Change in tissue concentrations of lipid hydroperoxides, vitamin C and vitamin E in rats with streptozotocin-induced diabetes

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ABSTRACT

The tissue concentration of lipid hydroperoxides, which was determined by a specific method involving chemical derivatization and HPLC, increased significantly in the heart, liver, kidney and muscle of diabetic rats 8 weeks after the intraperitoneal injection of streptozotocin compared with that of the control group. These results demonstrate that an enhanced oxidative stress is caused in these tissues by diabetes. Vitamin C concentrations of the brain, heart, lung, liver, kidney and plasma of the diabetic rats decreased significantly after 8 weeks compared with those of the control group. Vitamin E concentrations of the brain, heart, liver, kidney, muscle and plasma of the diabetic rats increased significantly after 4 weeks compared with the control group. After 8 weeks, an elevation in vitamin E concentration was observed in the heart, liver, muscle and plasma of the diabetic rats.

INTRODUCTION

Diabetes mellitus is characterized by a series of complications that affect many organs. Oxygen free radicals have been implicated in the pathogenesis of diabetes mellitus as well as its complications [1]. Glucose autoxidation and non-enzymic protein glycation are shown to produce oxygen free radicals [2,3]. In spite of the spreading interest in radical reactions, the method to evaluate oxidative stress is still controversial. The most commonly used indicator of lipid peroxidation is TBARS (thiobarbituric acid-reactive substances) [4]. TBARS has limited usefulness, even for the peroxidation of a simple oil [5]. Recently we developed a specific and sensitive method to determine the total level of lipid hydroperoxides involving chemical conversion of lipid hydroperoxides into aromatic phosphine oxide followed by measurement of the oxide with HPLC [6]. In this paper, we applied the method to evaluate the oxidative stress in diabetic rat tissues.

On the other hand, a variety of antioxidants scavenge free radicals and prevent oxidative damage to biological structures. The primary defence against oxidative stress in the cell rests with antioxidants, including vitamins C and E, glutathione (GSH), etc. [7]. There is evidence of a decrease in the concentration of vitamin C in the plasma, liver and kidney of diabetic rats [8–15]. Concerning the level of vitamin E in plasma, kidney, liver and heart ventricle during experimental diabetes, controversial results have been reported [11,13–19]. We therefore determined the concentration of vitamins C and E in seven tissues of rats with experimentally induced diabetes to investigate systematically the effect of diabetes on these vitamins.

Key words: diabetes, hydroperoxide, vitamin C, vitamin E.
Abbreviation: TBARS, thiobarbituric acid-reactive substances.
Correspondence: Dr Shosuke Kojo.
MATERIALS AND METHODS

Animals
Guidelines from the Prime Minister’s Office of Japan (No. 6 of 27 March 1980) for the care and use of laboratory animals were followed. Six-week-old male rats (SLC: Wistar strain) were obtained from Japan SLC Co. (Hamamatsu, Shizuoka, Japan). Animals were fed commercial laboratory chow (MF, Oriental Yeast Co., Osaka, Japan) and water ad libitum. Diabetes was induced in overnight-fasted rats by intraperitoneal injection of streptozotocin at a dose of 40 mg/kg body weight dissolved in citrate buffer (0.1 M, pH 4.5). Control rats received an equivalent amount of the buffer. The animals were housed in a room with a temperature of 24 ± 2 °C and a 12-h light/12-h dark cycle. Body weights were recorded daily.

Analytical methods
After fasting overnight, rats were anaesthetized with diethyl ether and killed by collecting the blood from the inferior vena cava using a syringe containing sodium heparin as an anticoagulant. After perfusion of ice-cold saline through the portal vein, organs were removed. The excised tissue was homogenized in 5 volumes of PBS (10 mM, pH 7.4) under ice cooling. All determinations were made in duplicate experiments with 4 to 7 animals in each group. Lipid hydroperoxide was measured as described [6]. The determination of vitamin C was made according to a specific and sensitive method [20,21] involving chemical derivatization and HPLC. The concentration of α-tocopherol (vitamin E) was determined by HPLC [22]. The conditions of HPLC and fluorescence detection (Shimadzu RF-535, Kyoto, Japan) were reported previously [5]. Blood was centrifuged at 13000 g for 5 min at 4 °C to separate plasma. Plasma glucose was measured using a commercially available diagnostic kit (Wako Pure Chem. Co., Osaka, Japan).

Protein concentrations were determined according to the method of Lowry et al. [23] using BSA as the standard.

Data were expressed as means ± S.D. and analysed by ANOVA using StatView software (Abacus Concepts, Berkeley, CA, U.S.A.). Differences between group means were considered significant at P < 0.05 using the Bonferroni/Dunn Procedure generated by this program.

RESULTS

Body weight
At the start of the experiment, the body weight of the control group was 134.7 ± 10.2 g and that of the diabetic group was 136.6 ± 11.3 g. The body weight of the control rats increased steadily and the rate of the increase was much higher than that of the diabetic rats. Already after 1 week, a significant difference (P < 0.01) between the two groups was observed (182.8 ± 12.0 g and 162.5 ± 18.9 g for the control and diabetic groups respectively). After 4 weeks, the body weight of the diabetic group was 166.1 ± 21.3 g and started decreasing, whereas that of the control group (256.3 ± 17.2 g) was significantly higher (P < 0.01). After 8 weeks, the body weight of the diabetic rats was 123.9 ± 11.1 g which was also significantly lower (P < 0.01) than that of the control rats (297.2 ± 21.8 g). The profile of body weight change was similar to that reported previously [24].

Plasma glucose
The plasma glucose levels of the control rats at 4 and 8 weeks were 158.1 ± 11.2 and 161.2 ± 26.3 mg/dl respectively. The levels in the diabetic rats were 539.7 ± 66.3 and 688.7 ± 138.1 mg/dl respectively, significantly higher (P < 0.01) than those of the control group.

Concentration of tissue lipid hydroperoxides
The concentrations of tissue lipid hydroperoxides are shown in Table 1. Lipid hydroperoxide levels in the liver and kidney of the diabetic rats were already significantly higher than those of control rats at 4 weeks. Hydroperoxide concentrations of the liver, kidney, heart and muscle of diabetic rats were significantly higher than those of the control group at 8 weeks.

Significant differences were not observed in the brain or lung after 4 and 8 weeks of diabetes (Table 1). Significant differences were not observed between control groups at 4 and 8 weeks.

Vitamin C
The tissue concentrations of vitamin C in diabetic rats were significantly decreased 4 weeks after the injection of streptozotocin in plasma, heart, lung, liver and kidney compared with the control group (Table 2). After 8 weeks, the level of vitamin C in the brain of diabetic rats also decreased significantly compared with the control group (Table 2). The vitamin C concentration of muscle was not affected by diabetes for 8 weeks (Table 2).

Vitamin E
Among the tocopherols (α, β, γ and δ), only the α-homologue was present in rat tissues of the present experiment. At 4 weeks, the vitamin E concentration of diabetic rats increased significantly in plasma, brain,
Table 1  Levels of lipid hydroperoxides in the control and diabetic rats after 4 and 8 weeks
Diabetes was induced in male Wistar rats by an intraperitoneal injection of streptozotocin (40 mg/kg body weight). After 4 and 8 weeks, tissue concentrations of lipid hydroperoxides were determined as described in the text. Values are means ± S.D. The number of rats is shown in parentheses. Asterisks indicate significant difference from the corresponding control (ANOVA Bonferroni/Dunn Procedure, *P < 0.05 and **P < 0.01).

<table>
<thead>
<tr>
<th>Lipid hydroperoxides (pmol/mg protein)</th>
<th>After 4 weeks</th>
<th>After 8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diabetes (6)</td>
<td>Control (7)</td>
</tr>
<tr>
<td>Brain</td>
<td>140.1 ± 62.8</td>
<td>125.7 ± 40.0</td>
</tr>
<tr>
<td>Heart</td>
<td>523.1 ± 114.1</td>
<td>601.2 ± 98.8*</td>
</tr>
<tr>
<td>Lung</td>
<td>348.7 ± 138.0</td>
<td>255.9 ± 108.6</td>
</tr>
<tr>
<td>Liver</td>
<td>407.2 ± 81.9*</td>
<td>396.4 ± 69.8**</td>
</tr>
<tr>
<td>Kidney</td>
<td>338.2 ± 54.2*</td>
<td>317.8 ± 25.0**</td>
</tr>
<tr>
<td>Muscle</td>
<td>155.8 ± 74.5</td>
<td>240.7 ± 106.2*</td>
</tr>
</tbody>
</table>

Table 2  Tissue concentrations of vitamin C in the control and diabetic rats after 4 and 8 weeks
Diabetes was induced in male Wistar rats by an intraperitoneal injection of streptozotocin (40 mg/kg body weight). After 4 and 8 weeks, tissue concentrations of vitamin C were determined as described in the text. Values are means ± S.D. The number of rats is shown in parentheses. Asterisks indicate significant difference from the corresponding control (ANOVA Bonferroni/Dunn Procedure, *P < 0.05 and **P < 0.01).

<table>
<thead>
<tr>
<th>Vitamin C (nmol/g tissue)</th>
<th>After 4 weeks</th>
<th>After 8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>1996 ± 205 (6)</td>
<td>1514 ± 369* (5)</td>
</tr>
<tr>
<td>Heart</td>
<td>421.0 ± 72.5 (6)</td>
<td>325 ± 0.5** (4)</td>
</tr>
<tr>
<td>Lung</td>
<td>1209 ± 176 (6)</td>
<td>246 (7) ± 81.5** (4)</td>
</tr>
<tr>
<td>Liver</td>
<td>912.9 ± 148.0 (6)</td>
<td>262 ± 16.5** (4)</td>
</tr>
<tr>
<td>Kidney</td>
<td>152.1 ± 14.0 (4)</td>
<td>116.3 ± 54.5 (5)</td>
</tr>
<tr>
<td>Muscle</td>
<td>34.4 ± 5.7 (5)</td>
<td>20.2 ± 6.4** (5)</td>
</tr>
<tr>
<td>Plasma</td>
<td>57.8 ± 22.9</td>
<td>32.1 ± 7.1</td>
</tr>
</tbody>
</table>

Table 3  Tissue concentrations of vitamin E in the control and diabetic rats after 4 and 8 weeks
Diabetes was induced in male Wistar rats by an intraperitoneal injection of streptozotocin (40 mg/kg body weight). After 4 and 8 weeks, tissue concentrations of vitamin E (α-tocopherol) were determined as described in the text. Values are means ± S.D. The number of rats is shown in parentheses. Asterisks indicate significant difference from the corresponding control (ANOVA Bonferroni/Dunn Procedure, *P < 0.05 and **P < 0.01).

<table>
<thead>
<tr>
<th>Vitamin E (nmol/g tissue)</th>
<th>After 4 weeks</th>
<th>After 8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>37.5 ± 4.8* (6)</td>
<td>39.6 ± 6.2 (5)</td>
</tr>
<tr>
<td>Heart</td>
<td>28.8 ± 6.5** (6)</td>
<td>53.8 ± 13.4** (6)</td>
</tr>
<tr>
<td>Lung</td>
<td>16.0 ± 5.6 (6)</td>
<td>16.3 ± 4.5 (6)</td>
</tr>
<tr>
<td>Liver</td>
<td>44.4 ± 10.1** (5)</td>
<td>95.8 ± 23.0** (6)</td>
</tr>
<tr>
<td>Kidney</td>
<td>13.0 ± 1.6** (6)</td>
<td>15.4 ± 5.4 (6)</td>
</tr>
<tr>
<td>Muscle</td>
<td>24.0 ± 4.8** (6)</td>
<td>30.8 ± 1.9** (6)</td>
</tr>
<tr>
<td>Plasma</td>
<td>16.8 ± 3.2** (5)</td>
<td>80.7 ± 49.9**</td>
</tr>
</tbody>
</table>

Heart, liver, kidney and muscle compared with that of the control rat (Table 3). The vitamin E concentration of the lung was not changed by diabetes. After 8 weeks, significant differences between the control and diabetic groups in tissue vitamin E were observed for plasma, heart, liver and muscle (Table 3). A significant difference was not observed for vitamin E level in the brain, lung and kidney after 8 weeks (Table 3). Significant differences were not observed between control groups at 4 and 8 weeks.
**DISCUSSION**

**Increase of tissue lipid hydroperoxides as an index of oxidative stress**

Although radical reactions receive much attention in relation to pathogenic disorders such as diabetes mellitus [1], atherosclerosis [25], cancer [25], ageing [26] and so on [25], the search for a reliable indicator of lipid peroxidation in animal tissues is still an important activity. Conventional indicators of radical reactions are classified into three main categories, which are products of lipid peroxidation such as malondialdehyde [27], TBARS [4], modified proteins [28] and DNA [29], decreased antioxidants like vitamins C [20] and E [5], and GSH [30], and activity change of antioxidant enzymes including superoxide dismutase [31] and glutathione peroxidase [32]. As another kind of index, the oxidative mediator, rather than the products of peroxidation, may be determined. Lipid hydroperoxide is a probable candidate for such an oxidative mediator, because it is formed by radical reactions, and has sufficient lifetime to migrate and generate reactive radicals to peroxidate protein and DNA.

The determination of lipid hydroperoxides has been hampered by their instability and extremely low levels. The chemiluminescent detection [33] of hydroperoxides combined with the separation of the lipid components with HPLC is very sensitive, but cannot be applied to the most fundamental determination, i.e. to the quantification of total hydroperoxides in biological samples, because endogenous compounds such as ubiquinol and tocopherols interfere seriously with the chemiluminescence reaction [34].

Recently we developed a specific and sensitive method [6] to determine the level of lipid hydroperoxides in animal tissues. The efficiency of lipid hydroperoxides as an index of oxidative stress has been confirmed by their increase in a typical case of enhanced oxidative stress such as aged [35], vitamin C-deficient [21] and vitamin E-deficient [36] animal tissues.

In the diabetic rat, the lipid hydroperoxide concentration in the liver and kidney was increased 4 weeks after the injection of streptozotocin (Table 1). This suggests that these organs are sensitive to the diabetic condition and that hydroperoxides, a mediator of radical reactions, increased after only 4 weeks. Elevation of renal TBARS, i.e. aldehydic products, in diabetic rats was reported by Kakkar et al. [37]. These results support the view that enhanced oxidative stress is involved in the aetiology of diabetic nephropathy.

After 8 weeks, lipid hydroperoxides increased in the heart and muscle along with the liver and kidney. These results support the hypothesis that radical reactions are a factor causing cardiomyopathy, which often develops in patients with diabetes [38]. This idea is consistent with reports that have described an increase of TBARS in the heart, aorta and the heart ventricles of diabetic rats [18,24]. For patients with Type 2 (non-insulin-dependent) diabetes mellitus, an increase in the plasma hydroperoxide level was reported [39].

**Change in the concentration of vitamin C**

Ascorbate has received much attention as a reducing agent since its discovery and recently it has been recognized as an outstanding plasma antioxidant [40]. In diabetic rats, a decrease of vitamin C in plasma [8–11,13,15], liver [8,12,15] and kidney [8,14] has been reported. In the present experiment, vitamin C concentrations in the brain, heart, lung, liver, kidney and plasma of diabetic rats were shown to decrease significantly (Table 2). These results suggest that tissue vitamin C in diabetic rats decreases in the whole body, although ascorbate is not a vitamin to Wistar rats unlike the inherently scorbutic rats [21]. The decrease in vitamin C may be ascribed to its enhanced consumption by elevated oxidative stress caused by diabetes as evidenced by augmentation of tissue lipid hydroperoxides as described above. The decrease may also be due to the reduced activity of glucose-6-phosphate dehydrogenase which produces NADPH to regenerate ascorbate from dehydroascorbate as suggested by Bode at al. [12]. However, this point remains to be explored, since the contribution of the enzyme in the metabolism of vitamin C is not clear at present. It is conceivable that the decrease in vitamin C leads further to the enhancement of radical reactions, and vice versa.

**Change in the concentration of vitamin E**

Vitamin E is a major lipid-soluble chain-breaking antioxidant. Unlike vitamin C, contradictory results have been reported concerning the plasma vitamin E concentrations in diabetes. For example, the vitamin E level in diabetic rat plasma was reported to increase [16,19], decrease [13] or to be unchanged [11]. This is also the case for the plasma vitamin E concentration in patients with diabetes [39,41–43]. The discrepancies among reports seem to be explained on the grounds that vitamin E concentration depends on the experimental conditions such as duration and stage of diabetes.

In the present experiment, vitamin E concentrations in brain, heart, liver, kidney, muscle and plasma increased after 4 weeks, i.e. at an early stage of diabetes. The dose of streptozotocin was 40 mg/kg body weight, which was less than that used in previous reports [8–14,16–19]. The elevation of tissue tocopherol may be partly explained by the fact that plasma vitamin E may be increased by mobilization with lipids from the liver resulting from hyperlipidaemia accompanied by diabetes.

After 8 weeks, the vitamin E level in plasma, muscle,
liver and heart of the diabetic rat was still increased, whereas in other tissues it did not increase significantly. In the brain and kidney, a significant difference between the diabetic and control groups at 4 weeks disappeared after 8 weeks. This may be caused by increased consumption of the vitamin by prolonged oxidative stress of diabetes.

In summary, we report that tissue lipid hydroperoxides, a mediator of radical reactions, increase in the heart, liver, kidney and muscle of diabetic rats and this result supports a view that radical reactions are involved in diabetic complications such as nephropathy and cardiomyopathy. The vitamin C level in the brain, heart, lung, liver, kidney and plasma of diabetic rats decreased and this could be evidence of enhanced oxidative stress in diabetes. In an early stage of diabetes, vitamin E concentrations in plasma, brain, heart, liver, kidney and muscle were found to increase. The tissue level of vitamin E seemed to depend on the stage of diabetes.

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