New biomarkers for oxidative damage, were used to identify whether hyperglycemia caused oxidative stress in diabetic Akita mice. At 13 weeks of age, the tissues of these mice were obtained, and the levels of N\(^{\text{e}}\)-(hexanonyl)lysine (HEL) and dityrosine (DT) were measured, these being related to lipid peroxide-derived protein covalent modification and protein cross-linking. The levels of HEL and DT in the kidneys of Akita mice were significantly increased compared with the control mice without any accumulation of thiobarbituric acid reactive substances and 4-hydroxy-2-nonenal-modified protein. Immunopositive staining was clearly observed in the kidneys of the Akita mice when using the anti-HEL antibody or anti-DT antibody. These results suggest that hyperglycemia in Akita mice induced oxidative stress and increased these markers in the kidneys.

**Key words:** diabetes; oxidative stress; N\(^{\text{e}}\)-(hexanonyl)lysine; dityrosine; lipid peroxidation

The excessive production of reactive oxygen species (ROS) is thought to cause cellular damage *in vivo*. This damage is implicated in the process of many diseases. To evaluate the extent of oxidative stress, some oxidative stress markers have been developed and are recognized as useful;\(^{10}\) for example, 8-hydroxydeoxyguanosine, which is produced by the oxidation of DNA, is commonly used as a marker of oxidative DNA damage. HNE (4-hydroxy-2-nonenal) is one of the typical aldehyde products, and is also used as a marker of lipid peroxidation. N\(^{\text{e}}\)-(hexanonyl)lysine (HEL)\(^{2,3}\) and dityrosine (DT)\(^{4,5}\) have recently been developed as new biomarkers of oxidative stress and have been detected in the development of such diseases as atherosclerosis.

Lipid hydroperoxides are formed during the initial stage of lipid peroxidation. During the process of membrane lipid peroxidation, various aldehydes are subsequently formed as the final products after lipid hydroperoxides have been broken down. HNE is formed by the lipid peroxidation of omega-6 unsaturated fatty acids, and HNE-modified biomolecules are accumulated in some tissues in various diseases.\(^{1}\) Kato *et al.* have reported that HEL was formed by the reaction of 13-hydroperoxyoctadecadienoic acid, a model for lipid hydroperoxides, with a lysine residue during the early stage of lipid peroxidation. They confirmed the presence of HEL in oxidized LDL by using the specific anti-HEL antibody. Furthermore, positive staining was immunohistochemically observed in human atherosclerotic lesions.\(^{2}\) HEL has also been detected after exercise-induced oxidative stress in rat skeletal muscle.\(^{3}\) These results indicate that HEL could be an early marker of oxidative damage to biological molecules *in vivo*.

The oxidation of tyrosine generates a tyrosyl radical, and DT is then formed by the reaction of two tyrosyl radicals. DT is formed by ROS, enzymatic reactions, UV irradiation and lipid peroxidation.\(^{6-11}\) DT has been immunohistochemically detected in lipofuscin from an aged human brain\(^{9}\) and in atherosclerotic lesions of the aorta from Apo-E deficient mice.\(^{9}\) These results suggest that DT could be a useful marker for evaluating protein cross-linking caused by oxidative stress.

The "Akita mouse" is characterized as a spontaneously diabetic model with onset at an early age. Diabetes of the Akita mouse is inherited by an autosomal dominant trait, and characterized by a decrease in the
number of β-cells of the pancreatic islet. Furthermore, mutation of the insulin2 gene is clearly demonstrated in the Akita mouse, and is recognized as a major factor of hyperglycemia of this animal. Hydronephrosis has also been commonly observed in the kidneys of Akita mice. Hyperglycemia causes the autoxidation of glucose, glycation of proteins and activation of polyol metabolism. These events accelerate the generation of ROS, and result in an increase in the oxidative modification of lipids, DNA and proteins in various tissues. Oxidative stress may play an important role in the development of complications in diabetes. Ihara et al. have reported oxidative stress in the pancreas of diabetic GK rats. However, it has not been investigated whether Akita mice encounter oxidative stress resulting in the oxidative modification of biomolecules caused by hyperglycemia.

In this study, we investigate the relationship between hyperglycemia and oxidative stress by using hyperglycemic Akita mice. We show here for the first time that oxidative stress had already occurred in the kidneys of hyperglycemic Akita mice at 13 weeks of age. Oxidative stress was immunochemically detected as lipid hydroxide-modified protein with the anti-HEL antibody and as protein DT with the anti-DT antibody.

Akita mice (male, 5-week-old; Japan SLC, Hamamatsu, Japan) and C57BL/6CrSlc mice (male, 5-week-old; Japan SLC, Hamamatsu, Japan) were maintained until 13 weeks of age in accordance with the Guidelines for Animal Experimentation of Nagoya University. These mice were allowed free access to tap water and stock feed (CE-2, CLEA Japan, Tokyo, Japan) for 8 weeks. The mice were killed under anesthesia with diethyl ether after 8 weeks of feeding, and the serum and tissues (liver and kidneys) were obtained. No significant difference in growth was apparent between the Akita and control mice. There was, however, a significant increase in serum glucose in the Akita mice (at 6 weeks, 455.92 ± 117.8 g/dl; at 8 weeks, 511.93 ± 76.69 g/dl; at 10 weeks, 526.4 ± 61.80 g/dl) compared to the control group (at 6 weeks, 135.42 ± 18.88 g/dl; at 8 weeks, 168.91 ± 74.20 g/dl; at 10 weeks, 137.00 ± 18.01 g/dl). During the course of the experiment, the serum glucose concentration of the Akita mice was above 400 mg/dl, while in the control mice, it was less than 170 mg/dl. To investigate the extent of lipid peroxide-derived protein covalent modification in hyperglycemia, we firstly evaluated the HEL level in the kidneys of Akita mice by an enzyme-linked immunosorbant assay (ELISA) using the anti-HEL antibody. Each tissue sample (liver and kidney) was homogenized in 9 volumes of a 100 mM phosphate buffer (pH 7.0) with a Potter-Elvehejm device. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C and used as the sample for ELISA. Fifty µl of a sample (0.01 mg of protein/ml) was pipetted into each well of a microtiter plate and kept at 4°C overnight, and the HEL level then measured as described by Kato et al. The HEL level in the kidneys of the Akita mice was significantly higher than in those of the control mice (Fig. 1(A)). This result shows that hyperglycemia induced the production of lipid hydroperoxide-modified protein in the kidneys of the Akita mice. Furthermore, we examined the localization of HEL in the kidneys of the Akita mice. A part of the kidney was fixed in 4% paraformaldehyde, before the kidney specimen was embedded in paraffin and sectioned. The sections were then deparaffinized with xylene and ethanol. To detect the HEL-modified protein, the avidin-biotin complex (ABC) method was performed with a commercial kit (VECTASTAIN Elite ABC KIT, Vector Laboratories, Burlingame, CA, U.S.A.). The positive reactions were visualized by incubating with a commercial peroxidase substrate solution (FIRST DAB, Sigma) containing diaminobenzidine, urea, and H2O2. Nuclei were counterstained with methyl green. Immunopositive staining with a granular pattern in the cytoplasm of renal proximal tubules was clearly observed (Fig. 2(A)). On the other hand, only weak staining was observed in the kidneys from the control mice (Fig. 2(B)).

It is known that lipid peroxidation products can
modify biological materials such as lipids, DNA and proteins. Among the products, the reactivity of malondialdehyde (MDA) and HNE has been investigated in detail, and it has been shown that MDA or HNE-modified proteins and thiobarbituric acid reactive substances (TBARS) were increased in several tissues by diabetes. However, in this study, TBARS and HNE-modified proteins, which are both advanced lipid peroxidation end products, were not significantly increased in the kidneys of Akita mice compared to the levels in the control mice (data not shown). These results suggest that the accumulation of HEL in the kidneys represents the initial stage of oxidative stress in hyperglycemia. We think that HEL is a sensitive marker for detecting oxidative tissue damage in hyperglycemia.

In order to detect the protein cross-linking occurring with hyperglycemia, we evaluated the DT level in the kidneys of Akita mice by ELISA, using the same samples as those prepared for the HEL measurement. The DT level in the kidneys of the Akita mice was significantly higher than in those of the control mice (Fig. 1(B)). Figure 2(C) shows that the Akita mice revealed stronger immunostaining with a granular pattern by the anti-DT antibody in the cytoplasm of renal proximal tubules compared to the control mice (Fig. 2(D)). These results suggest that hyperglycemia increased protein oxidation in the kidneys of Akita mice.

In conclusion, hyperglycemia in Akita mice increased HEL and DT in the kidneys without any elevation of the HNE-modified protein and TBARS levels. At 22 weeks, although no significant difference in the levels of HEL and DT was observed between the control and Akita mice, HNE was increased in the Akita mice compared to the control mice (data not shown). These results suggest that oxidative stress had occurred in the kidneys of hyperglycemic Akita mice and that this stress could be detected by using the novel biomarkers. The use of HEL and DT as biomarkers is expected for detecting diabetic complications.

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References


