

Norepinephrine Release in the Heart Atria of Diabetic Rats

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Abstract. The content, release and uptake of norepinephrine (NE) in the sympathetic nerves of the rat heart atria were studied in the course of diabetes and in age-matched controls. Diabetes was induced by streptozotocin (STZ) and rats were subjected to further experiments 1, 4 or 7 months later (STZ1, STZ4, STZ7). Isolated atria were superfused with oxygenated Krebs-Henseleit (KH) solution. After equilibration, four 10-min fractions were collected: B1, basal release of NE; S1, potassium-evoked release (KER), where NE outflow was stimulated by depolarisation with 50 mmol/l KCl; B2, basal release of NE under the influence of the neuronal uptake blocker desipramine (DES); S2, KER under the influence of DES. The content of NE was measured by radioimmunoassay.

In STZ4 and STZ7 rats, NE concentrations were significantly lower in both atria compared to controls. B1 and S1 were significantly higher in STZ4 than in control atria. DES increased KER of NE in controls only. In contrast, DES caused a significant decrease in B2 and S2 in STZ4 atria, suggesting that a substantial portion of NE release was due to a calcium-independent carrier-mediated process. In experiments with calcium-free KH solution in fractions B2 and S2, KER in controls was nearly abolished. However, in STZ4 and STZ7 atria, S2 was still significantly higher than B2. In conclusion, the NE-releasing mechanism may be different in the chronically diabetic animals than in healthy subjects and may contribute to the decreased NE concentration in the STZ atria.

Key words: Norepinephrine — KCl-evoked release — Carrier-mediated release — Streptozotocin — Rat — Heart

Introduction

Diabetic autonomic neuropathy accompanies the later stages of diabetes mellitus and contributes significantly to the increased morbidity and mortality of diabetic patients (Burger et al. 1999). Multiple lines of evidence suggest that both sympathetic and parasympathetic branches of the autonomic nervous system are impaired

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in insulin-dependent diabetes mellitus (IDDM) in humans (Hilsted 1982; Eckberg et al. 1986; Lishner et al. 1987). Also in the well-defined experimental model of IDDM induced in rats by a single dose of streptozotocin, autonomic neuropathy has been clearly demonstrated by functional studies: Baroreceptor reflex-mediated tachycardia was shown to be blunted and reflex bradycardia enhanced (Kimball et al. 1992). In addition, resting bradycardia, a diminished circadian variation in the heart rate, and decreased cardiac sympathetic and parasympathetic control under resting conditions have been demonstrated in this animal model (Hicks et al. 1998).

However, quantitative data on the impact of diabetes on the sympathetic innervation of the mammalian heart seem to be controversial. Cardiac norepinephrine (NE) levels have been found to be increased (Paulson and Light 1981; Akiyama et al. 1989; Ganguly et al. 1987) or unchanged (Gando et al. 1993; Patel et al. 1997) in the earlier stages of the disease and decreased (Wisniewska and Wisniewski 1996; Schmid et al. 1999) or unchanged later (Felten et al. 1982; Vadlamudi and McNeill 1984; Zola et al. 1988). Also, NE turnover and/or release in the diabetic rat heart have been suggested to be enhanced (Ganguly et al. 1987), unchanged (Patel et al. 1997), or even reduced (Gando et al. 1993). Similarly, also the plasma levels of catecholamines have been reported to be increased (Paulson and Light 1981; Wisniewska and Wisniewski 1996) or unchanged (Christensen 1979). These apparent discrepancies may be related to the differences in experimental models, i.e., in the duration and severity of diabetes, in the gender, strain and age of animals, or in the differences in experimental protocols.

The purpose of this study was to evaluate how diabetes affects the content, release, and uptake of NE in cardiac sympathetic nerves in early and later stages of streptozotocin-induced diabetes in female rats, i.e., 1, 4 and 7 months after the onset of the disease. NE release from the heart atria was studied *in vitro* under basal conditions and after stimulation by a high extracellular potassium concentration.

Materials and Methods

Animals

Wistar female rats purchased from Velaz (Prague, Czech Republic) at the age of 50 days were used. The animals were housed five *per* cage, fed standard laboratory chow with free access to drinking water. All animals were left intact to adapt for 2 weeks before the initiation of the experiment and then were randomly divided into 6 groups. All experiments were approved by the University Committee for Experiments on Laboratory Animals and were conducted in accordance with the relevant Guidelines of the Czech Ministry of Agriculture for scientific experimentation on animals. Diabetes was induced by a single intravenous injection of streptozotocin (Sigma-Aldrich, USA; 65 mg/kg body weight) dissolved in citrate buffer (pH 4.5) and verified by severe hyperglycaemia. The plasma glucose

levels were measured before the administration of STZ and then weekly by an enzymatic method (Bio-La-Test, Lachema, Czech Republic). Only animals that had fasting glucose level continuously higher than 18 mmol/l were considered diabetic.

Three groups of diabetic rats, 1, 4 and 7 months after the onset of the disease (STZ1, STZ4 and STZ7, respectively) were studied. Control age-matched animals received a corresponding volume of vehicle and were designated Cont1, Cont4 and Cont7 for STZ1, STZ4 and STZ7 diabetic rats, respectively. NE concentrations were also measured in the control atria of 2-month-old rats (Cont0).

All chemicals, if not stated otherwise, were from Lachema (Czech Republic).

Tissue concentrations of NE

Rats were killed by cervical dislocation, their hearts were rapidly excised, rinsed with ice-cold saline and the right and left atria were dissected (RA, LA, respectively). Weighed samples were extracted in 0.4 mol/l HClO₄, centrifuged (5000 × g, 20 min) and NE concentrations were measured in supernatants by radioimmunoassay diagnostic kits (IBL, FRG).

Measurement of NE release in vitro

Rats were anaesthetised with ether and decapitated. The hearts were rapidly removed and placed into ice-cold oxygenated Krebs-Henseleit (KH) solution of the following composition (in mmol/l): NaCl 113, NaHCO₃ 25, KCl 4.75, CaCl₂ 2.5, MgSO₄ 1.19, glucose 11.1, Na₂ EDTA 0.029, ascorbic acid 0.289. Both atria were dissected, sliced, pooled (approx. 70–100 mg of tissue) and transferred into 0.5 ml perfusion chambers. Tissues were superfused at a rate of 0.15 ml/min with oxygenated (95% O₂ – 5% CO₂) KH solution. All perfusion experiments were performed at 37°C according to the scheme shown in Fig. 1A. NE release from the Cont0 atria in preliminary experiments is shown in Fig. 1B. After a 30 min equilibration period, seven 10-min fractions were collected at 0°C. Spontaneous release of NE was measured in fractions 1 and 5 (B1 and B2 respectively), i.e., in the course of perfusion by the KH solution containing 4.75 mmol/l KCl. After collection of B1 and B2 fractions, NE outflow was stimulated by depolarisation with a superfusion fluid containing 50 mmol/l KCl, in which the concentration of NaCl was reduced by 50 mmol/l, and NE concentrations were measured in fractions 3 and 7 (S1 and S2 respectively), i.e., in the course of perfusion by the modified KH solution containing 50 mmol/l KCl. NE concentrations in the superfusates were determined using radioimmunoassay diagnostic kits (IBL, FRG).

Effect of desipramine on NE release

The effect of the neuronal uptake blocker desipramine (DES) (Sigma-Aldrich, USA) on NE outflow was tested using a similar experimental protocol in all control groups and in STZ1 animals at two concentrations (10⁻⁷ mol/l and 10⁻⁶ mol/l), and in

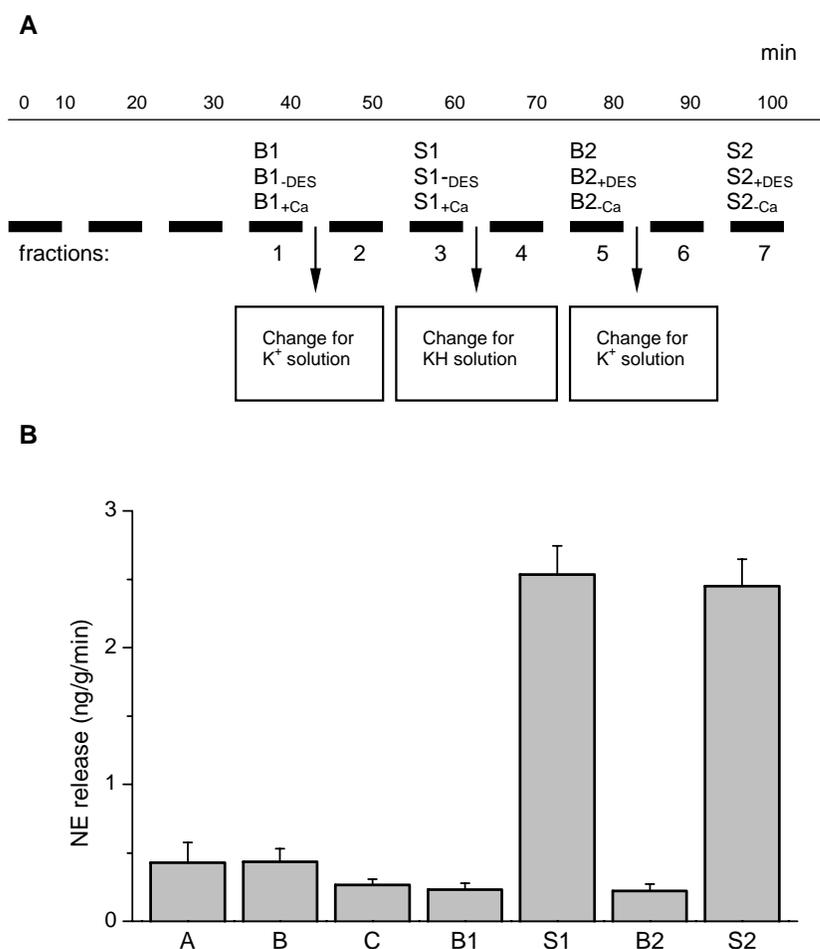


Figure 1. A. The scheme of the superfusion protocol. Perfusion chambers contained sliced atria of 2-month-old rats (70–100 mg tissue *per* chamber). After a 30-min equilibration period, 10-min fractions were collected. Immediately after the collection of fractions B1 and B2 (fraction No. 1 and 5), in which spontaneous norepinephrine (NE) outflow was measured, the superfusion fluid was changed for a solution containing 50 mmol/l KCl, and after 10-min mixed outflow (fraction No. 2 and 6), another 10-min stimulated fractions (S1 and S2) were collected (fraction No. 3 and 7). The *indices* describe substances present or absent (when in brackets) in the superfusion media: DES – desipramine (10^{-7} or 10^{-6} mol/l); Ca – calcium ions present (+) or absent (–) in the superfusion media. **B.** NE release from the heart atria in the control experiment. The three 10-min intervals during the equilibration period are designated A, B, and C.

STZ4 and STZ7 atria at a concentration of 10^{-6} mol/l. Spontaneous and stimulated NE outputs were measured in the absence (fractions B1_{-DES} and S1_{-DES}) and presence (fractions B2_{+DES} and S2_{+DES}) of DES in the superfusion fluid.

Release of NE under calcium-free conditions

In another set of experiments, calcium-free solutions were used in fractions B2 and S2 to differentiate between the calcium-dependent exocytotic NE release and the calcium-independent carrier-mediated release of NE. Spontaneous release under the calcium-free conditions (B2_{-Ca}) was measured using a modified KH solution of the following composition (in mmol/l): NaCl 118, NaHCO₃ 25, KCl 4.75, MgSO₄ 1.19, glucose 11.1, Na₂ EDTA 1, ascorbic acid 0.289. NE outflow in S2_{-Ca} fractions was determined by superfusion with the calcium-free solution, in which the concentration of KCl was increased to 50 mmol/l and that of NaCl was reduced correspondingly.

Data analysis

The NE outflow was then expressed in ng/g/min and NE tissue concentrations in ng/g wet weight. The composition of superfusion solutions did not interfere with the assay. Each value in the text and figures is the mean \pm S.E.M. of at least 4 experiments. The ratio between K⁺-evoked release (KER) in S1 and S2 fractions (S2/S1) was calculated for each experiment. Statistical analysis was performed by means of analysis of variance (ANOVA) with post hoc tests corrected for multiple comparisons by the Bonferroni's method, using the BMDP statistical package (ver. 2.0, Statistical Solutions, USA). The results were considered significantly different when $p < 0.05$.

Results*General*

There were no significant differences in body weights (BW) and blood glucose levels among all animal groups before STZ or vehicle injection. BW of control animals increased from 205 ± 4 g at the age of 2 months to 355 ± 10 g at the age of 9 months. BW of STZ rats did not significantly change in the whole course of experiment, being 195 ± 5 g at the age of 2 months (i.e. just before the STZ injection) and 198 ± 11 g at the age of 9 months (STZ7 group). Fasting blood glucose levels in control animals did not exceed 7 mmol/l. A substantial number of the diabetic rats either died (25%) or exhibited partial spontaneous recovery (35%) in the course of the study, which limited the numbers of rats in the experimental groups STZ4 and particularly STZ7 with respect to the control groups.

NE concentrations in the heart atria of control aging rats

The impact of age on NE concentrations in the heart atria in healthy animals is shown in Fig. 2.

In all age groups, NE concentrations were comparable in both atria. At the age of 2 months (Cont0), NE levels were 1594 ± 137 ng/g and 1552 ± 93 ng/g in the RA and LA, respectively. Compared to Cont0 values, NE tissue levels were not significantly different in any of the heart atria in 3- and 6-month-old animals (Cont1

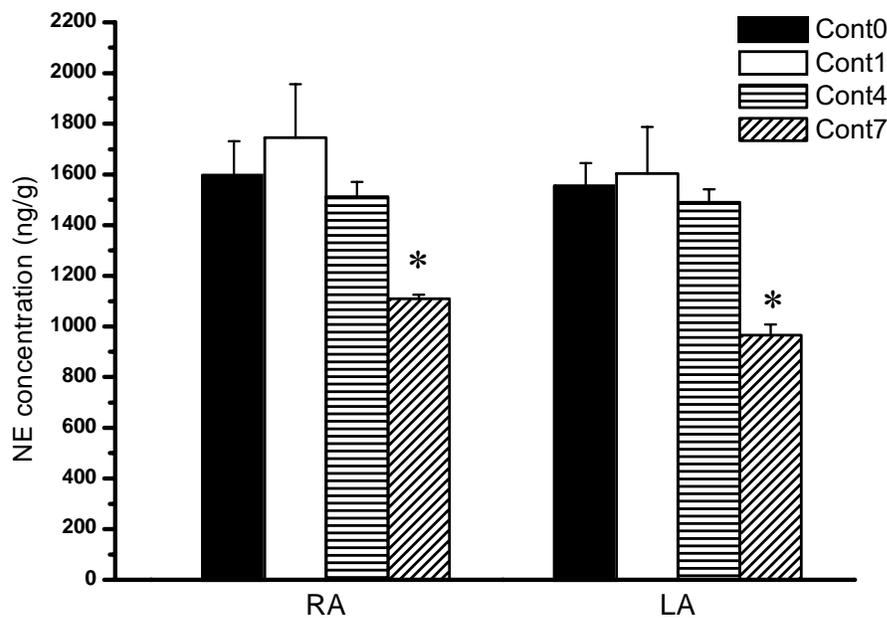


Figure 2. Norepinephrine (NE) concentrations in the heart atria of the control aging rats. RA – right atrium; LA – left atrium; Cont0, Cont1, Cont4, and Cont7 – intact 2-, 3-, 6; and 9-month-old rats. Error bars = S.E.M. * $p < 0.01$ compared to Cont0 values, $n = 6$ for each compartment and age.

and Cont4, respectively). However, NE concentrations in both atria significantly decreased at the age of 9 months (Cont7; $p < 0.05$, compared to Cont0).

NE concentrations in the heart atria of STZ-diabetic rats

The diabetic state significantly affected NE concentrations in the heart atria (Fig. 3). An initial significant increase in STZ1 atria ($p < 0.05$, compared to the respective controls) was followed by a sustained decline in tissue NE concentrations that became significantly different in STZ7 atria ($p < 0.05$, compared to Cont7 atria).

Spontaneous and stimulated NE release under control conditions

In the control experiments, the rate of spontaneous NE release (B1 and B2) did not change with age in all control groups and ranged between 0.199 ± 0.01 ng/g/min in Cont7 to 0.224 ± 0.01 in Cont1 atria. The rate of spontaneous NE release in STZ1 and STZ7 atria was not significantly different (0.239 ± 0.02 and 0.185 ± 0.03 ng/g/min) either from each other or from the control groups. In contrast, the rate of spontaneous NE release was significantly enhanced in STZ4 atrial samples (0.371 ± 0.03 ng/g/min).

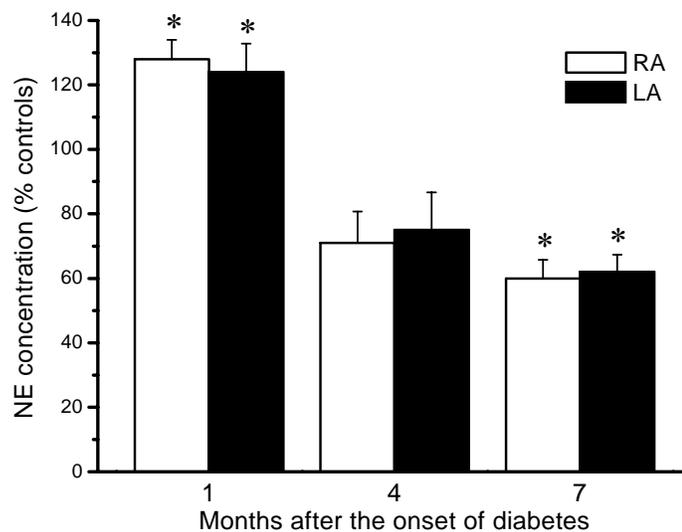


Figure 3. Norepinephrine (NE) concentrations in the atria of STZ-diabetic rats. NE concentrations in diabetic atria are expressed as % of the respective control values. Error bars = S.E.M. * $p < 0.05$ compared to the respective control, $n = 6$ for all controls and STZ1 and STZ4 animals, $n = 4$ for STZ7 animals.

The rate of KER of NE (S1 and S2) was significantly higher than the spontaneous output in all groups of control as well as diabetic animals (Fig. 4). The calculated ratios S2/S1 did not show any differences between control and STZ rats being 1.07 ± 0.1 in the control atria and 0.99 ± 0.2 in the STZ samples. However, there were significant differences in the absolute values of the release. In the STZ animals, KER of NE was significantly increased 1 and 4 months after the onset of the disease, and then it declined (when compared to age-matched controls).

Effect of DES on NE release

In the groups Cont1 and STZ1, two concentrations (10^{-7} and 10^{-6} mol/l) of DES were tested by adding the drug into the superfusion media at the time of collection of B2_{+DES} and S2_{+DES} fractions. In all preparations, DES did not influence the rate of spontaneous release of NE, but it dose-dependently enhanced KER of NE (S2_{+DES}) in both Cont1 and STZ1 atria. However, this effect was substantially smaller in STZ1 than in Cont1 samples, as indicated by the comparison of S2_{+DES}/S1_{-DES} ratios: S2_{+DES}/S1_{-DES} were 1.35 ± 0.09 and 1.89 ± 0.09 in Cont1 atria with DES concentrations 10^{-7} and 10^{-6} mol/l, respectively. In contrast, DES at a concentration of 10^{-7} mol/l was ineffective in STZ1 atria (S2_{+DES}/S1_{-DES} = 0.96 ± 0.14) and at a concentration of 10^{-6} mol/l it was less effective than in Cont1 atria (S2_{+DES}/S1_{-DES} = 1.55 ± 0.12). Nevertheless, DES at the higher concentration still significantly enhanced KER of NE in STZ1 atria.

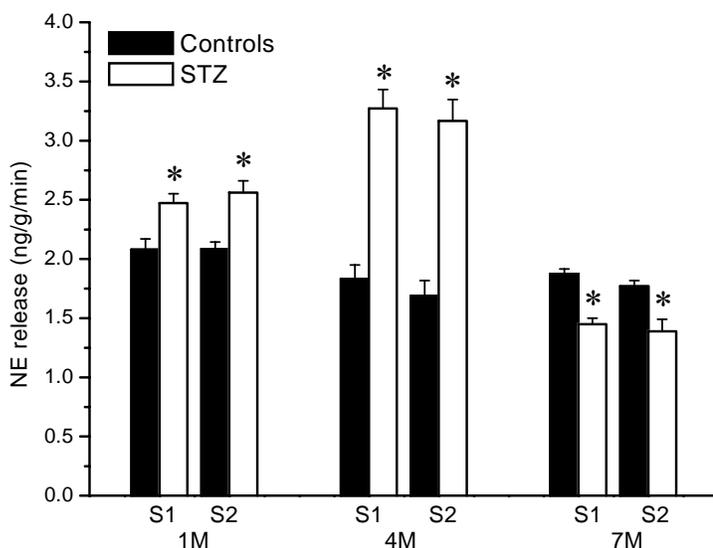


Figure 4. Norepinephrine (NE) release from the atria of STZ-diabetic rats. KER of NE during the first and second stimulation (S1 and S2, respectively) in preparations from STZ-diabetic rats 1, 4, and 7 months after the onset of diabetes (1M, 4M, 7M, respectively). Error bars = S.E.M. * $p < 0.05$ compared to the respective control value, $n = 5$ for all controls and STZ1 and STZ4 animals, $n = 4$ for STZ7 animals.

A completely different situation was observed in STZ4 samples, where DES (10^{-6} mol/l) significantly inhibited KER of NE. Interestingly, the spontaneous efflux of NE from STZ4 atria was significantly enhanced compared to controls (see the previous paragraph) and it was inhibited by the same concentration of DES. Spontaneous and KER of NE (B1_{-DES} and S1_{-DES}) as well as DES-influenced B2_{+DES} and S2_{+DES} from the Cont4 and Cont7 atria did not differ significantly from Cont1 samples. The S2_{+DES}/S1_{-DES} ratios were 2.11 ± 0.15 and 0.74 ± 0.07 for Cont4 and STZ4 samples, respectively. At months 7 of diabetes, DES failed to affect KER of NE (Fig. 5).

Release of NE under calcium-free conditions

KER of NE under calcium-free conditions in controls approached the values of the spontaneous release and did not exceed 11% of KER values in the whole course of experiment (S2_{-Ca}/S1_{+Ca} ranged from 0.06 ± 0.01 to 0.1 ± 0.01 in all control groups). In the diabetic atria, KER of NE under Ca-free conditions was slightly larger 1 month after the onset of the disease (S2_{-Ca}/S1_{+Ca} = 0.17 ± 0.01), reached its maximum after 4 months (S2_{-Ca}/S1_{+Ca} = 0.29 ± 0.02) and was still higher (S2_{-Ca}/S1_{+Ca} = 0.27 ± 0.02) than in the respective controls after 7 months lasting diabetes (Fig. 6).

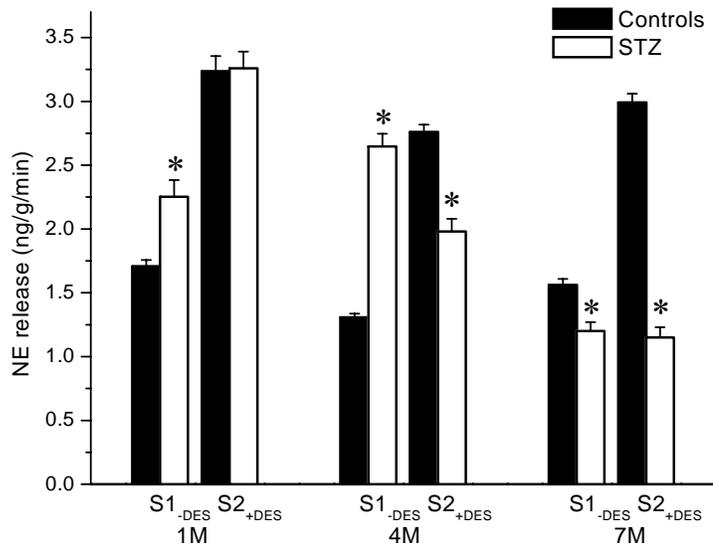


Figure 5. Norepinephrine (NE) release from the atria of STZ-diabetic rats in the presence or absence of DES. KER of NE during the first and second stimulation (S1_{-DES} and S2_{+DES}, respectively) in preparations from STZ-diabetic rats 1, 4, and 7 months after the onset of diabetes (1M, 4M, 7M, respectively). The neuronal uptake blocker DES (10^{-6} mol/l) was added into the superfusion fluid during the second stimulation period. Error bars = S.E.M. * $p < 0.05$ compared to the respective control value, $n = 5$ for all controls and STZ1 and STZ4 animals, $n = 4$ for STZ7 animals.

Discussion

The present study reports some new data concerning the function of the cardiac sympathetic innervation in the course of STZ-induced diabetes in female rats. NE tissue levels were determined in the separated heart atria 1, 4, and 7 months after the onset of the disease and in age-matched controls. NE concentrations in both atria decreased significantly with age, the most expressed decline was revealed between 6- and 9-month-old animals (40%). Our findings are in a good agreement with the data on the impact of age on NE levels in the rat heart in males (McLean et al. 1983).

Also, NE release and its uptake in various types of the male rats heart preparations have been reported to be diminished with age (Docherty 1996; Snyder et al. 1998a; Snyder et al. 1998b). In addition, Snyder et al. (1998c) suggested that the decline in the capacity of adrenergic nerve terminals to release NE is significantly smaller in female than in male rats. In our study, the age of animals had no impact on the release and/or uptake of NE in experiments using isolated sliced heart atria of female rats. Thus, in the present study the decrease in cardiac NE levels in female rats cannot be attributed to the impaired neuronal uptake mechanism.

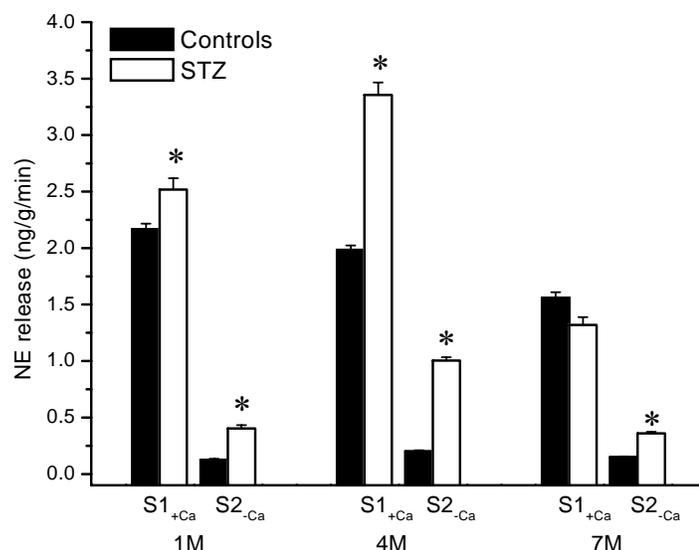


Figure 6. Norepinephrine (NE) release from the atria of STZ-diabetic rats in the presence or absence of calcium ions. KER of NE during the first and second stimulation (S1_{+Ca} and S2_{-Ca}, respectively) in preparations from STZ-diabetic rats 1, 4, and 7 months after the onset of diabetes (1M, 4M, 7M, respectively). The atria were superfused either in the presence (+Ca) or absence (-Ca) of calcium ions in the superfusion media. * $p < 0.05$ compared to the respective control value, $n = 5$ for all controls and STZ1 and STZ4 animals, $n = 4$ for STZ7 animals.

This study covers a relatively long period comparing concentrations of NE in the heart atria in the short- and long-term diabetic animals. The initial slight increase in NE levels (28%) in the atria of the STZ-diabetic rats was followed by a sustained decline, resulting in NE concentrations that in STZ-diabetic animals 7 months after the onset of the disease reached 60% of the age-matched control values. In human long-term diabetics, NE concentrations in the hearts obtained post-mortem were also found to be significantly decreased (Neubauer and Christensen 1976). It thus seems likely that STZ diabetes lasting less than 4 months cannot completely simulate the chronic diabetic conditions seen in diabetic humans.

NE release from the postganglionic sympathetic nerve endings has been extensively studied. The release of the neurotransmitter is initiated by a depolarisation-induced inward transport of calcium ions to the terminal that is followed by migration of synaptic vesicles towards the neuronal membrane and by exocytosis. The released NE is then removed from the synaptic cleft, particularly by re-uptake of the transmitter into nerve terminals by an active carrier mechanism linked to Na⁺,K⁺-ATPase (Nedergaard 1988).

Another mechanism of NE release from the nerve terminals is mediated by the

bi-directional neuronal carrier that normally transports NE back into the cell. The outward carrier-mediated transport may be induced by at least two mechanisms: by a decrease of the Na^+ concentration gradient across the neuronal membrane or by an increased cytosolic NE level (Langeloh et al. 1987; Trendelenburg 1990; Levi and Raiteri 1993). The neuronal carrier blocker, DES, inhibits the transport of NE in either direction (Levi and Raiteri 1993).

Depolarisation induced by high K^+ concentration in the extracellular fluid may influence KER of various transmitters differently. Okuma and Osumi (1986) reported that in rat brain slices, dopamine (DA) release induced by KCl was calcium-independent in contrast to DA output induced by electrical stimulation. However, both KCl- and electrical stimulation-induced releases of NE were calcium-dependent. Also in the rat heart, KER of NE has been reported to be calcium-dependent (Dumont et al. 1997). However, recent data indicate that KER of NE may induce both exocytotic and carrier-mediated release (Yamazaki et al. 1998). Our results obtained from *in vitro* release experiments in the presence or absence of calcium ions indicated that KER of NE was mediated by calcium-dependent exocytotic mechanism, as KER of NE was enhanced by DES in all control preparations.

NE turnover and release in the diabetic rat have been studied using various experimental protocols both *in vivo* and *in vitro*. Ganguly et al. (1986) reported an increased cardiac concentration and turnover of NE in the male Sprague-Dawley rats diabetic for 8 weeks. However, they suggested that intraneuronal storage granules of NE in the diabetic granules might be defective, which may account for an accelerated release of NE from its increased cytoplasmic pool (Ganguly et al. 1987). Defective exocytotic release from the left atria of male diabetic Wistar rats 8–12 weeks after STZ administration has been also reported (Gando et al. 1993).

In the present study, spontaneous release of NE from control and diabetic atria was not influenced by any experimental protocol with the exception of STZ4 atria, where it was significantly larger than in controls and at the same time it was inhibited by DES, suggesting involvement of carrier-mediated release. This increase in the spontaneous non-exocytotic release might contribute to the reduction in the NE tissue levels in STZ4 atria.

The absolute values of KER of NE were increased in STZ1 and STZ4 samples. However, if NE tissue concentrations (increased in STZ1 and decreased in STZ4) were related to the values of NE release, a greater proportion of NE was released in STZ4 atria only. At this stage of diabetes, NE release was significantly inhibited by DES and in relatively great proportion (30%) was calcium-independent. It thus seems to be clear that the carrier-mediated, calcium-independent release of NE may play an important role at the advanced stages of diabetes. Since intracellular Na^+ concentration was reported to be significantly increased in diabetes (Schaffer 1991; Doliba et al. 2000), the sodium gradient across the neuronal membrane may be substantially decreased and thus might influence the activity of the neuronal transporter. In addition, the shift in the intracellular NE distribution may increase the affinity of the carrier inside the membrane (Langeloh et al. 1987). In addi-

tion, insulin has been recently reported to modulate the activity of the NE uptake mechanism in SK-N-SH cells (Apparsundaram et al. 2001). However, the effect of long-term insulin deficiency on the activity of the carrier needs to be further investigated.

The absence of DES effect in the STZ7 atria is difficult to explain on the basis of the present data. Reports on the long-term STZ-diabetic rats are relatively rare, since only a small number of animals survives without at least partial compensation of diabetes. In a study using the radioactive analogue of NE, C-11 hydroxyephedrine, which is actively taken up by the sympathetic nerve terminals of the heart, a decreased retention of the tracer has been demonstrated in animals rendered diabetic for 6 and 9 months, suggesting defective neuronal uptake (Schmid et al. 1999). In addition, in long-term diabetes, severe morphological changes of the sympathetic nerves in the heart were shown, including defective *varicosities*, loss of the Schwann cell envelopes, and degeneration of terminals (Felten et al. 1982; Tomlinson and Yusof 1983; Addicks et al. 1993).

In conclusion, this is the first report on KER of NE in STZ rats. Our study suggests differential changes of NE-releasing mechanisms in the course of the disease. Increased KER of NE from the diabetic atria might be attributed to the abnormally working neuronal transporter.

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