Diabetes-Induced Alterations in Atrial Natriuretic Peptide Gene Expression in Wistar-Kyoto and Spontaneously Hypertensive Rats

Hiroaki Matsubara, Yasukiyo Mori, Jin Yamamoto, and Mitsuo Inada

We investigated the effects of streptozotocin-induced diabetes on atrial natriuretic peptide (ANP) synthesis, hemodynamic parameters, blood volume, and histopathology, as well as the reversibility of such effects with insulin therapy in Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). The biastral ANP messenger RNA (mRNA) levels in the diabetic WKY rats increased by 16–17% compared with those in the age-matched WKY rats at 12 weeks after the onset of diabetes, whereas their ventricular ANP mRNA levels showed increases of 190% in left ventricles and 160% in right ventricles at 8 weeks. In the diabetic SHRs, the left atrial ANP mRNA levels increased by 36% compared with those in the age-matched SHRs, as early as 4 weeks after diabetes onset. Their ventricular ANP mRNA levels also showed 80–82% increases in left and right ventricles at 4 weeks. In proportion to changes in cardiac ANP synthesis, the biventricular end-diastolic pressures were significantly elevated at 8 weeks in the diabetic WKY rats and at 4 weeks in the diabetic SHRs. The blood volume significantly increased at 8 weeks in the diabetic WKY rats and remained higher thereafter, whereas it did not change in the diabetic SHRs throughout the experimental period. The left ventricular peak dP/dt was depressed in the 8-week diabetic SHRs, whereas in the diabetic WKY rats, its depression was observed at 12 weeks after diabetes onset. Histopathological studies showed that diabetic changes in ANP synthesis and hemodynamic parameters described above occurred before the cardiomyopathic histological changes. Cardiac ANP synthesis in the diabetic rats completely reverted to control levels after insulin therapy, accompanied by normalization of hemodynamic parameters. The present study indicates that 1) ANP synthesis is significantly augmented in the streptozotocin-induced diabetic rat compared with that in the normal rat, and the combination of diabetes and hypertension produces an earlier and greater effect in stimulating cardiac ANP synthesis than does either disease alone; 2) an elevation in the intraventricular filling pressure that occurs before observable cardiomyopathic histopathological alterations might be involved partially in the augmented ANP synthesis; and 3) the reversibility with insulin therapy suggests that the streptozotocin-induced alterations observed in cardiac ANP synthesis and hemodynamics result from insulin-deficient diabetes mellitus, not from cardiac toxicity of streptozotocin. (Circulation Research 1990;67:803–813)

Atrial natriuretic peptide (ANP) contributes to fluid, electrolyte, and blood pressure homeostasis through its natriuretic and vasodilative actions. The main stimulus for ANP secretion is thought to be atrial stretch in response to volume expansion or pressure elevation.1

Diabetes mellitus is associated with abnormalities in fluid and electrolyte balance or alterations in related hormone levels.2,3 Diabetes causes cardiomyopathy with a depression in cardiac function and contractile protein enzymatic activity,4–6 and these alterations in hemodynamics and blood volume may affect cardiac ANP synthesis and secretion. However, circulating ANP levels are reported to be elevated7–9 or unchanged10 in rats with diabetes produced by streptozotocin (STZ), and the effects of diabetes on cardiac ANP synthesis have not been investigated yet.

Hypertension is present in 40–80% of patients with diabetes and aggravates the cardiovascular com-

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plication of diabetes. A combination of hypertension and diabetes leads to a greater myocardial degeneration than does either disease alone. Although hypertension induces a substantial increase in cardiac ANP messenger RNA (mRNA) levels, the effects of diabetes on cardiac ANP synthesis in the hypertensive state also have not been clarified.

The purpose of this study is to examine the effects of STZ-induced diabetes on ANP mRNA and ANP levels in atria and ventricles in relation to hemodynamic parameters, blood volume, and histopathology in Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). In addition, we also assessed the effect of insulin therapy on cardiac ANP synthesis and hemodynamic alterations in these STZ-treated rats.

Materials and Methods
Production of Diabetic Rats and Insulin Therapy

Six-week-old male WKY rats (n=12) and SHRs (n=12) (Charles River Japan, Atsugi, Japan) were fed food and tap water ad libitum throughout the study. At 8 weeks, the rats were anesthetized with ether and treated with 60 mg/kg STZ (Sigma Chemical Co., St. Louis). STZ was dissolved in ice-chilled 0.02 M citrate buffer (pH 4.5) immediately before use and was injected into the tail vein in the nonfasted rats, as previously described. Diabetic state was assessed by measuring nonfasting plasma glucose level (in tail vein blood obtained during ether anesthesia) 2 weeks after STZ administration. Only animals with glucose levels that exceeded 300 mg/dl were considered diabetic. Systolic blood pressure was measured once a week with the tail-cuff method, and body weight was checked.

Eight weeks after STZ injection, the diabetic WKY rats and SHRs were treated with insulin for 2–4 weeks. Age-matched normal and diabetic WKY rats and SHRs (n=12 for each group) served as controls and were assessed as the 0-, 4-, 8-, 10-, or 12-week diabetic controls in Figures 2, 3, and 4. Lente insulin was injected subcutaneously each evening (usually between 4 and 6 pm). For the first 3 days of therapy, either 3 or 4 units of insulin was administered daily according to blood glucose levels obtained during the morning. Blood glucose levels were measured at weekly intervals thereafter, and the insulin doses were adjusted to try to regulate the levels at 100–200 mg/dl (average daily dose, 2.6±0.2 units). At 4, 8, 10, and 12 weeks after STZ administration, or 2 and 4 weeks after insulin treatment, blood and heart samples were obtained, and hemodynamic studies were performed.

Determinations of Hemodynamic Parameters and Circulating Blood Volume

The rats were anesthetized with ether and placed supinely on a board with their legs taped. A polyethylene catheter (PE-50, Statham, Cleveland) was placed in the femoral vein, through which blood (2 ml) for the ANP, glucose, and thyroxine assays was withdrawn. The blood loss was immediately replaced with fresh blood obtained just before the experiment from other normotensive donor rats. Other catheters were placed into the left ventricle through the right carotid artery and into the right ventricle through the left jugular vein. Mean arterial pressure, heart rate, and left and right ventricular end-diastolic pressures were recorded by Statham transducers connected to a polygraph. The peak rate of rise of left ventricular pressure (peak dP/dt) was obtained by electronic differentiation of left ventricular pressure. Blood volume was then determined by dilution of Evans blue. Briefly, a 0.5% (wt/vol) solution in a volume of 0.1 ml was injected via the venous catheter, and 5 minutes postinjection, about 0.3 ml of an arterial sample was obtained. After centrifugation of the sample, three aliquots (50 μl) of plasma samples were obtained, and the Evans blue concentration was determined spectrophotometrically. Blood volume was calculated as blood volume=plasma volume/(1–hematocrit/100×0.8). On the completion of each experiment, the heart was excised, and the atria and ventricles were removed, washed, and blotted dry. The left ventricle, including the interventricular septum, and the right ventricular free wall were weighed separately.

Atrial Natriuretic Peptide Immunoreactivity

Blood samples were collected in aprotinin (500 KIU/ml) and Na2EDTA (1 mg/ml) and centrifuged; then plasma was extracted as previously reported. Briefly, plasma was diluted with 4% acetic acid and passed through a Sep-Pak C18 cartridge (Waters Associates, Milford, Mass.) prewashed with 10 ml methanol, washed with 10 ml 4% acetic acid, eluted with 90% methanol/4% acetic acid, evaporated to dryness, and stored at −20°C until assayed. Atrial tissues and the apical parts of ventricular tissues were immediately added into 1 M acetic acid/20 mM HCl and heated at 100°C for 10 minutes. The samples were then homogenized and centrifuged for 25 minutes at 12,000g. The resulting supernatant was stored at −20°C until assayed. Immunoreactive ANP (IR-ANP) of plasma samples and of extracts was determined using a commercially available α-ANP antibody (Peninsula Laboratories, Inc., Belmont, Calif.). The radioimmunoassay procedure was performed as previously described. Measurements of ANP immunoreactivity in plasma and cardiac tissues were performed individually for all samples obtained from each experimental group. Recovery of extracted ANP, as determined by addition of unlabeled ANP to plasma, was 82% (n=8). The intra-assay and interassay coefficients of variation were 5.4% (n=9) and 7.4% (n=9), respectively.

Preparation and Analysis of Atrial and Ventricular Atrial Natriuretic Peptide mRNA

Tissue samples for RNA extraction were immediately frozen in liquid nitrogen and stored at −80°C. The same tissue samples (n=3) were pooled, and we obtained four separate groups of pooled samples
from each experimental group. Total RNA was extracted from each group of pooled samples with the guanidine isothiocyanate–CsCl procedure.\textsuperscript{23} RNA was quantified spectrophotometrically by absorption at 260 nm. Northern blot hybridization analyses were carried out to detect the size of ANP mRNA, as described previously.\textsuperscript{24} For quantitative analyses of ANP mRNA in total RNA, we dotted in duplicate RNA samples diluted serially five times on the nitrocellulose filters;\textsuperscript{25} 0.0625–1 \( \mu \)g for total atrial RNA and 0.625–10 \( \mu \)g for total ventricular RNA were applied. The filters were air dried, baked at 80°C for 2 hours, divided into two parts for ANP and reference \( \beta \)-actin hybridization, and then prehybridized and hybridized to the radiolabeled rat ANP complementary DNA probe (782 bp, kindly provided by Dr. Shinzo Oikawa, Suntory Institute for Biomedical Research, Osaka, Japan)\textsuperscript{26} or chicken \( \beta \)-actin complementary DNA probe (760 base pairs, Oncor, Gaithersburg, Md.) that had been labeled to 8–10\( \times \)10\(^6\) cpm/\( \mu \)g with a multipriming DNA labeling system.\textsuperscript{24} Autoradiographic signals were measured by a scanning densitometer. Absorbances were plotted as a function of the amounts of dotted RNA, and slope was determined using linear regression analysis. Relative ANP mRNA levels were arbitrarily normalized for the slope of corresponding \( \beta \)-actin mRNA (i.e., as a ratio of the ANP/\( \beta \)-actin slope). There was no significant change in cardiac \( \beta \)-actin mRNA levels, which were used as an internal control, in response to diabetes. Dot blot hybridization was performed for four separate RNA samples obtained from each experimental group. The mean ratio of the ANP/\( \beta \)-actin slopes was taken as a quantitative index of relative ANP mRNA.

**Histopathological Studies**

The remaining ventricular tissues were immersed in 10% formalin solution. Rings 1–2 mm in thickness were cut perpendicular to the long axis of the ventricle. Ventricular rings were embedded in paraffin, and sections were examined with hematoxylin and cosin and trichrome stain. Two to three ventricular rings were examined from each diabetic WKY rat, diabetic SHR, and control (\( n = 9 \) for each group) at 4, 8, 10, and 12 weeks after STZ administration. Interstitial fibrosis was defined as collagen deposition between and around myocardial cells. The extent and degree of fibrosis was subjectively graded on the following scale: 0–1+, absent or minimally increased staining in a focal distribution; 2+, moderately intense staining in focal zones; and 3+, severe changes, with dense or highly cellular collagen deposition between groups of cells.

**Other Variables and Statistical Methods**

Blood glucose levels were determined using the glucose hydrogenase method.\textsuperscript{16,17} Total serum thyroxine levels were determined by radioimmunoassay (Eiken Chemical, Tokyo, Japan).\textsuperscript{17,18} Serum sodium, potassium, and creatinine levels were measured by an autoanalyzer (Hitachi Co., Tokyo, Japan). Protein content was determined by Lowry’s method with bovine serum albumin as standard.\textsuperscript{22} Statistical analysis was performed by two-way analysis of variance and the Newman-Keuls method. A value of \( p < 0.05 \) was considered statistically significant. All values are expressed as mean±SEM.

**Results**

**Body Weight, Hemodynamics, Blood Volume, Renal Function, and Thyroid Hormone Levels**

As shown in Table 1, the development of diabetes was proved in the STZ-treated rats by significantly higher blood glucose levels than those in the vehicle-treated control rats. The body weight significantly decreased in the diabetic WKY rats and SHRs at both 4 and 8 weeks after STZ administration. Four- and 8-week periods of diabetes resulted in significant decreases in heart rate and serum thyroxine levels in both rat strains, consistent with previous findings.\textsuperscript{17,18} Systolic blood pressure was not affected until 8 weeks after diabetes onset (data shown on only 4- and 8-week rats). Serum sodium, potassium, and creatinine levels were also unaltered. Between-strain comparisons provided no significant difference in heart rate and serum thyroxine levels.

As shown in Table 1, left ventricular peak dP/dt, both ventricular end-diastolic pressures, and blood volume in the 4-week diabetic WKY rats did not differ from the values in the control WKY rats. In the 8-week diabetic WKY rats, both ventricular end-diastolic pressures and blood volume significantly increased; however, left ventricular peak dP/dt was unchanged. Both ventricular end-diastolic pressures in the diabetic SHRs significantly increased over those in the age-matched SHRs at both 4 and 8 weeks. Left ventricular peak dP/dt was depressed in the 8-week diabetic SHRs compared with the control SHRs, but not in the 4-week diabetic SHRs. No significant difference in blood volume was observed between the diabetic and control SHRs at both 4 and 8 weeks.

**Plasma IR-ANP, Cardiac Weight, Cardiac IR-ANP, and ANP mRNA Levels**

As shown in Table 2, plasma IR-ANP levels in the diabetic WKY rats were similar to those in the control WKY rats at 4 weeks after STZ administration, but these levels were elevated at 8 weeks. In the diabetic SHRs, plasma IR-ANP levels increased already at 4 weeks and reached values more than twofold those of the control at 8 weeks. Heart weight in the diabetic WKY rats did not differ from the control at 4 weeks and decreased at 8 weeks. The diabetic SHRs showed a decrease in heart weight at both time periods compared with the control. The left ventricular weight-to-body weight ratio was higher in the diabetic WKY rats than in the control WKY rats at 8 weeks, whereas this ratio in the diabetic SHRs did not differ from that in the control SHRs at 4 and 8 weeks. The right ventricular weight—
Table 1. Diabetes-Induced Alterations in Body Weight, Hemodynamics, Renal Function, and Thyroxine Level in Wistar-Kyoto and Spontaneously Hypertensive Rats

<table>
<thead>
<tr>
<th></th>
<th>4 weeks diabetes</th>
<th>8 weeks diabetes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WKY</td>
<td>WKY + DM</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>292 ± 4</td>
<td>229 ± 6*†</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>140 ± 2</td>
<td>144 ± 4</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>472 ± 16</td>
<td>372 ± 13§</td>
</tr>
<tr>
<td>LV peak dP/dt (mm Hg/sec × 10³)</td>
<td>16 ± 0.8</td>
<td>16 ± 0.9</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>3.2 ± 0.2</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>RVEDP (mm Hg)</td>
<td>2.6 ± 0.2</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Blood volume (ml/kg wt)</td>
<td>61 ± 0.4</td>
<td>63 ± 0.5</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>118 ± 7</td>
<td>440 ± 12*‡</td>
</tr>
<tr>
<td>Serum sodium (meq/l)</td>
<td>146 ± 2</td>
<td>143 ± 1</td>
</tr>
<tr>
<td>Serum potassium (meq/l)</td>
<td>3.9 ± 0.2</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>43 ± 5</td>
<td>54 ± 7</td>
</tr>
<tr>
<td>Serum thyroxine (µg/dl)</td>
<td>4.3 ± 0.5</td>
<td>1.6 ± 0.6§</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; DM, diabetes mellitus; LVEDP, left ventricular end-diastolic pressure; RVEDP, right ventricular end-diastolic pressure.

* p<0.01 vs. age-matched WKY rats.
† p<0.05 vs. age-matched SHR.
‡ p<0.01 vs. age-matched SHR.
§ p<0.05 vs. age-matched WKY rats.

To-body weight ratio did not differ between the diabetic and control rats from either strain at 4 weeks; however, this ratio was higher in the diabetic WKY rats than in the controls at 8 weeks.

Left and right ventricular IR-ANP levels in the 8-week diabetic WKY rats increased by 160% and 120%, respectively, compared with data for the control WKY rats, whereas these levels were unaltered at 4 weeks (Table 2). In the diabetic SHR rats, left and right ventricular IR-ANP levels at 4 weeks increased by 60% and 70%, respectively, compared with the values in the control SHR rats. At 8 weeks, these IR-ANP levels further increased by 149% in left ventricles and by 81% in right ventricles. On the other hand, atrial IR-ANP levels in the diabetic SHR rats decreased by 42% in left atria at 4 weeks, and by 43% in both left and right atria at 8 weeks. In contrast, there were no such changes in the diabetic WKY rats.

To determine if the changes in IR-ANP levels reflect changes at ANP gene expression, we measured relative ANP mRNA levels in atria and ventricles. Northern blot analyses showed that total RNA extracted from atria or ventricles of diabetic WKY rats and SHR rats contained a single major band of the same size (approximately 950 base pairs) as atrial ANP mRNA from WKY rats (Figure 1 shows ANP mRNA accumulations in left ventricles). To quantify the ANP mRNA accumulations, the slope of the ANP mRNA levels in dot blot hybridization was normalized for that of corresponding β-actin mRNA. As shown in Table 2, although there was no significant change in biventricular ANP mRNA levels in the diabetic and control WKY rats at 4 weeks, 8 weeks of diabetes produced a 190% increase in left ventricular ANP mRNA levels and a 160% increase in right ventricular ANP mRNA levels in WKY rats. Interestingly, the combination of hypertension and diabetes induced an earlier and greater increase in the ANP mRNA levels than did either disease alone. The diabetic SHR rats had 80–82% increases in left and right ventricular ANP mRNA levels, respectively. In contrast, atrial ANP mRNA levels appeared to be less responsive. There were no changes in atrial ANP mRNA levels in the diabetic WKY rats at either time period. The diabetic SHR rats exhibited a 36% increase in left atrial ANP mRNA levels after 4 weeks, and at 8 weeks, this accumulation increased by 44% in the left atria and by 27% in the right atria.

Reversibility With Insulin Therapy

The blood glucose levels in the diabetic WKY rats and SHR rats were normalized by the effective insulin therapy (average daily dose, 2.6 ± 0.2 units). The 4-week insulin therapy reverted the decreased body and heart weight in the diabetic WKY rats and SHR rats to the levels of controls. Both the depressed left ventricular peak dP/dt in the diabetic SHR rats and the elevated blood volume in the diabetic WKY rats also were normalized by the insulin therapy (data not shown).
TABLE 2. Diabetes-Induced Alterations in Heart Weight and Cardiac Atrial Natriuretic Peptide Biosynthesis in Wistar-Kyoto and Spontaneously Hypertensive Rats

<table>
<thead>
<tr>
<th>4 weeks diabetes</th>
<th>8 weeks diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WKY</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.83±0.05</td>
</tr>
<tr>
<td>Ventricle/body weight (mg/g)</td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>0.53±0.02</td>
</tr>
<tr>
<td>Left</td>
<td>2.25±0.03</td>
</tr>
<tr>
<td>Plasma IR-ANP (pg/ml)</td>
<td>36±3</td>
</tr>
<tr>
<td>Atrial IR-ANP (μg/mg protein)</td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>3.9±0.2</td>
</tr>
<tr>
<td>Left</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>Ventricular IR-ANP (ng/mg protein)</td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>0.24±0.02</td>
</tr>
<tr>
<td>Left</td>
<td>0.27±0.03</td>
</tr>
<tr>
<td>ANP/β-actin mRNA</td>
<td></td>
</tr>
<tr>
<td>Right atrium</td>
<td>160±10</td>
</tr>
<tr>
<td>Left atrium</td>
<td>148±8</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>0.81±0.08</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>1.29±0.07</td>
</tr>
</tbody>
</table>

Values are mean±SEM. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; DM, diabetes mellitus; IR-ANP, immunoreactive atrial natriuretic peptide. RNA samples were dotted in duplicate, followed by hybridization to appropriate complementary DNA probes. Absorbances of autoradiographic signals by a scanning densitometer were plotted as a function of amounts of dotted RNA, and slopes were determined. Relative ANP mRNA levels were expressed by the arbitrary normalization for the slope of corresponding β-actin mRNA.

*p<0.01 vs. age-matched WKY rats.
†p<0.01 vs. age-matched SHRs.
‡p<0.05 vs. age-matched WKY rats.
§p<0.05 vs. age-matched SHRs.

As shown in Figure 2, the elevated right and left ventricular end-diastolic pressures in the diabetic WKY rats were significantly reduced by the first 2-week insulin therapy. The 4-week insulin therapy completely reversed these increases. In the insulin-treated diabetic SHRs, right ventricular end-diastolic pressure only partially, but significantly, decreased over the first 2-week therapy and was completely normalized by the 4-week therapy (Figure 3). The insulin therapy for 2 weeks did not affect the elevated left ventricular end-diastolic pressure in the diabetic SHRs; however, the elevation reverted to the control's level by the 4-week therapy.

Northern blot analysis showed that the insulin-treated diabetic WKY rats and SHRs had the same size of ANP mRNA (approximately 950 base pairs) as the normal or diabetic controls (Figure 4 reveals ANP mRNA accumulations in left ventricles). The right ventricular ANP mRNA levels in the 10- and 12-week diabetic WKY rats increased approximately

FIGURE 1. Northern blot analysis of left ventricular atrial natriuretic peptide (ANP) messenger RNA accumulations in Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) at 4 and 8 weeks after streptozotocin administration. Total left ventricular RNA (10 μg in each lane) was fractionated on 1.2% agarose gel, transferred to nitrocellulose filters, and hybridized to a [32P]cRNA complementary DNA probe. DM, diabetes mellitus.
by 145% and 193%, respectively, compared with the levels in the age-matched WKY rats (Figure 2). The left ventricular ANP mRNA levels in the 10- and 12-week diabetic WKY group increased by approximately 206% and 222%, respectively, compared with those in the age-matched WKY group (Figures 2 and 4). These increased ANP mRNA levels in biventricles in the diabetic WKY rats reverted to the levels of controls by the first 2-week insulin therapy. In the diabetic SHRs, the right and left ventricular ANP mRNA levels increased by approximately 126% and 137%, respectively, at 10 weeks, and by 133% and 155%, respectively, at 12 weeks, as compared with the age-matched SHRs (Figures 3 and 4). The increased ANP mRNA levels in biventricles reverted to the levels of controls over the 4-week insulin therapy. The 2-week insulin therapy also induced significant decreases in biventricular ANP mRNA levels, but these decreases were not sufficient to normalize the elevated levels.

In contrast to the decreases in atrial IR-ANP levels in the diabetic WKY and SHR groups, their atrial ANP mRNA levels significantly increased; however, the degree of the increase appeared to be relatively less than that in the ventricular ANP mRNA levels (Table 2). In the diabetic WKY rats at 12 weeks, biventricular ANP mRNA levels increased approximately by 16–17%, compared with the age-matched WKY rats. No significant increase was observed until 10 weeks after diabetes onset. This increase in the 12-week diabetic WKY rats was completely blocked by insulin therapy. In the diabetic SHRs, we observed

**FIGURE 2.** Effect of insulin therapy on biventricular end-diastolic pressures and atrial natriuretic peptide (ANP) messenger RNA (mRNA) levels in the diabetic Wistar-Kyoto (WKY) rats. Insulin therapy was initiated at 8 weeks after induction of diabetes, as indicated by the arrows, and was continued over the following 2- or 4-week period. Hatched columns represent untreated diabetic WKY rats; closed columns represent insulin-treated diabetic WKY rats; open columns represent normal WKY rats. Data at 4 and 8 weeks after induction of diabetes are the same as shown in Table 2. RVEDP, right ventricular end-diastolic pressure; LVEDP, left ventricular end-diastolic pressure. *p<0.05, compared with age-matched WKY rats; †p<0.05, compared with age-matched insulin-treated diabetic WKY rats.

**FIGURE 3.** Effect of insulin therapy on biventricular end-diastolic pressures and atrial natriuretic peptide (ANP) messenger RNA (mRNA) levels in the diabetic spontaneously hypertensive rats (SHRs). Insulin therapy was initiated at 8 weeks after induction of diabetes, as indicated by the arrows, and was continued over the following 2- or 4-week period. Hatched columns represent untreated diabetic SHRs; closed columns represent insulin-treated diabetic SHRs; open columns represent normal SHRs. Data at 4 and 8 weeks after induction of diabetes are the same as shown in Table 2. RVEDP, right ventricular end-diastolic pressure; LVEDP, left ventricular end-diastolic pressure. *p<0.05, compared with age-matched SHRs; †p<0.05, compared with age-matched insulin-treated diabetic SHRs.
an increase of approximately 14% and 42% in the right and left atrial ANP mRNA levels, respectively, at 10 weeks, and of 14% and 46% in the right and left atria, respectively, at 12 weeks. These increases reverted to the levels of controls by the 4-week insulin therapy; however, the 2-week insulin therapy was not sufficient to normalize the increased levels (atrial data in insulin therapy not shown).

**Correlations Between Biventricular End-diastolic Pressures and Cardiac IR-ANP**

As shown in Figure 5, there were significant correlations between ventricular end-diastolic pressures and ventricular IR-ANP levels (right ventricle, $r=0.63$, $p<0.001, n=72$; left ventricle, $r=0.70, p<0.001, n=72$) when the data of insulin-treated, age-matched diabetic, and normal WKY rats were included. We also

**Figure 4.** Northern blot analysis of insulin therapy on left ventricular atrial natriuretic peptide messenger RNA accumulations in diabetic Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). The diabetic WKY rats and SHRs at 8 weeks after streptozotocin administration were treated by insulin over 2 or 4 weeks. The age-matched diabetic WKY rats and SHRs as the control correspond to the rats at 10 or 12 weeks after streptozotocin injection, respectively. Total left ventricular RNA (10 μg in each lane) was fractioned on agarose gels. DM, diabetes mellitus.

**Figure 5.** Correlations between right and left ventricular end-diastolic pressures (RVEDP and LVEDP) and ventricular immunoreactive atrial natriuretic peptide (IR-ANP) levels in the insulin-treated (●), age-matched untreated diabetic (△), and normal (○) Wistar-Kyoto (WKY) rats or spontaneously hypertensive rats (SHRs).
found positive correlations in the insulin-treated, age-matched diabetic, and normal SHRs (right ventricle, \( r=0.72, p<0.001, n=72 \); left ventricle, \( r=0.75, p<0.001, n=72 \)). We could not observe significant relations between ventricular end-diastolic pressures and atrial IR-ANP levels in both rat strains.

**Histopathology**

To compare the changes in ANP synthesis and hemodynamic parameters with the histopathological changes in diabetic cardiomyopathy, the ventricular samples obtained at the time studied for ANP mRNA and IR-ANP were examined with hematoxylin and eosin and trichrome stain. We focused on the interstitial fibrosis and myocardial degeneration as the histological changes characteristic but nonspecific for the diabetic cardiomyopathy.12,13,27 Surprisingly, sections from the diabetic SHRs and the diabetic WKY rats showed virtually no cardiomyopathic changes over 12 weeks after STZ administration. In fact, the control rats could not be differentiated from the diabetic rats on the basis of morphological examination alone (data not shown), indicating that the diabetic changes in ANP synthesis and hemodynamics occurred before the recognizable cardiomyopathic histopathological changes.

**Discussion**

A 4-week period of diabetes in WKY rats did not produce any alteration in cardiac ANP synthesis. In the 8-week diabetic WKY rats, we observed a significant increase in biventricular ANP mRNA and IR-ANP levels accompanied by elevated plasma IR-ANP levels. The atrial ANP mRNA levels were unaltered until 8 weeks after STZ injection and then increased significantly. The biventricular end-diastolic pressures and blood volume also were significantly elevated at 8 weeks. In addition, we found a positive correlation between ventricular IR-ANP levels and ventricular end-diastolic pressures. However, the left ventricular peak dP/dt as an index of systolic ventricular function was unaltered until 10 weeks after diabetes onset, and it decreased significantly at 12 weeks. This suggests that an elevation in the intraventricular filling pressure, associated with expanded blood volume rather than depressed cardiac function, plays an important role in regulating the cardiac ANP synthesis in the diabetic WKY rats.

Elevated blood volumes have been observed in type I diabetic patients28 as well as diabetic rats.2 Örtoła et al7 recently reported the relation between elevated plasma IR-ANP levels and glomerular hyperfiltration in the early stage of STZ diabetic rats, and they attributed the elevated IR-ANP level to the increased blood volume. We expanded their study by actually measuring the blood volume and hemodynamic parameters and assessed cardiac ANP mRNA and IR-ANP synthesis as well as plasma IR-ANP levels. Because the serum creatinine level was not altered throughout the experimental period, we suggest that the increase in blood volume does not result from the compromised renal function but is associated with an attempt to achieve osmotic equilibrium with the body cells against the serum hyperosmolarity in the diabetic status.

Hypertension leads to a substantial increase in ventricular ANP mRNA levels.14–16 Our study indicated that SHRs had a significant increase in ventricular ANP mRNA levels along with the development of hypertension, consistent with a previous report.14 Interestingly, the combination of diabetes and hypertension induced an earlier and greater effect in stimulating cardiac ANP synthesis than did either disease alone. These changes in the combined diseases were evident as early as 4 weeks after the induction of diabetes and were more pronounced at 8 weeks. In proportion to the changes in ANP synthesis, the biventricular end-diastolic pressures also were significantly elevated at 4 weeks and remained higher thereafter. In contrast to the diabetic WKY rats, the left ventricular peak dP/dt in the diabetic SHRs was already depressed at 8 weeks, and their blood volume was not different from that in the age-matched control SHRs throughout the experimental period. In addition, we observed complete reversal of the elevated ANP synthesis and hemodynamic abnormality in the diabetic SHRs after effective insulin therapy. There was a positive relation between ventricular filling pressures and ventricular IR-ANP levels during insulin therapy. These findings suggest that the elevated intraventricular filling pressure, caused by the STZ-induced diabetic effect on hypertensive heart, is important in the regulation of cardiac ANP synthesis, as is the case in the diabetic WKY rats, and that the increased ANP synthesis is not a result of STZ-induced cardiac toxicity. However, unlike the diabetic WKY rats, it appears that the earlier depression in cardiac performance by the combination of diabetes and hypertension, rather than the changes in blood volume, is more closely involved in the elevated filling pressure. The lack of increase in blood volume found in the diabetic SHRs may be in part attributed to negative sodium balance caused by pressure-induced natriuresis, as observed in hypertension.29

Diabetes mellitus is known to result in myocardial abnormalities in both clinical and experimental settings. Isolated working hearts from diabetic rats showed a decreased ability to respond to increased filling pressures30; papillary muscles isolated from diabetic rats had a depressed velocity of shortening and a delayed onset of relaxation.5 Initially, it was thought that the cardiovascular disease was due to the high incidence of atherosclerosis affecting the large arteries and capillaries. Recently, however, various experimental studies have suggested that a specific cardiomyopathy might explain the increased mortality and morbidity of diabetes. The pathogenesis of this diabetic cardiomyopathy is complex and may depend on a depressed myocardial myosin and actomyosin–Ca2+-ATPase,31 shifting in the predominant myosin isozyme subtype from V1 to the less...
active V$_3$, and increased metabolism of fatty acids in the myocardium. In the present study, we were unable to identify histologically prominent degenerative lesions in the diabetic WKY rats. This finding is in agreement with the observations by Hashimoto$^{27}$ and Factor et al.$^{12,13}$ The first suggestion that hypertension may be causally related to diabetic cardiomyopathy came from the experiments of Factor et al$^{12,13}$ on two-kidney, one-clip renovascular hypertensive rats. They provided evidence that the combination of diabetes and hypertension of 8-week duration produces significantly greater interstitial fibrosis and myocyte degeneration than either disease alone. In contrast, Hashimoto$^{27}$ described significant fibrosis changes observed after 12 months, when diabetes was induced in the SHRs. Our study also shows that no cardiomyopathic histopathological changes are detected in the diabetic SHRs over 12 weeks after STZ administration and confirms this previous study. Our findings suggest that the changes in ANP synthesis and hemodynamic parameters occur before the recognizable cardiomyopathic histopathological changes. The biochemical changes such as the shifting in myosin isozyme$^{31,32}$ may be closely involved in the depressed cardiac function observed in the present study. Microangiopathy, which is not easily evaluated in histological sections and is aggravated by the presence of hypertension, may also be potentially responsible for the changes in the diabetic heart. Reversibility with insulin could support the notion that depressed function does not result from irreversible structural change in the myocardium.

In addition to the correlation between the ventricular end-diastolic pressure and ventricular ANP, it is of interest to note the time course of their changes during insulin therapy. As shown in Figures 2 and 3, 2-week insulin therapy induced a complete reversal of biventricular ANP mRNA levels in the diabetic WKY rats, despite a partial reversal of biventricular end-diastolic pressures. In the diabetic SHRs, although the left ventricular end-diastolic pressure was unchanged by 2-week insulin therapy, the left ventricular ANP mRNA level was significantly decreased. These findings may imply that ventricular ANP gene expression requires a persistent stimulation of elevated filling pressure, possibly ventricular wall stretching, and responds sensitively to small changes in intraventricular pressures. On the other hand, we should also note the relation between the left ventricular end-diastolic pressures and ANP mRNA levels in the 4-week diabetic WKY rats and normal SHRs; the pressure value is equal, but SHRs have the more augmented ANP mRNA and IR-ANP levels. Because ventricular hypertrophy develops in this stage of SHRs, as shown in the increase in left ventricular weight-to-body weight ratio (Table 2), such structural change may be related to the maintenance of normal ventricular end-diastolic pressures. Initial increase of plasma ANP levels in this stage also could affect the normal ventricular end-diastolic pressures via the natriuretic and vasodilative effects of ANP. However, we cannot negate the possibility that other factors related to the development of hypertrophy in SHRs contribute to the augmented ANP synthesis. Further studies are needed to clarify the mechanism(s) responsible for the ANP synthesis, especially in the ventricles.

In contrast to large changes in ventricular IR-ANP and mRNA detected in diabetic rats, very small changes were observed in atrial levels in the diabetic WKY rats and SHRs. This finding is in agreement with the recent observation by Arai et al$^{14}$ that ventricular ANP mRNA levels in the SHR–stroke prone rat strains increase 30 times more than those in the WKY rats, whereas no significant change is observed in their atrial ANP mRNA and IR-ANP levels. The relatively smaller response in atrial ANP mRNA levels, as compared with those in ventricles, is shown in other experimental ventricular hypertrophic rats$^{15,34}$ and cardiomyopathic hamsters. In cardiomyopathic hamsters with severe congestive heart failure, atrial IR-ANP levels are decreased by 50%, whereas at the ultrastructural level, their atrial cardiocytes show a pattern of intense secretory activity. Thus, the modest changes in atrial IR-ANP and mRNA levels may reflect the condition in which atrial cardiocytes are maximally stimulated, possibly because of rapid generation and release, and gene expression is not increased to a level proportional to demand. Whether the small change in ANP gene expression is due to a different response of atrial and ventricular ANP in the diabetic heart or to a negative feedback effect of increased plasma ANP levels on atrial ANP remains to be determined.

We observed decreased thyroid hormone levels in the diabetic rats, thereby confirming earlier data. Previous studies documented that thyroid hormones stimulated cardiac ANP synthesis and secretion in cultured atrial and ventricular cardiocytes. The plasma IR-ANP levels were reduced in patients with hypothyroidism, and correcting hypothyroidism normalized the plasma levels. Thus, because the thyroid hormone may be important in stimulating ANP synthesis and secretion in vivo and in vitro, further studies would be needed to assess the exact role of this hormone in ANP synthesis in the diabetic state. Total RNA extracted from either the atria or ventricles of diabetic WKY rats and SHRs contained the same size of a hybridizing RNA band as did that of the controls. Our gel chromatographic analyses revealed that the major portion of IR-ANP in either atria or ventricles of diabetic WKY rats and SHRs represented the high molecular weight prohormone (data not shown), indicating that the heart in diabetic WKY rats and SHRs is capable of synthesizing and storing the prohormone.

In summary, our study demonstrates that cardiac ANP synthesis in the diabetic WKY rats is stimulated by elevated intraventricular filling pressures caused by both expanded blood volume and depressed cardiac performance. The combination of diabetes and hypertension induces an earlier and greater effect in
stimulating cardiac ANP synthesis than does either disease alone, which is caused mainly by the depression in cardiac performance and occurs before the recognizable cardiomyopathic histopathological change. Reversibility with insulin therapy indicates that the STZ-induced diabetic alterations observed in cardiac ANP synthesis and hemodynamic parameters result from insulin-deficient diabetic cardiomyopathy, not from cardiac toxicity of STZ. This suggests that the cardiac ANP system is associated with the abnormality in fluid and electrolyte balance in diabetes mellitus and that it functions as a cardiac compensatory mechanism against elevation in intra-ventricular filling pressures via its natriuretic and vasodilative actions.

References


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