Effect of diabetes mellitus on levels of atrial natriuretic hormone in plasma and the right atrium in the non-obese diabetic mouse

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Abstract. Atrial natriuretic hormone (ANH) levels in plasma and the right atrium of non-obese diabetic mice in the decompensated diabetic state were investigated by radioimmunoassay and histological examination. Severely diabetic mice, with blood glucose levels over 33.6 mmol/l showed significantly higher hematocrit, plasma sodium and calculated plasma osmolarity than the age-matched normoglycemic mice. The plasma ANH levels in diabetic mice were significantly lower (17.5 pmol/l) than those in normoglycemic mice (43.6 pmol/l). The ANH concentrations in the right atrium were 26.6 mg/g protein in the diabetic and 11.2 mg/g protein in the normoglycemic mice. The right atrium in the diabetic mice showed much wider immunohistochemical staining by anti-human ANH antiserum compared with the normoglycemic mice. An increase in the number of atrial specific granules in the diabetic mice was observed by transmission and scanning electron microscopy. Morphometrical analysis indicated that the number of granules increased to more than twice that in the normoglycemic mice. These findings indicate that plasma ANH decreases in diabetic mice. The store of right atrial ANH may be increased to compensate for the marked dehydration in severe diabetes. Disturbances of water and electrolyte metabolism have been observed in decompensated diabetes mellitus (1-3). Changes in the renin-angiotensin-aldosterone system and vasopressin secretion have been shown both in patients with diabetes mellitus (4,5), and in rats treated with the diabeticogenic agent, streptozotocin (STZ) (6-8). Atrial natriuretic hormone (ANH) is a hormone released by atrial myocytes in response to acute (9) and chronic (10) extracellular volume expansion. Changes in ANH levels both in the plasma and the atrium can be expected in severe diabetes mellitus. Recently, plasma ANH levels in patients with diabetes mellitus and in STZ-induced diabetic rats have been studied (11-14); however, the results are inconclusive, and there have been no reports on atrial ANH levels in the severely hyperglycemic and hyperosmolar state.

To investigate the effect of severe diabetes on ANH levels, we used female non-obese diabetic mice as an animal model of insulin-dependent diabetis. This model is characterized by insulitis being present at 30 days of age, and by 80% of the females and 20% of the males developing diabetes at 210 days of age (15). They die one or two months after development of diabetes owing to marked hyperglycemia. We determined plasma and right atrial ANH levels by radioimmunoassay (RIA), and observed morphological changes in the right atria by immunohistochemistry, transmission and scanning electron microscopy.

Material and Methods
Female non-obese diabetic mice were obtained from our own breeding colony at the Institute of Experimental Animals, Shimane Medical University. They were housed at 24°C under 12 h light, 12 h dark cycles (08.00 to 20.00 h) and given free access to water and chow pellets, which contain 60% carbohydrate, 15% fat, and 27% protein (Oriental Kobo Co, Tokyo, Japan).
From 15 weeks of age the urinary glucose levels of the mice were examined every three to four days by using Diastix (Miles-Sankyo Co., LTD, Tokyo). Their body weights were measured. Some animals showed high urinary glucose (>5.6 mmol/l) after 20 weeks and the number of animals showing high urinary glucose increased with aging.

At 25 weeks of age the animals were anesthetized with an ip injection of sodium pentobarbital (0.1 mg per gram body weight). The blood was drawn into a syringe from the left carotid artery and transferred into cooled test tubes containing 15 mg EDTA and aprotonin (1000 kallikrein inhibitor units). Plasma glucose levels were measured by the glucose oxidase method (Glu-Lq, Iatron Lab, Inc, Tokyo). We also measured the hematocrit. Plasma sodium was determined by using a flame photometer (type 750 Hitachi Koki Co, LTD, Tokyo). Plasma osmolality (P_\text{osmol}) was estimated by the following formula:

\[ P_{\text{osmol}} = 2[Na^+] + [\text{glucose}]. \]

The mice were divided into three groups according to urinary and plasma glucose concentrations and weight loss. Group 1 was the control group and was normoglycemic. The urinary glucose in this group had remained negative prior to experiment, and at that time plasma glucose concentrations were less than 11.2 mmol/l. Group 2 included mice whose urinary glucose had been positive (>14 mmol/l) within the last month prior to sacrifice and whose plasma glucose levels at sacrifice was over 11.2 mmol/l. The percent weight loss was calculated with the formula (Maximum body weight-body weight at sacrifice/Maximum body weight \times 100). The percent weight loss was less than 20% in Group 2 mice. Group 3 mice had high plasma glucose concentrations, >33.6 mmol/l, and their percent weight loss was over 20%.

The right atria were divided into 4 pieces. The first piece was used for immunohistochemistry by the peroxidase-anti-peroxidase (PAP) method (17) using anti-human ANH antisera (Peptide Institute Inc, Minoo, Japan) diluted to 1:400. After processing with the PAP method, the specimens were stained with hematoxylin.

The second piece of each atrium was cut into small specimens for transmission electron microscopic study. The specimens were immersed into cold 2.5% glutaraldehyde and 2.0% paraformaldehyde with a 0.1% phosphate buffered solution at pH 7.4 for 2 h at 4°C. They were then postfixed in 1.0% osmium tetroxide (OsO_4) for 2 h at 4°C. After dehydration in a sequence of graded ethanol solutions, they were embedded in epon. They were cut into ultrathin sections of about 80 nm, and the sections were stained with uranyl acetate and lead citrate and examined with transmission electron microscope (JEOL, 1200 EX). The atrial specific granules in the Group 1 and Group 3 mice were counted in accordance with methods used by Cantin et al. (18). Only longitudinal sections framed on both sides by myofilaments and containing the centre part of a nucleus and at least one Golgi complex were photographed, without taking into account the number of specific granules present. Twenty photographs fulfilling these criteria from each of 4 mice in Group 1 and Group 3, total 80 myocytes in each group, were used. The granules were counted on 13 cm \times 18 cm prints at a final magnification of 22 000. The granules were divided into three groups as described by Berger et al. (19).

The third piece of the atrium was prepared for scanning electron microscope. The pieces were processed by the Aldehyde-Osmium-DMSO-Osmium method (20). They were then dehydrated in a sequence of graded ethanol solutions. After treatment with isomyl acetate, they were dried in a critical point dryer (Hitachi HCP-2). The dried samples were coated with platinum using an ion-coater with a rotating stage and observed in a high-resolution field emission scanning electron microscope (Hitachi S-800).

The last pieces were immediately frozen and stored at -80°C for RIA. The ANH concentration was measured by a RIA as described by Nakao et al. (21). The atrial ANH levels in Group 1 and Group 3 mice were measured with the anti-human ANH antisera which was used for the immunohistochemistry. The anti-human ANH antisera was used at a final dilution of 1:25 000. Synthetic rat ANH (Peptide Institute Inc) was used as a standard. Protein content of atrial samples was measured by the method of Lowry et al. (22). For plasma RIA, anti human/rat ANH antisera kindly donated by Dr Nakao, Kyoto University, was used at a final dilution of 1:200 000, because plasma ANH could not be detected using the antisera from the Peptide Institute. Plasma ANH was measured directly. Intra- and inter-assay coefficients of variation in the present study were 4.5 and 7.8%, respectively.

The data are expressed as means ±SD. The statistical analysis of the data was performed by one-way analysis of variance, and the significance was determined by the Scheffe's method for the two comparisons. The Student's unpaired t-test was used for analysis of atrial ANH levels, and the number of granules between Group 1 and Group 3. The statistical significance was defined as p<0.05.

**Results**

Body weights and plasma glucose concentrations of the three groups are shown in Table 1. The mice in Group 3 lost almost 30% of their body weight and their plasma glucose concentrations were 5 times as higher than those of the Group 1 mice. In the mice in Group 2, body weights were not different from those of the Group 1 mice, but the plasma glucose concentrations were higher. Plasma glucose concentrations in the mice in Group 3 were markedly higher than in the mice in Group 2.
Table 2 shows the hematocrit, plasma sodium, and calculated plasma osmolarity of the three groups. Hematocrit in Group 3 was higher than in both Group 2 and Group 1. Plasma sodium was higher in Group 3 than in Group 1 mice. In Group 2, the value was greater than in Group 1, but not statistically different from that in Group 3. Calculated plasma osmolarity was higher in Group 2 and Group 3 than in Group 1.

The perinuclear area of the bilateral atrial cells was stained brown by the PAP method with anti-human ANH antiserum (Fig. 1). In Group 3, the right atrial cells appeared to be stained much wider than in Group 1 (Fig. 1a). In Group 1, the specimens had relatively narrow regions which were deeply stained (Fig. 1b). The area of the staining in Group 2 mice varied among sections and animals.

In the transmission electron microscope study, atrial specific granules with a limiting membrane and sometimes with a lucent area between the limiting membrane and the core were observed in the right atrial cells. The granules were distributed mainly at the perinuclear region. The Golgi complex was developed at the perinuclear region of right atrial cells in both Group 3 and Group 1 mice (Fig. 2a and b). For morphometrical analysis, the granules were divided into three groups (19). A-granules possess a very electron-dense core that

**Fig. 1.**
Immunohistochemical staining of the right atrium in the Group 3 (Fig. 1a) and the Group 1 mouse (Fig. 1b). Most myocytes, particularly near the pericardium exhibit an intense reaction. Immunoreactive material is accumulated around the nucleus. In the Group 3 mouse, the tissue appears to be stained much wider than in the Group 1 mouse. Bar=100 μm.
Table 1.
General characteristics of three groups of the non-obese diabetic mice.

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<thead>
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<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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<tbody>
<tr>
<td>Number of animals</td>
<td>20</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Body weight</td>
<td>25.0±4.0</td>
<td>26.3±3.7</td>
<td>18.8±4.0</td>
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<td>Plasma glucose (mmol/l)</td>
<td>9.1±2.0</td>
<td>33.4±9.3</td>
<td>46.4±10.1</td>
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Group 1: normoglycemic, Group 2: moderately hyperglycemic, Group 3: hyperglycemic non-obese diabetic mice. Values are means ± sd. 1: p<0.01. Each value was compared with Group 1 (one-way analysis of variance). 2: p<0.05. 3: p<0.01. Values were compared with Group 2 (one-way analysis of variance).

Table 2.
Plasma osmolarity and related variables in the non-obese diabetic mice.

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<th>Group 2</th>
<th>Group 3</th>
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<tbody>
<tr>
<td>Number of animals</td>
<td>15</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>36.3±1.5</td>
<td>37.5±2.9</td>
<td>42.6±1.2</td>
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<tr>
<td>Plasma-sodium (mmol/l)</td>
<td>140.9±4.5</td>
<td>157.8±9.5</td>
<td>163.0±9.8</td>
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<tr>
<td>P_osmol (mmol/l)</td>
<td>288.5±6.6</td>
<td>347.1±15.3</td>
<td>368.5±9.9</td>
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Group 1: normoglycemic, Group 2: moderately hyperglycemic, Group 3: hyperglycemic non-obese diabetic mice. Values are means ± sd. 1: p<0.01. 2: p<0.05. Values were compared with Group 1 (one-way analysis of variance). 3: p<0.05. Values were compared with Group 2 (one-way analysis of variance).

is sometimes slightly retracted from the inner part of the membrane, leaving a thin halo around the core. B-granules are characterized by a pale, fibrogranular core. D-granules have a relatively small core.
diameter and possess a dense core which resembles those in the norepinephrine-containing granules in adrenergic nerve endings (22). The numbers of each type of granules per myocyte were significantly increased in Group 3 over those in Group 1 mice (Table 3). The ratio of the numbers of granules of Group 3 mice to Group 2 mice was 2.43 for A-granules, 2.08 for B-granules, 2.29 for D-granules, and 2.27 for total.

The atrial specific granules were observed three-dimensionally by scanning electron microscopy. Group 3 mice had more granules in the perinuclear area than Group 1 mice, which is consistent with the results obtained by immunohistochemistry and transmission electron (Fig. 3a and b).

Table 4 shows plasma and right atrial ANH levels in the three different glycemic states. Plasma ANH levels were lower in Group 3 and Group 2 mice than in Group 1. On the other hand, the right atrial

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<th>Group 3</th>
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<tbody>
<tr>
<td>Number of animals</td>
<td>15</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Plasma ANH (pmol/l)</td>
<td>43.6±2.8</td>
<td>27.9±1.8</td>
<td>17.5±3.1</td>
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<tr>
<td>Atrial ANH (mg/g)</td>
<td>11.2±1.4</td>
<td>26.6±1.4</td>
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Group 1: normoglycemic, Group 2: moderately hyperglycemic, Group 3: hyperglycemic non-obese diabetic mice. Values are means ± sd. 1: p<0.01. 2: p<0.05. Values compared with Group 1 (one-way analysis variance). 3: p<0.05. Values compared with Group 1 (unpaired Student's t-test).

Fig. 3.
Scanning electron micrographs of mouse atrial cardiocytes. In the atrial myocyte of a Group 3 mouse, numerous atrial granules (arrowheads) in the central core of the myocyte are seen (Fig. 3a). In the atrial myocyte of a Group 1 mouse, only few atrial granules are present and their number is less than in the Group 3 mouse (Fig. 3b). G: Golgi complex; N: nucleus; M: mitochondria. Bar=1 μm.
ANH concentration per tissue protein content in Group 3 mice increased to more than twice those in Group 1 mice.

Discussion

The plasma ANH level measured by RIA ranged between 10-20 pmol/l in normal humans, and 60-130 pmol/l in rats (21,23). In BALA/c mice, plasma ANH level was reported to be 43 pmol/l (21). The plasma ANH level in normoglycemic non-obese diabetic mice was 43.6±2.8 pmol/l in the present study, which is consistent with the ANH levels in humans, rats and BALB/c mice.

There have been controversial reports regarding the plasma ANH levels in the diabetic state (11-14). Ortola et al. (12) reported that the plasma ANH levels doubled in diabetic rats which had been iv injected with STZ (60 mg/kg) 2 weeks prior to experiment. They speculated that moderate hyperglycemia, with its attendant chronic volume expansion, stimulates atrial ANH release, and that elevated plasma ANH levels might contribute to the hyperfiltration observed in early diabetes (10,24). In contrast, Hebden et al. (13) demonstrated that plasma ANH levels were unchanged in rats at 1, 3, 6 and 12 weeks after STZ injection (55 mg/kg). In another report, they observed slight elevation of plasma ANH levels in diabetic rats 6 weeks after STZ injection (55 mg/kg) compared with the control rats (14). Our results do not agree with their studies. This may be due to species difference, or to the difference between the drug-induced and spontaneous animal models. While Ortola et al. and Hebden et al. examined plasma ANH levels in diabetic rats, we used severely or moderately ill diabetic mice with hyperosmolarity. Thus, the magnitude of hyperglycemia might play an important role in the regulation of plasma ANH levels.

The non-obese diabetic mice showed sudden onset of diabetes, marked hyperglycemia, polydipsia and polyuria (15). In addition, the calculated osmolarity, plasma sodium and hematocrit were significantly higher in the hyperglycemic than in the normoglycemic mice. These results suggest that hyperglycemic and the moderately hyperglycemic mice were profoundly dehydrated. The reduction of plasma ANH levels observed in the two groups of hyperglycemic mice may be the result of a homeostatic mechanism which protects animals from water depletion owing to severe osmotic diuresis. Such a marked depletion of body fluid may cause significant decreases in atrial pressure. It has been demonstrated that plasma ANH levels were markedly decreased in dehydrated rats (24,25). The reduction of plasma ANH levels in the two groups of hyperglycemic mice may result from either reduction of release of ANH from the atrium, increased breakdown of circulating ANH, or an increase in clearance receptor (26).

A recent study by Zisfein et al. (27) showed that in the rat atrial ANH content measured by RIA did not change even with marked volume depletion. In our study, we found an increase in right atrial ANH levels in the diabetic mice as revealed by RIA and immunohistochemical staining of atria. A marked increase in atrial specific granules in the hyperglycemic mice was also observed in transmission and scanning electron microscope studies. An increase in granules is consistent with the wider distribution of immunoreactive substances in the right atrium. Berger & Bencosmo classified atrial specific granules to three types (19). All three types of granules were increased 2-fold in hyperglycemic mice. It is unclear, however, whether the increase in specific granules was the result of reduction of release or active synthesis of ANH induced by the reduction of plasma ANH by some breakdown or clearance mechanism.

In conclusion, this study demonstrated that severely ill non-obese diabetic mice have decreased circulating ANH levels and increased atrial ANH levels. The store of atrial ANH may be increased to adjust or counterbalance the marked dehydration in the decompensated diabetic state.

Table 3.
Various types of granules in the right atrium.

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<tr>
<td>Number of animals</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Number of cells</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>A-granules</td>
<td>15.2±10.9</td>
<td>37.0±16.3</td>
</tr>
<tr>
<td>B-granules</td>
<td>12.9±9.5</td>
<td>26.8±14.0</td>
</tr>
<tr>
<td>D-granules</td>
<td>8.3±6.8</td>
<td>19.0±10.7</td>
</tr>
<tr>
<td>Total</td>
<td>36.5±19.5</td>
<td>82.8±32.9</td>
</tr>
</tbody>
</table>

Group 1: normoglycemic, Group 2 hyperglycemic non-obese diabetic mice.
A, B, D-granules: number of granules per myocyte.
Values are means ± sd. 1: p<0.001. Each value compared with Group 1 (unpaired Student’s t-test).
References


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