Downregulation of the atrial natriuretic peptide/natriuretic peptide receptor-C system in the early stages of diabetic retinopathy in the rat

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Purpose: Atrial natriuretic peptide (ANP) is a known vascular antipermeability and antiangiogenic factor, but its possible alteration during the early stages of diabetic retinopathy has not yet been explored. The present study sought to investigate the expression of ANP and its receptors using a model of streptozotocin (STZ) induced diabetes in the rat.

Methods: Diabetes was induced in male Wistar rats by an intraperitoneal injection of STZ. Age matched animals served as control. One and 3 months after the onset of diabetes, the expression of ANP mRNA and that of its receptors (NPRA, NPRB, NPRC) and the immunoreactive ANP was quantified in retinal tissue by quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) and radioimmunoassay, respectively. The locations of ANP and glial fibrillary acidic protein (GFAP) in normal and diabetic retinas were also established by immunohistochemistry.

Results: No alteration in the gene expression of the retinal natriuretic peptide system was noted after 1 month of diabetes. However, 3 months after the onset of diabetes, significantly diminished ANP and NPRC mRNA levels were detected in the retina of diabetic rats compared to controls, while NPRA, NPRB mRNA levels remained unchanged. At this time point, retinal ANP concentrations were significantly diminished in the diabetic rats compared to control rats. However, at 1 month retinal ANP concentrations in diabetic retina were similar to control rats. Diabetes caused the downregulation of ANP protein expression in the layers of the retina at 3 months after the induction of diabetes. ANP immunoreactivity was detected in the cell bodies of the astrocytes and in their processes enveloping vessels.

Conclusions: The downregulation of ANP and NPRC in retinas of diabetic rats suggests a role for this peptide in experimental diabetic retinopathy. Further studies should address the possible involvement of the ANP/NPRC system in the pathophysiology of diabetic retinopathy.

Diabetic retinopathy is the most common microvascular complication suffered by patients with long standing type 1 diabetes [1]. Clinically, diabetic retinopathy can be divided into the stages: background and proliferative retinopathy. Background diabetic retinopathy is characterized by increased vascular permeability and progressive vascular occlusion [2]. Retinal ischemia due to capillary nonperfusion causes the compensatory formation of new blood vessels that enter the vitreous space. This second proliferative stage of diabetic retinopathy leads to visual impairment through bleeding or retinal detachment by accompanying fibrous tissue [3].

Currently, there are no good animal models of human diabetic retinopathy [2,4] although there are some similarities at the molecular level. Rodents are a poor but acceptable [2,4] in vivo model of nonproliferative diabetic retinopathy. In both human and rodents, the diabetic retinal vasculature upregulates cell adhesion molecules and shows an increased number of leukocytes, which adhere to the retinal vessels and trigger capillary occlusion, endothelial cell injury and death, and blood-retinal barrier breakdown [5-8].

Recent findings related to the atrial natriuretic peptide (ANP) suggest this protein might be worth evaluating in models of diabetic retinopathy. In vitro, ANP exerts an antigrowth effect on endothelial cells [9]. ANP also acts as an anti-permeability factor by inhibiting the signalling functions of vascular permeability factors such as vascular endothelial growth factor (VEGF) and by preserving the functional morphology of endothelial cell tight junctions [10]. Moreover, ANP was one of the first described endogenous inhibitors of both the synthesis and angiogenic function of VEGF in cultured endothelial cells [11,12]. ANP inhibits VEGF transcription and protein production in cultured human vascular endothelial cells via the NPRC receptor [11]. Moreover, ANP has been found to inhibit the activation of several key signaling molecules (these include ERK, JNK, and p38 members of the MAP kinase family) with a role in VEGF induced angiogenesis, mediating their actions through both NPRA and NPRC receptors [12].

ANP is a member of the natriuretic peptide (NP) family, a group of cardiovascular cyclic peptide hormones with diuretic, natriuretic, and vasodilatory properties [13]. Three main natriuretic peptide receptors (NPR) have been cloned and named alphabetically: natriuretic peptide receptor A (NPRA), natriuretic peptide receptor B (NPRB) and natriuretic peptide receptor C (NPRC) [13]. Most of the biological effects of ANP are mediated by the guanylate cyclase coupled A-receptor
Wistar rats of 7 to 8 weeks of age and weighing 200-250 g. A total of eighty male animals and experimental diabetes:

1. The expression of ANP and its receptors, (NPRA, NPRB, and NPRC) in relation to expression profiles in nondiabetic rats warrants evaluation.
2. In the eye, the presence of immunoreactive ANP has been demonstrated in rat and rabbit retinas and in rabbit choroid tissue by radioimmunoassay (RIA) [17,18]. ANP has been immunohistochemically detected in the outer and inner plexiform layers of the retina in the rat and rabbit [19]. Moreover, ANP binding sites have been identified in rat and rabbit retinas by binding assay and autoradiographic procedures [20,21]. The presence of mRNA transcripts encoding the three natriuretic peptide receptors (NPRA, NPRB, and NPRC) has also been demonstrated in the retina of the rat and rabbit by polymerase chain reaction (PCR) [22]. Very recently, we reported the expression of NPR transcripts along with ANP, BNP, and CNP mRNA in the human retina and were able to detect NPs in the neural retina, glial, and vascular elements of the normal adult retina [23]. We have also found that the levels of ANP in the vitreous humor of diabetic patients with and without proliferative diabetic retinopathy were significantly higher than those in nondiabetic subjects. ANP was localized within epiretinal proliferative tissue in diabetics [24]. Glial reactivity is a frequent nervous system response to injury [25]. A common marker of reactive gliosis is the well described increased expression of glial fibrillary acidic protein (GFAP) [26]. In the Müller cells of the retina, de novo GFAP expression was found in experimental [27] or spontaneous human diabetic retinopathy [28]. It is well known that astrocytes, the glia confined to the innermost region of the retina, produce factors capable of modulating blood flow, vascular permeability, and cell survival [29]. In a previous study, we demonstrated the presence of ANP and GFAP in the astrocyte cell body and in astrocyte processes enveloping vessels in the innermost layers of the healthy human retina [23]. Given that retinal astrocytes produce ANP, the possibility of altered ANP and GFAP immunoreactivities in the retinal glial cells of diabetic rats warrants evaluation.

To explore some of the issues described above, we used a streptozotocin (STZ) induced diabetes model in the rat to evaluate: (1) the expression of ANP and its receptors, (NPRA, NPRB, and NPRC) in relation to expression profiles in nondiabetic rats by real time quantitative reverse transcription-polymerase chain reaction (RT-PCR) in the retina; (2) the ANP protein expression in control and diabetic retina by radioimmunoassay; (3) the influence of the diabetic state on these expression profiles in the retina; and (4) the distribution patterns of ANP and GFAP immunoreactivity in the different retinal layers of the control and diabetic rat by immunohistochemical techniques.

METHODS

Animals and experimental diabetes: A total of eighty male Wistar rats of 7 to 8 weeks of age and weighing 200-250 g (Charles River Laboratories, Wilmington, MA) were used for these experiments. Principles of laboratory animal care were followed (NIH publication number 85-23, revised 1985; Public Health Service Policy on Humane Care and Use of Laboratory Animals and Spanish “Real Decreto 223/1988 de 14 Marzo; BOE 18 Marzo 1988”). All experimental protocols were approved by the Research Committee at the Hospital Clínico San Carlos (Madrid, Spain) and the Dirección General de Agricultura de la Comunidad de Madrid. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich, Co. St. Louis, MO, 75 mg/kg body weight), and the control animals received the buffered vehicle (0.1 M citrate, pH 4.5). The criteria for diabetes included persistent hyperglycemia (>25 mM), glycosuria (when the glucose is detected in urine), polyuria (>150 ml/day), and impaired growth (reduction of 28% and 52% body weight at 1 and 3 months after the induction of diabetes).

After the injection of STZ, decapitation was used to kill 17 diabetic rats at 1 month, and 17 diabetic rats at 3 months. Seventeen control rats were also killed at the end of each period. Since pentobarbital was reported to elicit changes in the rat plasma and atrial ANP system, the rats were killed by decapitation. After the decapitation, the right common carotid artery was clamped and then, it was dissected and cannulated in order to obtain the plasma samples from the right common carotid artery from the brachiocephalic trunk. The blood samples were collected into prechilled plastic test tubes containing 100 µl 7.2% EDTA and proteases inhibitors at the following final concentrations: 10 µM phenyl-methyl-sulphonylfluoride (PMSF), 5 µM pepstatin A and 10 µM Trasylol. Just before killing the animal, blood glucose levels were measured by the glucose-oxidase method to confirm diabetic status. The eyes were enucleated and the retinas were quickly excised under a surgical microscope. The retinal tissue from each rat was placed in an eppendorf tube, snap frozen in liquid nitrogen, and stored at -80 °C until total RNA extraction and the rest was used for measurement of ANP by RIA.

In addition, six 3-month diabetic rats and six age matched nondiabetic rats were anesthetized by intramuscular injection of a “cocktail” made up of ketamine (50 mg/ml, Ketolar; Parke Davis, Grupo Phizer, Madrid, Spain), Diazepam (10 mg/ml, Valium, Roche, Barcelona, Spain), and atropine (1 mg/ml, Braun, Barcelona, Spain) in the following volume proportions: 1:1:0.5, respectively. The cocktail was diluted 1:1 with NaCl (0.9%) before injection. The dose used was 10 ml/kg body weight. Animals were perfused transcardially with cold, 50 mM phosphate buffered saline (pH=7.4), followed by 4% neutral buffered paraformaldehyde. Perfusion pressure was kept between 80 and 85 mm Hg. The eyes were enucleated, postfixed in 4% neutral buffered paraformaldehyde for 4 h, cut perpendicularly to the vitreal surface, dehydrated and embedded in paraffin. The tissue was sectioned at 4 µm and dried on snowcoat X-tra slides (Surgipath, Winnipeg, Manitoba, Canada) for immunohistochemistry.

Measurement of ANP in retinal tissue and plasma samples: Pooled retinas from one rat were homogenized in 0.1 M ace-
tic acid containing proteases inhibitors at the following final concentrations: 10 μM PMSF, 5 μM pepstatin A, and 27 μM EDTA. After centrifugation at 10,000 x g for 20 min at 4 °C, the supernatant was stored at -70 °C. The blood samples were centrifuged at 3,000 rpm for 20 min at 4 °C and the plasma was aspirated and stored at -70 °C until assayed. ANP immunoreactivity was measured by a specific and sensitive RIA, as outlined previously [30]. The intra- and interassay coefficients of variance were 10.2% and 18.7%, respectively.

**Protein assay:** The protein content of the retinal tissue was determined by the method of Lowry [31] using albumin as standard.

**Statistical analysis:** The Kolmogorov-Smirnov and Shapiro-Wilk test were used to confirm the assumption of normality of variables. Body weight, glucose levels and ANP concentrations in plasma and retina samples were displayed as mean±standard deviation. Data were statistically analyzed with the Mann-Whitney U tests for comparison of diabetic and control groups at 1 and 3 months after injection of STZ. SPSS (version 11.0; SPSS Inc., Chicago, IL) was used for the statistical analysis.

**RNA extraction:** Total RNA was extracted from both retinal and auricular tissues. The RNA concentration was spectrophotometrically determined.

**Real-time quantitative RT-PCR:** Following DNase treatment, first-strand cDNA was synthesized using 2.5 μM random hexamers and 1.25 U/μl multiscrIBE reverse transcriptase (RT; Applied Biosystem; Foster City, CA) according to the manufacturer’s instructions. Real-time quantitative RT-PCR analysis was performed using an automated sequence detection instrument (Prism 7700 Sequence Detector; Applied Biosystems) for the real-time monitoring of nucleic acid green dye fluorescence (SYBR Green I; Applied Biosystems Inc.). Total RNA from the auricle was used for the positive control (Primer Express, Applied Biosystems). Details of the primers and the GenBank Accession Numbers are given in Table 1.

PCR was performed as previously described [23] using a kit (Applied Biosystems Inc. Foster City, CA). All reactions were performed in duplicate. For each primer pair, nontemplate controls were included to check for significant levels of contaminants and the formation of primer-dimers, which interfere with quantification when SYBR Green is used as the fluorescence dye. Agarose gel electrophoretic analysis was used to check whether the amplified products corresponded to the size predicted for cDNA fragments of the ANP and its receptors (NPRA, NPRB, and NPRC).

**Data analysis:** Data were analyzed using the relative standard curve method as described in the PE Applied Biosystems User Bulletin number 2. Standard curves were generated in parallel for the target (ANP, NPRA, NPRB, and NPRC) and for β-actin (endogenous control) using cDNAs synthesized from serial 1:10 dilutions of a RNA sample, prepared by pooling a fraction of the RNAs of all individual samples included in this study. For each experimental sample, the amount of target and endogenous reference is determined from the appropriate standard curve. The normalized values for ANP and NPR mRNA were calculated by dividing the mean amount of target by the mean β-actin value for each sample. Relative ANP_N, NPR_N mRNA values for the target samples were plotted as a fraction of the normal retina calibrator value, which was arbitrarily set at 1. The results were confirmed in a minimum of three consecutive experiments. Test for statistical significance were performed using Mann-Whitney U tests for two independent samples.

**Immunohistochemical detection of ANP and GFAP:** Immunohistochemical staining was performed as previously described [23]. Rabbit polyclonal antibody to rat ANP (the generous gift of Dr. J. Gutkowska, Montreal, Canada), and a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP, clone GA5, Sigma-Aldrich) were used. Briefly, deparaffinized and hydrated sections were incubated in blocking solution TBT (Tris Base Saline (TBS) 0.5 M, pH 7.4, containing 3% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Triton X-100) for 30 min at room temperature to reduce nonspecific binding. The tissue sections were subjected to a preliminary heat induced antigen retrieval step. This involved pressure cooker heating in a 0.01 M sodium citrate solution prior to incubation with the primary antibody. Sections were incubated overnight at 4 °C in a humidified chamber with the anti-ANP rabbit polyclonal antibody at 1:200 dilution and an anti-glial fibrillary acidic protein (GFAP) mouse monoclonal

<p>| Table 1. Sequence of PCR primer pairs for the ANP and its receptors |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
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<tr>
<th>Primer name</th>
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<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Amplicon length (bp)</th>
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<td>CAATGGAACAGACCTCCTAA</td>
<td>CACGTTGGCCAAGGCGCTGT</td>
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<td>NPRC</td>
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<td>CTTGTTAAACCGGCGAATGAA</td>
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<td>NPRA</td>
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<td>ANP</td>
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<td>CAATGGAACAGACCTCCTAA</td>
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<td>94</td>
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<tr>
<td>β-actin</td>
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<td>CTGCGGAAAGGAACGCTCA</td>
<td>GCTGCTGCTGCCCTGGAT</td>
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**Table 2. Body weight, plasma glucose concentrations, and plasma IR-ANP concentrations**

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<tr>
<th>Experimental group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Glucose (mM)</th>
<th>IR-ANP (pg/ml)</th>
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<tr>
<td>1 month after STZ injection</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>STZ diabetic rats</td>
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<td>223.7±5.2</td>
<td>26.4±0.8</td>
<td>56.8±11.3</td>
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<td>Age matched control rats</td>
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<td>390.6±5.1</td>
<td>6.5±0.9</td>
<td>53.8±7.8</td>
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<tr>
<td>3 months after STZ injection</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ diabetic rats</td>
<td>12</td>
<td>277.5±8.9</td>
<td>30.4±0.8</td>
<td>60.0±12.5</td>
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<tr>
<td>Age matched control rats</td>
<td>12</td>
<td>501.3±2.6</td>
<td>7.2±0.7</td>
<td>63.3±15.1</td>
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</table>

Body weight, plasma glucose concentrations, and plasma IR-ANP concentrations at both 1 and 3 months after injection of 75 mg STZ/kg body weight or vehicle in diabetic and control rats. The values are mean±standard deviation. Asterisks (*) indicate statistical significance (p<0.001, Mann-Whitney U test) for the comparison to age matched control rats.
antibody at a 1:400 dilution. The slides were washed for 5 min in TBS. Immunodetection was performed using biotinylated anti-rabbit and anti-mouse immunoglobulins followed by alkaline phosphatase conjugated streptavidin and a fuchsin chromogen (kit LSAB2; DAKO Corp., Carpinteria, CA) for red staining. The sections were lightly counterstained with Mayer’s hematoxylin. Final mounting was done in the water soluble medium Glicergel (DAKO).

Negative control slides were incubated either with primary antibody preabsorbed with 10 nM ANP, 10 nM GFAP or with normal rabbit serum instead of the primary antibody. Specimens of atrium were used to assess the immunohistochemical detection of ANP.

Image analysis: Images were captured using Leica Qwin image processing and analysis software (Leica Microscopy Systems, Heerbrugg, Switzerland) on a personal computer linked to a high resolution video camera (Leica DC100) mounted on a microscope (Zeiss, Germany). The degree and pattern of immunostaining both within and between specimens was assessed by two masked observers (both of whom obtained similar results). The intensity of staining was graded qualitatively as background (corresponding to the level of staining seen in the negative controls), weak, moderate, or intense (corresponding to the highest level of immunoreactivity), each of these being recorded as 0, 1, 2, and 3, respectively. For each retinal specimen, staining intensity was recorded with NFL representing nerve fiber layer, GCL representing ganglion cell

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Figure 1. Detection of NPRs and ANP gene expression in the rat retina. Messenger RNA was extracted from the rat retina, reverse transcribed, and amplified by quantitative real-time-PCR. Gene specific primers in retinal tissue were used for NPRA (lane 2), NPRB (lane 3), NPRC (lane 4), and ANP (lane 5). Single bands of the predicted molecular size for NPRA (98 bp), NPRB (149 bp), NPRC (71 bp), and ANP (94 bp) transcripts were detected. The gel also shows a DNA molecular marker (lane 1) and a nontemplate control (lane 6).

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Figure 2. Amounts of ANP and NPR mRNA in the STZ diabetic rat retinas relative to the age matched control rat retinas. Relative quantification of ANP (A), NPRA (B), NPRB (C), and NPRC (D) gene expression in control and diabetic rat retinas 1 and 3 months after the administration of citric buffer or streptozotocin (STZ). Estimates of ANP and NPR mRNA levels were obtained from 12 rat retinas from each group (total of 48 retinas) using real-time quantitative RT-PCR. The normalized values for ANP and NPR mRNA were calculated by dividing the mean target mRNA quantity by the mean β-actin value for each retina. Relative ANP_{n} and NPR_{n} mRNA values for the target samples are plotted as a fraction of the normal retina reference value, which was arbitrarily set to 1. Error bars represent standard deviations based upon 3 separate experiments. Asterisks (*) mark statistically significant differences from control (p<0.05, Mann-Whitney U test).
layer, IPL representing inner plexiform layer, INL representing inner nuclear layer, OPL representing outer plexiform layer, ONL representing outer nuclear layer, IS representing photoreceptor inner segments, and OS representing photoreceptor outer segments. An average score was then calculated for each retinal layer within each group.

RESULTS

Body weight, blood glucose levels, and ANP plasma concentrations: Table 2 shows the variables determined. The initial body weights for the two groups were not significantly different (control, 200.8±9 g, n=12; diabetes, 204.5±9 g, n=12; Mann-Whitney U test, p>0.05). However, at the established time points of 1 and 3 months after diabetes induction, the body weight of the diabetic rats was significantly lower than that recorded for the age matched controls (Mann-Whitney U test, p<0.001). Blood glucose levels at 1 and 3 months were significantly higher in the diabetic rats compared to the controls rats (Mann-Whitney U test, p<0.001). In contrast, diabetic and control animals showed similar plasma ANP concentrations at both time points (Mann-Whitney U test, p<0.05).

ANP retinal concentrations: Retinal ANP concentrations at 3 months after diabetes induction were significantly diminished in the diabetic rats (48.0±3.9 pg/mg protein; n=5) compared to control rats (64.8±4.1 pg/mg protein; n=5). However, at 1 month, retinal ANP concentrations in diabetic rat retinas (63±3.2 pg/mg protein, n=5) were similar to control rats (70±4.6 pg/mg protein, n=5; Mann-Whitney U test, p<0.05).

Quantification of ANP, NPRA, NPRB, and NPRC mRNAs in the retina: To validate real-time PCR, standard curves with r>0.98 and slope values in the range -3.1 to -3.4 were required, indicating a near 100% reaction efficiency (E). The real-time detection of dsDNA enabled us to construct a dissociation curve at the end of the PCR run, by ramping the sample temperature from 60 °C to 95 °C while continuously collecting fluorescence data. The curves of the melting profiles for the natriuretic peptide receptors and the housekeeping gene indicated no accumulation of primer dimers (data not shown). Figure 1 shows the bands obtained after electrophoretic separation of the PCR products of the three NPR (NPRA, NPRB, and NPRC) and ANP mRNAs. These bands were the expected sizes (98 bp, 149 bp, 71 bp, and 94 bp, respectively).

Figure 3. ANP and GFAP immunoreactivities in the control and diabetic rat retinas. Photomicrographs of control retina showing ANP (A) and GFAP (C) immunostaining and of diabetic retina after 3 months of diabetes showing ANP (B) and GFAP (D) immunostaining. ANP and GFAP immunoreactivities could be observed on astrocytes and their processes around blood vessels in adjacent sections of the retina (A-C, arrows). Negative controls for the immunohistochemical detection of ANP (E) were free of labeling. All the sections were counterstained with haematoxylin and photographed at a magnification of 160x. The photoreceptor outer segments (OS), photoreceptor inner segments (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), and nerve fiber layer (NFL) are labeled in E.
Our results showed that all the NP receptors were expressed in rat retinal tissue. The NPR expression profile normalized for β-actin was fairly uniform in the retinal specimens. No alteration in the gene expression of the retinal NP system was noted after 1 month of diabetes. However, 3 months after the onset of diabetes, significantly diminished ANP and NPRC mRNA levels (<2.0 fold; Mann-Whitney U test, p<0.05) were detected in the retina of diabetic rats compared to controls, while NPRA, NPRB mRNA levels remained unchanged (Figure 2).

Localization of ANP and GFAP immunoreactivity in the retina: In control animals, ANP was specifically and intensely immunolabeled in the nerve fiber, ganglion cell and inner and outer plexiform layers of the retina. Labeling was prominent in the cytoplasms of ganglion cells and astrocytes of the inner retina. ANP labeling was also detected in the inner nuclear layer and in scattered cells of the outer nuclear layer. Because of the high cell density in the outer and inner nuclear layers, the morphology of individual cells could not be reliably assessed (e.g., amacrine cells, horizontal cells, and bipolar cells). The inner and outer segments of the rods and cones were not labeled (Figure 3A). Cells of the retinal pigment epithelium showed intense ANP immunolabeling in the normal retina (data not shown). At three months, both control and diabetic rats showed intense ANP immunoreactivity in the cytoplasm of endothelial cells lining the blood vessels of the innermost retinal layers. Diabetes caused the downregulation of ANP immunolabeling in all the retinal layers (Figure 3B). Table 3 provides details of our immunohistochemistry results.

Normal rat retinas revealed GFAP immunolabeling in cells and their processes of the inner layer of the retina, especially in the inner limiting membrane, nerve fiber and ganglion cell layers and around blood vessels. According to their location and morphology, these GFAP positive cells were interpreted as being retinal astrocytes. (Figure 3C). In an adjacent section (Figure 3A), ANP immunoreactivity was also detected in cell bodies of the astrocytes and in their processes enveloping ves-Table 3. Mean intensity of ANP staining in the retina

<table>
<thead>
<tr>
<th>Retinal layer</th>
<th>Control retina</th>
<th>Diabetic retina</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFL</td>
<td>2.10±0.60</td>
<td>1.10±0.79</td>
</tr>
<tr>
<td>GCL</td>
<td>2.43±0.45</td>
<td>1.00±0.74</td>
</tr>
<tr>
<td>IPL</td>
<td>1.95±1.0</td>
<td>0.99±0.80</td>
</tr>
<tr>
<td>INL</td>
<td>1.84±0.98</td>
<td>1.21±0.54</td>
</tr>
<tr>
<td>OPL</td>
<td>1.75±1.0</td>
<td>1.14±0.60</td>
</tr>
<tr>
<td>ONL</td>
<td>0.95±0.74</td>
<td>0.80±0.58</td>
</tr>
</tbody>
</table>

The intensity of retinal ANP staining was graded qualitatively as background (0; the level of staining seen in the negative controls), weak (1), moderate (2), or intense (3; the highest level of immunoreactivity). The values in the table are the mean intensity with standard deviation. For each retinal specimen, staining intensity was recorded for nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL). An average score was then calculated for each retinal layer within each group.

DISCUSSION

The findings of this study indicate that STZ induced diabetes in rats caused a time dependent decrease in the gene expression of both ANP and NPRC accompanied by diminished protein expression of ANP in retinal tissue.

No significant differences were observed in the ANP immunoreactivity of plasma samples from diabetic and nondiabetic rats. In patients with type I diabetes, plasma ANP concentrations are increased [32] or unaltered [33] compared to nondiabetic subjects. In rats with chronic STZ induced diabetes, plasma ANP levels are increased [34] or unaltered compared to nondiabetic rats [35]. However, the plasma ANP levels recorded in diabetic patients and in rat models of diabetes in the different studies have been inconsistent, probably because of differences in metabolic conditions [32-35].

Herein, we detected the gene expression of ANP in the normal rat retina for the first time. In previous molecular studies on the rat retina [22,36], we were able to identify BNP and CNP mRNA but not ANP mRNA by RT-PCR. These differences in our findings most likely reflect the different methodologies used. Indeed, the present study differs from the report by Gaspar et al. [36] in that here we used real time quantitative RT-PCR, whose sensitivity is presumably higher than that of conventional RT-PCR. Moreover, this study demonstrates that in addition to ANP, its receptors NPRA, NPRB, and NPRC are also expressed in the normal rat retina. In agreement, Fernandez-Durango et al. [22] identified mRNA transcripts encoding the three NP receptors in the retina, choroid and ciliary processes of the rat and rabbit eye using the conventional RT-PCR technique.

Our real time quantitative RT-PCR findings indicated that a 1-month period of STZ induced diabetes was insufficient to modify the expression of the retinal NP system. However, 3 months after the onset of diabetes, ANP and NPRC mRNA levels were found to be significantly reduced in the retina of animals with diabetes. Moreover, at this same time point, retinal NPRA, NPRB mRNA levels were unaltered. In agreement, immunoreactive ANP concentrations in retinal tissues were significantly diminished in diabetic rats only after 3 months of STZ injections compared to age matched control rats. Those results suggest that ANP expression is decreased in rat diabetic retinas 3 months after the onset of diabetes.

Our immunohistochemistry results confirm that ANP is located in the nerve fiber, ganglion cell, and inner and outer plexiform layers of the control retina. ANP labeling was also detected in the inner nuclear layer and in scattered cells of the outer nuclear layer. The inner and outer segments of the rods
and cones were not labeled. Cells of the retinal pigment epithelium showed intense ANP immunolabeling in normal retinas. In previous studies performed on human retinas, we observed similar ANP location [23]. ANP immunoreactivity has previously found in the inner and outer plexiform layers of the rat retina [19,37]. However, Cao et al. [37] did not show ANP immunoreactivity in ganglion cells and astrocytes and their process around blood vessels in the inner retina in rats. The differences among results might be accounted for by different fixing procedures and the utilization of different antisera. As ANP is a very labile peptide with a high rate of degradation [13], perfusion of the rat before killing was performed in the present study to obtain staining in neural tissue. The tissue sections were also subjected to a heat induced antigen retrieval step to unmask the antigen in the sections.

Three months after the onset of diabetes, we observed the downregulation of ANP immunolabeling in all the layers of the retina, but most prominently in the nerve fiber and ganglion cell layers. Thus, STZ induced diabetes led to significantly reduced ANP mRNA levels in the retina accompanied by diminished ANP immunostaining in its innermost layers. The mechanism and causal factors responsible for this decrease in ANP expression in the retina are unclear. One possibility is that the decrease could be related to the well documented neuronal, glial and vascular cell loss through apoptosis that occurs in the retina of humans with diabetes mellitus and rats with experimental diabetes [38,39]. Moreover, ANP induces apoptosis in rat cardiac myocytes [40] and endothelial cells [41], while ANP was found to protect rat PC 12 cells from apoptosis [42], suggesting the highly cell-type specific regulation of apoptotic processes exerted by ANP. Further work is needed to establish whether the decreased expression of retinal ANP in STZ induced diabetes is related to retinal cell apoptosis.

In the early stage of diabetic retinopathy, reduced ANP expression observed here is in clear contrast to the increased expression of other vasoactive peptides such as endothelin-1 (ET-1) [43] and VEGF [44]. The finding that in cultured endothelial cells, ET-1 and endothelin-3 (ET-3) upregulate VEGF production and that VEGF increases ET-1 mRNA and protein expression [11,45] suggests that interaction between both peptides may contribute to the vascular alterations of human and experimental diabetes. These data must be reconciled with recent reports indicating that ANP acts through both NPRC and GC receptors (NPRA and NPRB) to inhibit the synthesis and angiogenic function of VEGF in cultured bovine aortic endothelial cells [11,12]. ANP also inhibits the production and secretion of ET-1 from cultured aortic endothelial cells while prolonging the half life of the ET-1 transcript, via NPRC [46]. We could thus hypothesize that the decrease in the expression of both ANP and its receptor NPRC in the retina could contribute to an increase in both ET-1 and VEGF expression that occurs in the retina of diabetic rats [43,44]. Indeed, it has been reported that the exposure of A-10 smooth muscle cells to ET-1 leads to the downregulation of NPRC and associated adenyl cyclase signalling [47].

ANP inhibits adenylate cyclase through NPRC receptor [15]. Moreover, increased cAMP levels block the pro-angiogenic effects of several factors, such as migration, proliferation and in vitro angiogenesis of human umbilical vein endothelial cell (HUVEC) induced by VEGF and basic fibroblast growth factor (bFGF) [48,49], and in vivo angiogenesis stimulated by bFGF in the chick chorioallantoic membrane (CAM) assay [50,51]. The capacity of cAMP to block angiogenesis stimulated by both types of factors implies that this second messenger affects a common mechanism for angiogenesis, such as the reorganization and migration of the endothelial cells. Further studies will need to investigate the possible alterations of the adenyl cyclase system and its possible correlation with the changes in ANP/NPRC system in the diabetic retina.

On the other hand, exogenous ANP acts as an anti-permeability factor, inhibiting the signalling functions of vascular permeability factors such as VEGF, and preserving endothelial cell tight junction functional morphology in “in vitro” assays [10]. Indeed, transgenic mice overexpressing ANP show significantly reduced vascular permeability factor induced kinase activation and vascular permeability compared to nontransgenic littermates [10]. Retinal vascular permeability is increased in diabetic rat 1-2 weeks after STZ injection [52]. However, our results showed that the decrease in gene expression of both ANP and NPRC in retinas occurs at three months after the onset of STZ induced diabetes. Therefore, it is not reasonable to think that those changes could be involved in the development of the early diabetic blood-retinal barrier breakdown in experimental diabetes. Further studies will need to investigate the possible role of ANP in blood-retinal barrier breakdown in early diabetes.

Our immunohistochemical tests showed that GFAP immunoreactivity in control rat retinas was limited to the astrocytes [27] whereas in diabetes, Müller cells acquire prominent GFAP immunoreactivity through the extension of their processes [28]. We also report ANP immunoreactivity in retinal astrocytes for the first time suggesting that ANP may play a role in glial function. Similarly, we previously detected ANP immunoreactivity in the cell bodies of the astrocytes and in their processes enveloping the vascular elements of the normal human retina [23]. ANP and BNP binding are found on primary astrocytes and glial cell lines leading to cyclic GMP production [53]. ANP immunoreactivity is detected in astrocytes and neurons of the human cerebral cortex, cerebellum, and inferior olivary complex [54]. Interestingly, the finding that glial ANP (mainly in astrocytes) is increased in brain infarction [55] led to the suggestion that this peptide could regulate cerebral blood flow, blood-brain barrier permeability, or cerebrospinal fluid volume. Given the similarity of the blood-brain and blood-retinal barriers, future studies will be needed to establish whether ANP is able to regulate retinal blood flow and blood-retinal barrier permeability. Indeed, astrocytes increase both vascular endothelial cell barrier function and tight junction protein synthesis, most likely due to the release of soluble heat labile factors [56].
In summary, the expression of ANP and its receptors in control retinas and the location of immunoreactive ANP in different layers of the retina and in astrocytes suggest a physiologic role for ANP in this tissue. Furthermore, the significantly diminished ANP and NPRC mRNAs levels detected in the retina of diabetic rats three months after the onset of STZ-induced diabetes compared to controls suggest that retinal ANP and NPRC downregulation could play a crucial role in the development of diabetic retinopathy.

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