

Opposite regulation of brain and C-type natriuretic peptides in the streptozotocin-diabetic cardiopathy

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ABSTRACT

C-type natriuretic peptide (CNP), a recent addition to the family of natriuretic peptides including atrial and brain natriuretic peptide (ANP, BNP), is believed to be an endothelium-derived vasodilator and to have an antimitotic effect. ANP and BNP concentrations are increased in conditions such as congestive heart failure, but cardiac CNP concentrations have not been investigated in this connection. Diabetes mellitus also involves myocardial dysfunctions without coronary artery disease or systemic hypertension. We therefore investigated the cardiac expression of CNP mRNA compared with that of BNP mRNA in streptozotocin (STZ)-diabetic rats. STZ-diabetic male Wistar rats ($n=6$) were studied in comparison with controls ($n=6$). The animals were characterised by their mean arterial blood pressure and plasma glucose concentrations. After extraction of total cardiac RNA, a specific cDNA probe of BNP was used for northern blot analysis, whereas myocardial CNP

expression was analysed by an RNase-protection assay. Twelve weeks after diabetes was induced, the rats were normotensive (96.4 ± 2.0 compared with 95.1 ± 1.9 mmHg) and hyperglycaemic (615 ± 61 compared with 165 ± 21 mg/dl; $P < 0.001$). Left ventricular pressure was significantly impaired (76.8 ± 6.4 compared with 51.2 ± 3.6 mmHg). STZ-diabetic rats had a 3.2-fold increase in cardiac BNP expression compared with controls. In contrast, cardiac CNP mRNA concentrations were decreased 2.6-fold.

CNP seems to be downregulated like other peptides with antimitotic and vasodilator activities (nitric oxide, prostacyclin, kinins). This may contribute to cardiac dysfunction in diabetes mellitus and suggests that stimulation of CNP expression could provide cardiac protection in such cases.

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INTRODUCTION

Diabetes mellitus (DM) shows a markedly increased incidence of cardiovascular pathology that leads to hypertension, endothelial micro- and macroangiopathy, renal failure and myocardial dysfunction (Litwin *et al.* 1990). Some of these changes may be related to a dysregulated synthesis and release of vasoactive peptides in the blood vessel wall (Roth *et al.* 1983). Vasoconstrictor systems such as the renin-angiotensin system (RAS) (Brown *et al.* 1997) are believed to be activated during DM. Their most important pathophysiological role may be in the remodelling of cardiac and vascular tissue by

inducing hypertrophy, vascular hyperplasia and the deposition of extracellular matrix proteins, involving direct mitogenic effects (Weber *et al.* 1994).

The natriuretic system regulating salt and water balance and opposing the action of the RAS and/or endothelin system has been investigated for many years (Francis 1997). Plasma concentrations of the atrial natriuretic peptide (ANP) – the first member of the natriuretic peptide family – were found to be increased in patients with cardiac failure and/or DM (Tanabe *et al.* 1995). With respect to DM, in streptozotocin (STZ)-induced diabetic rats, ANP mRNA is upregulated in the cardiac ventricles (Wu

et al. 1998). In contrast, plasma concentration of brain natriuretic peptide (BNP) seems to be unaffected in acute hyperinsulinaemia (Tanabe *et al.* 1995). Nothing is known about cardiac BNP and the C-type natriuretic peptide (CNP) – the third member of this family – under diabetic conditions.

In man, CNP is highly expressed in distinct areas of the brain (Komatsu *et al.* 1991). CNP mRNA is also expressed in endothelium and weakly in human kidney and heart (Ogawa *et al.* 1995). In contrast to ANP and BNP, which are secreted in an endocrine fashion mainly from the heart, CNP is released in an autocrine/paracrine fashion from endothelial cells as an endothelium-derived vasodilator (Ogawa *et al.* 1995). Nevertheless, the systemic function of CNP is rather unclear. Patients with essential hypertension or congestive heart failure do not show altered plasma concentrations of CNP (Wei *et al.* 1993) whereas, in dogs, intravenous administration of CNP decreases the blood pressure (Clavell *et al.* 1993). As specific interaction between ligand and the guanylyl cyclase-coupled receptor B (GC-B) generates cGMP, mediating vasodilatation (Cerra 1994), CNP may have an important role in controlling vascular tone and vascular remodelling.

The aim of this study was to investigate the cardiac expression of CNP in DM and to compare its regulation with that of BNP in diabetic cardiopathy.

MATERIALS AND METHODS

Animal experiments

Experiments were performed in male Wistar rats weighing 300–330 g (Dr Karl Thomae, Biberach/Riss, Germany) and conformed with the Guidelines on the Handling and Training of Laboratory Animals.

DM was induced by a single intraperitoneal injection of STZ (70 mg/kg, diluted in 0.4 ml sodium citrate buffer (0.1 M, pH 4.5; Sigma, München, Germany) and hyperglycaemia was confirmed 48 h later by a reflectancemeter (Acutrend, Boehringer, Mannheim, Germany). We used only rats with blood glucose concentrations of more than 300 mg/dl 3 days after STZ injection ($n=6$). Rats treated with a single intraperitoneal injection of vehicle ($n=6$) were used as controls. For the measurement of mean arterial blood pressure (MAP) the animals were anaesthetised and a polyethylene catheter (PP10) was inserted through the femoral artery into the abdominal aorta at the 12th week after STZ injection.

In addition, for measurement of left ventricular pressure (LVP), the right carotid artery was cannulated with a specially constructed pig-tail catheter consisting of a PP20 tube. The pig-tail at the end was curved so as to guide the catheter into the ascending aorta. During the cannulation, the catheter was connected to a transducer (TD 860, Medex, Berlin, Germany) and the output signals were recorded by a personal computer-based recording and analysing system (MacLab/8s, WissTech, Spechbach, Germany). At the end of cannulation, blood samples were collected to determine the number of leucocytes, blood glycosylated haemoglobin (HbA_{1c}) content, haematocrit and plasma concentrations of sodium, glucose and total protein. Finally, the hearts were excised and rapidly frozen in liquid nitrogen and stored at -80°C .

Northern blot assay

Total RNA was extracted using TriZol reagent (LifeTechnologies, Karlsruhe, Germany) and following the manufacturer's directions. For each blot, 20 µg total RNA were loaded per lane and electrophoresed on a 0.8% agarose gel containing formaldehyde, transferred to Hybond-N membranes (Amersham, Freiburg, Germany) and immobilised by baking for 2 h at 80°C . For northern blotting the ^{32}P -labelled rat BNP-cDNA probe was used, with a probe for rat β -actin as a housekeeping gene. The density of the specific DNA bands were analysed on a Fujix BAS2000 (Fuji, Düsseldorf, Germany) phospho-imager system. The values obtained for β -actin by means of densitometry were used to correct data with respect to RNA loading.

Ribonuclease-protection assay

To detect cardiac CNP expression, an RNase-protection assay (RPA) was performed using the Ambion RPA II kit (ITC Biotechnology, USA), following the manufacturer's directions. Anti-sense RNA probes were generated by T7-polymerase transcription using linearised plasmids that contained fragments of CNP cDNA, or β -actin cDNA (ITC Biotechnology) as an internal control. The probes were radiolabelled with [^{32}P]UTP and approximately 5×10^4 c.p.m. of each probe were hybridised together with 30 µg total RNA per sample. Thus during RNase A/T1 digestion 369 bp and 150 bp respectively were protected from the CNP and β -actin RNA. The hybridised fragments were separated by electrophoresis on a denaturing gel. The density of the specific DNA bands were

analysed and calculated as in the northern blot analysis.

Statistical analysis of data

All data are expressed as means \pm s.e., and were analysed by Student's *t*-test. *P* values <0.05 were accepted as significant.

RESULTS

Characteristics of diabetic rats

Characteristically for this animal model, throughout the 12-week study period, STZ-treated rats displayed severe hyperglycaemia (615 ± 61 mg/dl compared with 165 ± 21 mg/dl in controls; $P < 0.001$), accompanied by a mild ketonuria. They also exhibited polydipsia (97.9 ± 4.4 compared with 22.1 ± 3.1 ml/24 h; $P < 0.001$), hypoproteinaemia (4.2 ± 0.6 compared with 6.2 ± 0.3 g/dl; $P < 0.01$), a loss of body weight (275 ± 10 compared with 489 ± 28 g; $P < 0.01$) and an increase in plasma HbA_{1c} concentrations (9.2 ± 0.8 compared with $2.6 \pm 0.5\%$; $P < 0.01$). No differences in plasma sodium (140.1 ± 2.2 compared with 139.3 ± 1.4 mM), haematocrit (41.6 ± 0.2 compared with 40.8 ± 0.3 vol%) and leucocytes (4.8 ± 0.2 compared with 5.1 ± 0.3 /nl) were observed between diabetic and control rats, indicating that all animals were euvoalaemic and that symptoms of infection or inflammation were absent. No change in MAP between the two groups was observed (96.4 ± 2.0 compared with 95.1 ± 1.9 mmHg), but the LVP was significantly impaired (76.8 ± 6.4 compared with 51.2 ± 3.6 mmHg; $P < 0.01$) in diabetic rats.

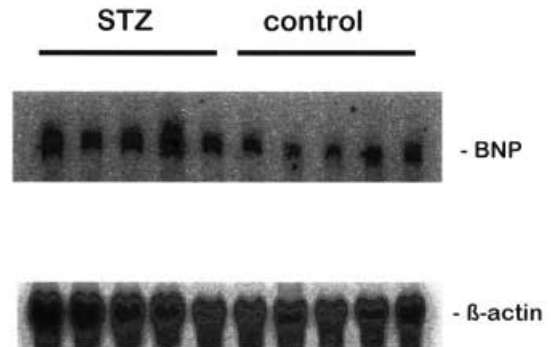
Cardiac BNP expression

BNP and β -actin mRNA were detectable in all samples of the northern blot, as shown in Fig. 1A. Quantification of the autoradiographic signals by densitometry and calibration using β -actin revealed an approximately 3.2-fold, significant, increase in the concentration of BNP mRNA in diabetic rats compared with the level of expression in control rats (STZ, 8.17 ± 1.24 ; controls, 2.52 ± 0.34 PSL/S Radiation density; $P < 0.01$) 12 weeks after STZ injection (Fig. 1B).

Cardiac CNP expression

Analysis of the RPAs demonstrated first an expression of CNP in normal rat hearts and secondly a significant decrease in the expression of

A



B

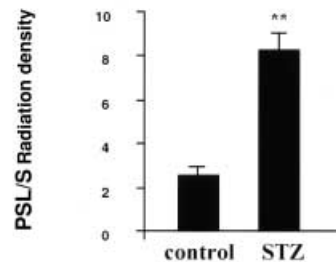


FIGURE 1. Increased myocardial expression of BNP at week 12 after the induction of diabetes. (A) Northern blot analysis from control and diabetic (STZ) animals showing the BNP band and β -actin as internal control. (B) Quantification of BNP expression in heart of control and diabetic rats after autoradiographic signal analysis. Data are shown as multiples after normalisation to β -actin mRNA levels ($n=5$ for both groups; values are means \pm s.e.M.). ** $P < 0.01$ compared with control group.

this CNP in STZ-diabetic rats compared with control rats (Fig. 2A). In contrast to BNP, CNP mRNA levels were decreased 2.6-fold (STZ, 2.31 ± 0.29 ; controls, 5.88 ± 1.12 PSL/S Radiation density; $P < 0.01$) 12 weeks after STZ injection (Fig. 2B).

DISCUSSION

The present study was conducted to investigate the influence of STZ on the regulation of two members of the natriuretic peptide family in the rat heart. Surprisingly, it revealed that STZ-induced diabetes caused an opposite regulation of BNP and CNP in

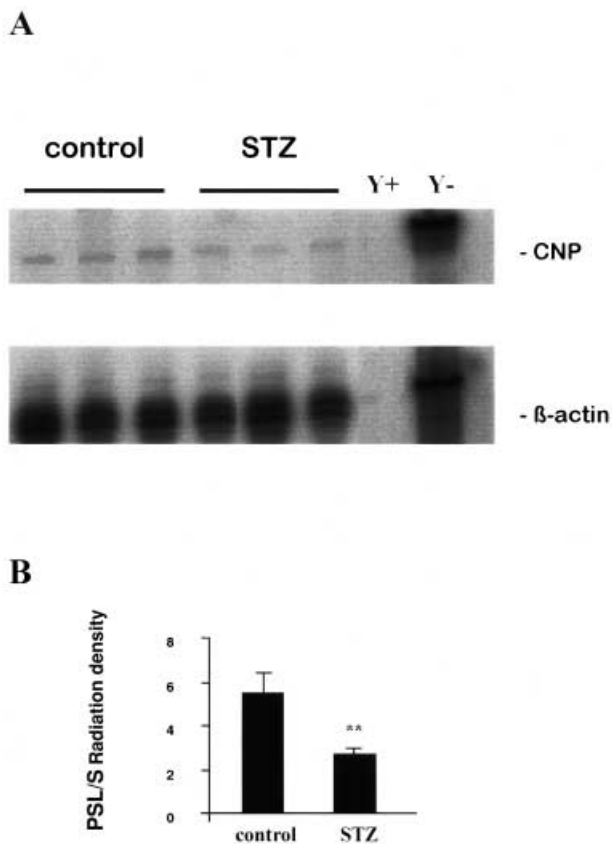


FIGURE 2. Reduced myocardial expression of CNP at week 12 after the induction of diabetes. (A) Representative RNase-protection assay analysis from control ($n=3$) and diabetic (STZ) ($n=3$) animals, showing expression of CNP (369 bp) and β -actin (150 bp). Y+, cDNA probes incubated with yeast RNA and RNase; Y-, cDNA probes incubated with yeast RNA without RNase. (B) Quantification of CNP expression in heart of control and diabetic rats after autoradiographic signal analysis. Data are shown as multiples after normalisation to β -actin mRNA levels ($n=5$ per group, values are means \pm s.e.m.). ** $P<0.01$ compared with control group.

the heart. Thus our findings of impaired synthesis of myocardial tissue CNP and an upregulation of BNP under diabetic conditions exclude the possibility that the three members of the natriuretic peptide family are acting equally in the heart.

Plasma concentrations of ANP and BNP are known to be increased in individuals with hypertension and/or cardiac failure. In tandem with ANP, BNP forms a dual control system, which has both diuretic and vasodilator properties. The principal stimulus to their release is right atrial distension or stretch, not pressure (Francis 1997). STZ-induced diabetes is not correlated with hypertension, but

with cardiac failure, including increased wall thickness (Fein *et al.* 1980). Thus the increase in BNP mRNA in our study was to be expected.

In congestive heart failure, plasma CNP concentration is unchanged compared with that in normal subjects (Wei *et al.* 1993). In addition, Shin *et al.* (1988) demonstrated an upregulation of the renal cortical and medullary CNP mRNA and urinary CNP excretion rates in STZ-induced diabetic rats that began 14 days after injection of STZ. As cardiac CNP cannot be associated with the postulated function of renally synthesised CNP as responsible for alterations in water and electrolyte homeostasis under diabetic conditions (Shin *et al.* 1988), other functions of CNP in the diabetic heart – for instance antihypertrophic effects – are likely and correlate with our findings. Thus our data and those of Shin *et al.* (1988) show an independent regulation of CNP in kidney and heart under hyperglycaemic conditions. Igaki *et al.* (1996) found that insulin suppresses endothelial secretion of CNP in cell culture. As we demonstrated a down regulation of cardiac CNP mRNA in STZ-induced insulin-deficient rats, our results also clearly show a heart-specific regulation of the peptide independent of the vascular system. However, we cannot distinguish between the impaired LVP or hyperglycaemia as the reason for the decreased expression of CNP mRNA.

An impaired cardiac production or function of the potent endogenous vasodilators in the systemic and coronary vascular beds such as the kinins, prostaglandin I_2 or nitric oxide have been suggested to contribute to disturbances in the regulation of coronary flow and the increased risk of myocardial infarction in DM. Therefore, endothelium-dependent relaxation based on the production of nitric oxide and subsequent increase in cGMP in the vascular smooth muscle cells is a prominent result of the interaction between CNP and its receptor. Impaired cardiac expression of CNP mRNA may contribute to these regulatory disturbances and may also be responsible for the more severe outcome of ischaemic heart disease in diabetic patients. However, further studies are necessary to clarify the role of cardiac CNP under diabetic conditions.

In conclusion, our results have shown that the cardiac natriuretic peptide-forming system is unequally regulated in untreated severely hyperglycaemic STZ-diabetic rats, indicating clearly the existence of two axes of the natriuretic peptide system. We suggest that these alterations in the natriuretic peptide system contribute to myocardial dysfunction in DM and that a stimulation of CNP expression could provide cardiac protection in cases of DM.

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