Augmented Natriuretic Peptide-Induced Guanylyl Cyclase Activity and Vasodilation in Experimental Hyperglycemic Rats

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ABSTRACT—The present study was aimed to investigate whether hyperglycemia may alter the regulation of vascular natriuretic peptide receptors (NPR). The hyperglycemia was induced in rats by the treatment with streptozotocin (50 mg/kg, i.v.). The expression of different subtypes of NPR was determined in the thoracic aorta by reverse transcriptase-polymerase chain reaction and quantitative in vitro receptor autoradiography. The isometric tension and the guanylyl cyclase activity of the isolated thoracic aorta in response to natriuretic peptides were also determined. Following the treatment with streptozotocin, the plasma concentration of atrial natriuretic peptide (ANP) was significantly increased. The expression of NPR-A was increased, while that of NPR-C was reduced. The receptor binding study demonstrated an increased maximal binding capacity of NPR, with its affinity not significantly altered. The magnitude of vasodilation and guanylyl cyclase activity in response to ANP was significantly increased. On the other hand, the vasodilator response as well as the tissue formation of cGMP in response to acetylcholine or sodium nitroprusside was significantly reduced. These results indicate that the hyperglycemia may cause an altered regulation of vascular NPR.

Keywords: Hyperglycemia, Atrial natriuretic peptide, Natriuretic peptide receptor, Guanylyl cyclase activity

Diabetes mellitus may be complicated by various vascular dysfunctions. An impairment of nitric oxide (NO)-dependent vasodilation has been demonstrated in rats with experimental diabetes induced by streptozotocin treatment (1, 2). The regulation of vascular natriuretic peptide (NP) is also altered in association with diabetes (3, 4). The biological action of NP is mediated by three distinct subtypes of NP receptors (NPR), i.e., NPR-A, NPR-B and NPR-C. NPR-A and NPR-B are linked to particulate guanylyl cyclase, resulting in a secondary formation of cGMP (5, 6), whereas NPR-C acts in the metabolic clearance of NP (7). Therefore, the overall activity of the NP system may be determined by the tissue expression of different subtypes of NPR as well as the tissue levels of NP. However, mechanisms underlying the altered vascular NP system have not been established.

The present study was aimed to examine whether there is an altered regulation of vascular NPR in association with hyperglycemia. The hyperglycemia was induced in rats by the treatment with streptozotocin, and the expression of NPR-A and NPR-C mRNA was determined in the thoracic aorta by the reverse transcription-polymerase chain reaction (RT-PCR). The guanylyl cyclase activity and isometric tension in response to NP were also determined. In vitro quantitative autoradiography was performed to characterize NPR.

MATERIALS AND METHODS

Animals
Male Sprague Dawley rats (180 – 250 g) were injected intravenously with streptozotocin (50 mg/kg). The rats treated with vehicle served as the control. The animals were killed by decapitation at 2, 4, 7 and 14 days after the injection, and the trunk blood was collected for plasma atrial natriuretic peptide (ANP) determination. All procedures were approved by the Institutional Ethics Committee on Experimental Animal Care and Use.

NPR-A and NPR-C mRNA expression
The thoracic aorta taken was quickly stored at −80°C until the assay. Total RNA was isolated from the aorta according to the protocols of the Ultraspec™ RNA isolation system (Biotec Laboratories, Houston, TX, USA). RNA concentration was determined by the absorbance
read at 260 nm. The mRNA expression of NPR-A was determined by RT-PCR. For RT, 1 μg total RNA was incubated with reverse transcriptase (Gibco BRL, Grand Island, NY, USA; 200 U), RNASin (10 U), dNTP mix (10 mmol/L), DTT (0.1 mol/L), MgCl₂ (25 mmol/L), oligo(dT) (0.5 μg/μl) and reaction buffer [200 mmol/L Tris-HCl (pH 8.4), 500 mmol/L KCl] in a final volume of 20 μl at 42°C for 50 min. After a final denaturation at 72°C for 5 min, 4 μl cDNA was subjected to PCR amplification.

PCR was conducted in a final volume of 20 μl containing 10 pmol of each primer, dNTP mix (250 μmol/L), MgCl₂ (1.5 mmol/L) and Taq polymerase (1 U) using a thermal cycler (MJ Research, Watertown, MA, USA). The profiles of PCR consisted of 30 cycles at 95°C for 30 s, 62°C for 30 s and 1 min elongation step at 72°C for NPR-A and NPR-C. For β-actin, 35 cycles at 94°C for 45 s, 56°C for 45 s and a 1 min 30 s elongation step at 72°C. The final extension was ended with 5 min of elongation at 72°C. Their primers were adopted as described by previous investigators (5, 8). The PCR products were size fractionated by 2% agarose gel electrophoresis and visualized under ultraviolet light with ethidium bromide staining. ANP, NPR-A and β-actin cDNAs were quantified by ImagerTM 1D MAIN (Bioneer, Cheongwon, Korea). The level of NPR-A or NPR-C cDNA was normalized by that of β-actin cDNA.

**Binding studies**

Receptor binding studies were performed in cryostat sections. After freezing the aortic preparations with liquid nitrogen, serial 20-μm sections were made, thaw-mounted onto gelatin-chrome-alum coated slides, and stored at −20°C until used. [125I]-Labeled rat ANP was prepared as described by Cho et al. (9). For localization of ANP binding sites, the binding of [125I]-ANP to the aorta sections was done as described previously in the kidney (10, 11). The sections were rinsed with 150 mM NaCl – 0.5% acetic acid (pH 5.0) at room temperature for 10 min in order to remove the endogenous natriuretic peptides (12). After preincubation for 50 min in 30 mM phosphate buffer (pH 7.2) containing 120 mM NaCl and 1 mM phenanthroline, the sections were then incubated with [125I]-ANP in fresh preincubation buffer containing 40 μg/ml bacitracin, 100 μg/ml phenylmethylsulfonyl fluoride and 0.5% bovine serum albumin at room temperature for 60 min. In order to characterize NPR, the competitive inhibition of [125I]-ANP binding to the aortic sections was examined in consecutive sections by co-incubating with various concentrations (1 or 10 pM to 10 μM) of unlabeled ANP. After incubation, the sections were rinsed in cold distilled water at 4°C and quickly dried under a stream of cold air.

Autoradiographic images were generated by exposing the slides with dried [125I]-ANP-labeled sections to Hyperfilm-3H (Amersham, Buckinghamshire, UK) and analyzed with Power Macintosh 8100/80AV computer. The optical densities of whole aortic cross-sectional areas were measured in every section and displayed into specific binding densities in amol/mm².

**Cell membrane preparation and guanylyl cyclase activity measurement**

Membrane preparation of the thoracic aorta was obtained and the particulate guanylyl cyclase activity was assayed as described previously (13). The protein concentration was determined by bicinchoninic acid assay kit (BioRad, Hercules, CA, USA). Rat ANP, porcine brain NP (BNP) or C-type NP (CNP) was added to the membrane protein preparations. After 15 min, the reaction was stopped with cold solution containing 50 mM sodium acetate (pH 5.0) and centrifuged at 1,000×g for 10 min at 4°C. cGMP contents were measured in the supernatant. Results were expressed as pmol·min⁻¹·mg protein⁻¹.

The ring preparations were exposed to the indicated drugs for 5 – 20 min, and they were homogenized in 1 ml of 10% trichloroacetic acid and centrifuged at 2,500×g at 4°C. The pellet was used for protein assay and the supernatant was extracted with water-saturated ether. The [125I]-radioimmunoassay kit (New England Nuclear, Boston, MA, USA) was used to determine cGMP contents.

**Recording isometric tension**

The thoracic aorta was taken and its ring preparation was made to record the isometric tension. It was precontracted with 10⁻⁴ M phenylephrine, and the responses to vasodilators including ANP, acetylcholine and sodium nitroprusside (SNP) was examined.

**Drugs and statistics**

Rat ANP, porcine BNP and CNP were purchased from Peninsula Laboratories (Belmont, CA, USA). All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless stated otherwise. The number of ligand-binding sites of different affinities and their apparent dissociation constants (Kd) and maximal binding capacities (Bmax) were derived from Scatchard analysis using the LIGAND iterative model-fitting computer program (14). Data are presented as means ± S.E.M. in each group. Comparisons between the groups were made by Student’s t-test.

**RESULTS**

**Expression of NPR-A and NPR-C**

Following the streptozotocin-treatment, the blood glucose concentration was increased and the body weight progressively decreased (Table 1). The plasma concen-
tration of ANP measured at day 14 was significantly higher in the experimental hyperglycemic rats than that in the control (37.1 ± 3.9 vs 25.2 ± 3.4 pg/ml, n = 6 each, P < 0.05). The vascular expression of NPR-A mRNA was significantly increased, while that of NPR-C mRNA was markedly reduced (Fig. 1).

**Characterization of 125I-ANP binding**

Figure 2 demonstrates the binding of 125I-ANP in the thoracic aorta. The unlabeled ANP displaced the 125I-ANP binding. Non-specific binding was less than 10% of the total binding. Peptides other than ANP, such as angiotensin II and arginine vasopressin, did not affect the 125I-ANP binding (data not shown).

Increasing concentrations of unlabeled ANP resulted in a monophasic displacement of 125I-ANP binding (Fig. 3). Following the treatment with streptozotocin, the maximal binding capacity was progressively increased with the binding affinity unaltered (Fig. 3, Table 2).

**ANP-induced vasodilation and particulate guanylyl cyclase activity**

Figure 4 shows the vasodilation responses to ANP. The degree of vasodilation was augmented in the experimental hyperglycemic rats, in which IC50 decreased from the basal value of 15.9 ± 4.6 to 11.2 ± 2.4, 4.2 ± 0.9, 2.7 ± 0.4 and 1.9 ± 0.3 nM at days 2, 4, 7 and 14, respectively.

ANP increased the guanylyl cyclase activity in a dose-dependent manner, the magnitude of which was greater in the experimental hyperglycemic rats (Fig. 5). However, EC50 did not significantly differ among the groups: 11.0 ± 2.9, 19.2 ± 4.6, 14.8 ± 5.3, 12.1 ± 7.2 and 21.9 ± 3.7 nM at days 0, 2, 4, 7 and 14, respectively. BNP (10−6 M) and CNP (10−6 M) also increased the guanylyl cyclase activity, the magnitude of which was increased in the experimental hyperglycemic rats (Fig. 6).

### Table 1. Blood glucose levels and body weight after injection of streptozotocin

<table>
<thead>
<tr>
<th>Days</th>
<th>Blood glucose (mg/dl)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>101 ± 12</td>
<td>238 ± 8</td>
</tr>
<tr>
<td>2</td>
<td>523 ± 55*</td>
<td>232 ± 5</td>
</tr>
<tr>
<td>4</td>
<td>541 ± 38*</td>
<td>224 ± 8</td>
</tr>
<tr>
<td>7</td>
<td>325 ± 26*</td>
<td>214 ± 10</td>
</tr>
<tr>
<td>14</td>
<td>540 ± 29*</td>
<td>198 ± 7*</td>
</tr>
</tbody>
</table>

Numbers of rats are 6 each. *P < 0.05, compared with day 0.

**Fig. 1.** Representative fluorographs showing the ethidium bromide-stained agarose gels containing reverse transcription-polymerase chain reaction products of NPR-A and NPR-C. Lower panels show the mean ± S.E.M. of the densities. C, control rats; E, experimental hyperglycemic rats. Numbers of rats were 6 each. *P < 0.05, **P < 0.01, compared with the control.
Acetylcholine- and SNP-induced vasodilation and cGMP increase

Acetylcholine caused a relaxation of phenylephrine-precontracted aortic rings in both control and experimental hyperglycemic groups, the degree of which was attenuated in the latter (IC$_{50}$: 1.8 ± 0.4 vs 12.8 ± 2.4 nM, Fig. 7). SNP also caused a relaxation of the phenylephrine-precontracted aortic rings, of which the degree was significantly reduced.
in the experimental hyperglycemic rats (IC\textsubscript{50}: 2.3 ± 0.6 vs 10.0 ± 2.5 nM, Fig. 7). Both acetylcholine and SNP increased the tissue cGMP contents, the degree of which was, however, significantly reduced in the experimental hyperglycemic rats (Fig. 8).

**DISCUSSION**

The plasma concentration of ANP was increased following the treatment with streptozotocin. Accordingly, the NP-induced vasodilation and tissue accumulation of cGMP
was augmented. RT-PCR revealed an increased expression of NPR-A mRNA, and quantitative in vitro receptor autoradiography demonstrated an increased binding capacity of NPR. Therefore, the increased particulate guanylyl cyclase activity may reflect an upregulation of biologically-active NPR. The markedly reduced expression of NPR-C mRNA, on the other hand, suggests that NPR-A and NPR-C should be differentially regulated. In addition, the decreased expression of NPR-C as well as the increased expression of NPR-A may contribute to the augmented vasodilator response to NP.

Our finding may be contradictory to that of Kamata et al. (2), in that the vasodilator response to ANP was not significantly altered in the diabetic rats. However, in their experiment, the extent and duration of hyperglycemia was greater than that in the present study. During the initial hyperglycemic stage, there may have been a transient increase of vascular response to ANP.

On the contrary, the vascular relaxation and cGMP production in response to SNP, an endothelium-independent vasodilator, were significantly reduced. While some previous investigators demonstrated a reduced response to SNP as in the present study (12, 15, 16), others observed an unaltered vasodilation response (2, 17). The discrepancy may be accounted for by the differences in diabetic duration and vascular species.

It has been suggested that the vascular cGMP accumulation mainly depends on NO/soluble guanylyl cyclase activity and only to a minor extent on NP/particulate guanylyl cyclase activity (18). Although the role of particulate guanylyl cyclase activity may be minor in contributing to the overall vascular accumulation of cGMP, it may become apparent in the presence of a primary decrease of the NO/soluble guanylyl cyclase system. It was observed that the renal response to ANP was increased when NO/soluble guanylyl cyclase system was chronically inhibited (19). Similarly, in deoxycorticosterone acetate-salt-induced hypertension, the activity of particulate guanylyl cyclase...
was augmented, while that of soluble guanylyl cyclase was attenuated (20). On the contrary, in two-kidney, one clip hypertension, the particulate guanylyl cyclase activity remained unaltered, while soluble guanylyl cyclase activity was increased (20). More recently, Hussain et al. (21) also observed a sensitive response to ANP of the aorta from endothelial NO synthase-knockout mouse and an enhanced ANP response in the rat chronically treated with inhibitors of either NO synthase or soluble guanylyl cyclase. Taken together, a primary impairment of soluble guanylyl cyclase activity may result in an augmentation of particulate guanylyl cyclase activity.

In summary, the vascular expression of NPR-A is up-regulated, while that of NPR-C is downregulated in experimental hyperglycemic rats, along with an enhanced guanylyl cyclase activity and vasodilation in response to ANP. Its pathophysiological significance remains to be further determined.

Acknowledgments

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REFERENCES