urinary TBA reactant levels have been found to be due to adducts of malonaldehyde with amino acids or amines,^{22–27} and the amount of red pigment is similar to that of the malonaldehyde derivatives estimated by the DNPH method as shown in a previous study²⁷ and in the present one. Because the urinary TBA reactants may be the final metabolites of lipid peroxidation in the whole body, measuring urinary TBA reactant levels may offer more advantages compared with plasma or serum levels in studying lipid peroxidation in the whole body. However, the possibility that urinary TBA reactant levels reflect kidney metabolism cannot be excluded.

Expression of Urinary TBA Reactant Levels Urinary TBA reactant levels are expressed by the concentration of red pigment in nmol/ml urine. However, this expression is not adequate because the volume of urine excreted varies and thus the concentration of TBA reactants may vary with the volume of urine. The daily TBA reactant level, red pigment in nmol/d,^{34,35} or the reactant level normalized by creatinine output, red pigment in nmol/mg creatinine,³⁰ may be better. When the creatinine output of a subject was kept almost constant, the TBA reactant level of that subject exhibited a 2-fold variation (Fig. 1). In the present study, we adopted two expressions: the TBA reactant level within a day, red pigment in nmol/kg·h, and the daily TBA reactant level, red pigment in nmol/kg·d, which may reflect the urinary TBA reactant level more accurately.

Estimation of Urinary TBA Reactant Levels Measurement of urinary TBA reactant levels in relation to the increased intake of dietary *n*-3 fatty acids has been carried out by several groups. Piche *et al.*³⁴ have reported that the daily reactant level in subjects fed a normal diet (0.69–1.31 μ mol/d) was slightly increased by consuming *n*-3 fatty acids for 50 d, but they concluded that this may be caused by the lipid peroxidation products formed in

food before its consumption. Nelson *et al.*³⁵⁾ have reported that the daily reactant level (4–6 $\mu\text{mol/d}$) was slightly increased by consuming *n*-3 fatty acids for 40 d. It has been shown by Knight *et al.*³⁰⁾ that the reactant level, normalized relative to creatinine output (0.18–1.38 nmol/mg creatinine), in male urine is higher than that in female urine.

Our previous paper²⁷⁾ demonstrated that the daily urinary TBA reactant level (26–95 nmol/kg $\cdot$ d) was not dependent on age and sex. In the present study, we have found that the reactant level in each subject within a day exhibited a 2–3-fold variation and the daily level exhibited a 1.5–3-fold variation, when the subject kept to his usual life-style. Because of a large inter-individual variation in the reactant level of the 10 subjects tested in the present study, comparison of the reactant levels between subjects is difficult. One of the factors causing an increase in the reactant level may be physical activity, because the reactant level in all the subjects in the afternoon or in the evening was higher than in the morning. The average daily TBA reactant level in the previous 12 Japanese subjects studied²⁷⁾ and the present 10 Japanese subjects (the total number of determinations was 92), was 50 ± 40 nmol red pigment/kg $\cdot$ d. The amounts of TBA reactants excreted daily in urine corresponded to 20–25% of the reactants in the whole body plasma calculated from the reactant level in plasma (3 nmol red pigment/ml).⁹⁾ Hence, it is suggested from this evidence that a large amount of TBA reactants is removed daily from the circulation into the urine.

It is likely that stress and exercise cause an increase in urinary TBA reactant levels, because the TBA reactant levels of a subject after remaining awake all night or performing hard exercise are dramatically increased. The reactant levels within a day for a subject increased 5.5-fold several hours after all night, and the daily level increased 3-fold the day after remaining awake all night (Fig. 3, series 1). The reactant level within a day for a subject increased 22-fold several hours after performing hard exercise, and the daily level increased 7-fold when compared with the level on the day before the hard exercise (Fig. 4, series 1 and 2). Because of large individual variations in the urinary TBA reactant levels, the effect of stress and exercise on the levels should be carefully monitored with many different individuals and under a variety of conditions using a strictly controlled experimental design. In the present study, the type of food taken for short periods, alcohol and smoking affected the urinary TBA reactant levels to some extent.

Excretion of the TBA reactants into urine may be rapid and occur within half a day after physical activity and stress. Increased physical activity and stress may rapidly enhance lipid peroxidation in the whole body, and lipid peroxidation products, consisting of malonaldehyde derivatives, may be rapidly removed from the blood circulation into the urine. While it has been shown that vigorous exercise causes a rapid but minor (20%) rise in the TBA reactant level in plasma,⁷⁾ we found in the present study that such exercise causes a rapid and more dramatic

increase in the urinary TBA reactant level.

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