

Parasympathetic dysfunction is associated with baroreflex and chemoreflex impairment in streptozotocin-induced diabetes in rats

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Abstract

This study explored physiological mechanisms of diabetic dysfunction in baroreceptors and chemoreceptors-mediated hemodynamic responses, and cholinergic neurotransmission in 30-day diabetic rats ($n=14$) and controls ($n=14$). Basal hemodynamic data and vagal response to electrical stimulation and methacholine injection were also evaluated. Muscarinic receptors were characterized using a radioligand receptor binding assay ($[^3\text{H}]$ N methylscopolamine). Experimental diabetes (50 mg/kg of STZ, i.v.) decreased systolic, diastolic, and mean arterial pressure and basal heart rate. Heart rate (HR) responses to vagal electrical stimulation (16, 32, and 64 Hz) were 15%, 11%, and 14% higher in diabetics vs non-diabetics, as were HR responses to methacholine injection (-130 ± 24 , -172 ± 18 , -206 ± 15 bpm vs. -48 ± 15 , -116 ± 12 , -151 ± 18 bpm, $P<0.05$). Muscarinic receptor density was higher (267.4 ± 11 vs 193.5 ± 22 fmol/mg/prot, $P<0.05$) in the atria of diabetic rats than in those of controls; the affinity was similar between groups. Diabetes-induced reduction of reflex responses to baro- (reflex bradycardia: -3.4 ± 0.3 and -2.7 ± 0.2 bpm/mm Hg; reflex tachycardia: -1.6 ± 0.1 and -1.4 ± 0.07 bpm/mm Hg, in control and diabetics, $P<0.05$) and chemoreceptor stimulation, enhancement of HR responsiveness to cardiac vagal electrical stimulation and methacholine stimulation, plus an increase in the number of atrial muscarinic receptors indicates reduced parasympathetic activity, which is probably derived from central nervous system derangement.

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1. Introduction

It is well known that diabetes mellitus produces changes in blood vessel structure and function, involving sensory, motor, and autonomic nervous systems. Significant increased morbidity and mortality, represented by orthostatic

hypotension, painless myocardial infarction, and sudden death, is observed in patients diagnosed with cardiovascular autonomic neuropathy (Ewing et al., 1980; Hilsted, 1982; Sampson et al., 1990). Extensive studies had been performed to clarify the mechanisms underlying such changes. Pathologic conditions, such as cardiomyopathy (Makino et al., 1987; Tahiliani and McNeill, 1986), increased lipid peroxidation (Tada et al., 1992), metabolic disturbances (Rodrigues and McNeill, 1992), and sympathetic nervous system structural and functional changes (Monckton and Pehowich, 1980; Takiguchi et al., 1988) are all contributors to this cardiovascular dysfunction.

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Streptozotocin (STZ)-induced diabetes in rats causes hypotension and bradycardia, probably related to pacemaker cell dysfunction (Maeda et al., 1995) associated with a depression in cardiac function (Jackson and Carrier, 1983; Shimabukuro et al., 1996). Indeed, baroreflex-mediated bradycardia and tachycardia in response to arterial pressure changes was found to be attenuated in diabetic rats after STZ (Dall'Ago et al., 2002) or alloxan (McDowell et al., 1994) administration. This impairment has been attributed, at least in part, to parasympathetic dysfunction, because early depressed vagal tonus has been consistently demonstrated in diabetic rats (Yagihashi, 1995). Moreover, evidence suggests that cardiovascular responses evoked by chemoreflex activation induced by potassium cyanide (KCN) in conscious rats mostly depends on cardiac vagal activation with some contribution of the cardiac-sympathetic activation in determining the final pressor responses (Franchini and Krieger, 1993). It was also showed before that STZ-diabetic rats have impaired cardiovascular responses evoked by KCN (Dall'Ago et al., 1997).

Chronotropic responses to cholinergic agonists were evaluated in isolated, spontaneously beating atria, but not in the conscious diabetic rat (Aronstam and Carrier, 1989; Carrier et al., 1984). Diminished muscarinic receptor population in cardiac tissue was observed in experimental diabetes (Aronstam and Carrier, 1989; Carrier et al., 1984; Kofo-Abayomi and Lucas, 1987), a change that is reversible by insulin (Aronstam and Carrier, 1989) and aldose reductase inhibitor (Kofo-Abayomi and Lucas, 1987) treatment, suggesting a metabolic cause for this abnormality. The relationship between hyperglycemia and cardiovascular dysfunction has already been demonstrated (Schaan et al., 2004). Although depressed vagal function can be associated with changes in peripheral or central nervous systems, no systematic functional studies have been undertaken to determinate the role of the parasympathetic efferent pathway on heart function in diabetic rats.

The aim of the present study was to evaluate the role of 30-day STZ-induced diabetes on the baro- and chemoreflex control of circulation by evaluating the heart rate responses to: (1) vagal electrical stimulation and (2) intravenous methacholine injection, a muscarinic receptor agonist, in rats. Atrial muscarinic receptor density and affinity were also quantified.

2. Methods

2.1. General procedures

Experiments were performed on 28 age-matched male Wistar rats, housed in individual cages, with free access to food and water. They were kept in a temperature-controlled room (22 °C) with a 12–12-h dark–light cycle. The rats were randomly assigned to 1 of 2 groups: control (C, $n=14$, body weight 224.2 ± 5 g) and diabetic (D, $n=14$, body weight 226.8 ± 6 g). Rats were injected with a single intravenous (i.v.)

injection of STZ (50 mg/kg, Sigma Chemical Company, St. Louis MO, USA) dissolved in citrate buffer (0.01 M, pH 4.5, D) or only citrate buffer (C) into the lateral tail vein. To prevent severe hypoglycemia in the first few hours after injection, both STZ- and citrate buffer-treated rats were given a 5% (wt/vol) dextrose solution to drink overnight. Experiments were performed in conscious rats whenever the procedures could be performed in this way (basal hemodynamic records, baro- and chemoreflex sensitivity evaluation).

2.2. Basal hemodynamic status

Thirty days after STZ administration, 2 catheters (PE10) filled with heparin in normal saline were installed in the animal, under general anesthesia (IP ketamine, 9 mg/kg, Parker, Davis, Brazil and xylazine, 1 mg/kg, Bayer, Brazil), these catheters being placed into the abdominal aorta and inferior vena cava through the left femoral artery and vein, respectively, tunneled subcutaneously and exteriorized at the back of the neck. The catheters were used for direct measurement of mean arterial pressure (MAP) and drug administration, respectively. One day after catheter placement, cardiovascular records were obtained. The arterial catheter was attached to a 20-cm (PE90) polyvinyl tube connected to a strain-gauge pressure transducer (P23 Db, Gould Statham, Oxnard, CA, USA). Blood pressure signals were recorded for 40 min with a microcomputer equipped with an analog-to-digital converter board (CODAS, 1 kHz, Dataq Instruments, and Akron, OH, USA). The recorded data were analyzed on a beat-to-beat basis to quantify MAP and heart rate (HR) at rest.

2.3. Baroreflex and chemoreflex sensitivity

After basal MAP recording, baroreflex-mediated changes were measured during peak increases or decreases in MAP after phenylephrine (0.02–0.32 μ g) or sodium nitroprusside (0.05–0.16 μ g) injections, and the corresponding peak reflex changes in HR was recorded after each dose of the drugs. The changes of MAP were within the range of 10–30 mm Hg. The maximum changes in MAP and HR were measured, and baroreflex sensitivity was determined as the slope of the MAP/HR ratio (bpm/mm Hg). The slopes obtained for each group of rats evaluated were then statistically compared.

Chemoreflex sensitivity was tested 30 min after the end of the baroreflex test session, by the administration of increasing intravenous doses of KCN (60, 100, 140, and 180 μ g/kg) (Dall'Ago et al., 1997; Franchini and Krieger, 1993). Mean AP and HR were measured continuously for 10 s before and for 15 s after the injections of KCN.

2.4. Bradycardic responses to vagal nerve stimulation and methacholine injection

The vagal electrical stimulation and muscarinic responses to methacholine were tested 24 h after baroreflex and

chemoreflex evaluation. The decrease in HR produced by the electrical stimulation (5 V, 2 ms, 2–64 Hz, for 10 s) of the cut right vagus nerve distal portion was studied in anesthetized rats (thiopental, 35 mg/kg, i.v.). The interval between stimuli was determined by the time required for HR to return to prestimulation levels. After vagal stimulation, the sensitivity of the heart muscarinic receptors was tested by evaluating HR responses to intravenous injections of increasing doses of methacholine (5, 7.5, and 10 μ g/kg) in the same rats. Body temperature was maintained at 37°C by external heating.

2.5. Muscarinic receptor binding assay

Saturation binding experiments were performed in membrane preparations of diabetic and control rats' atria. The procedures for membrane protein preparation have been described previously (Carrier et al., 1984). Briefly, the rats were killed by cervical dislocation, and the atria were removed from the heart, weighed, and washed with ice-cold phosphate-buffered saline (PBS) buffer. The tissues were then homogenized with a Polytron Homogenizer (Glenn Mills, Clinton, NJ, USA) in 10-ml of ice-cold lysis buffer containing 5 mM Tris-HCl, 2 mM EDTA, pH 7.4, and a protease inhibitor cocktail consisting of 5 mg/ml phenylmethylsulfonyl fluoride, 10 mg/ml benzamidine, and 5 mg/ml soybean trypsin inhibitor. The homogenate was centrifuged at 500 \times g for 15 min at 4°C. The pellets were then homogenized as before, spun again, and the supernatants pooled. The supernatants were centrifuged at 45,000 \times g for 15 min, and the pellets washed twice in the same buffer. The membrane fractions were resuspended in a buffer containing 50 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, and 5 mM EDTA. The protein content was determined with a Bio-Rad Protein Assay kit (Bio-Rad, Mississauga, ON, Canada), using bovine serum albumin as the standard. A muscarinic acetylcholine receptor non-selective antagonist [*N*-methyl-³H]scopolamine methyl chloride ([³H]NMS, 82 Ci/mmol) ranging from 10⁻¹¹ to 10⁻⁸ M was used in the absence (total binding) and presence (nonspecific binding) of atropine sulphate (Sigma Chemical Company, St. Louis, MO, EUA) for 90 min. Nonspecific binding was defined as that measured in the presence of 1 μ M atropine. Specific binding was determined by subtracting nonspecific from total binding. Binding measurements were obtained in duplicate for each experiment. Incubations at a volume of 1 ml (90 min at room temperature) were terminated by rapid filtration with Whatman GF/C filters (Xymotech, Montreal, PQ, Canada), and radioactivity was counted with an LS6000 Scintillation Counter (Beckman, Fullerton, CA).

2.6. Blood analysis

Arterial blood samples were obtained from the catheter placed in the femoral artery to determine blood glucose (test strips, Advantage, Roche, Indianapolis, IN, USA).

All experimental procedures described above were approved by the Ethical Committee for Animal Research of the Federal School Foundation of Medical Sciences of Porto Alegre.

2.7. Statistical analysis

Data are reported as means \pm S.E.M., and the unpaired Student's *t*-test was used for comparison between groups. Baroreflex sensitivity was evaluated by regression analysis of different groups, and the differences between the slopes of the relation between AP and HR response to pressor and depressor agents were evaluated for statistical significance using the Student's *t*-test for unpaired data. Differences in MAP and HR responses to KCN and the HR changes obtained by vagal stimulation and methacholine injections between groups were compared by analysis of variance for repeated measures, post hoc Student–Newmann–Keuls test. Binding data were analyzed using curve-fitting functions in GraphPad Prism software (GraphPad Software, San Diego, CA). Linear regression was performed on the percentage of bound versus the ratio of bound over free ligand, and only data with a regression coefficient of ≥ 0.9 were used for analysis. One- and two-site models were tested for all data sets, and the model yielding the least residual sum of squares was taken to describe the data. Probability levels < 0.05 were considered significant for all tests.

3. Results

3.1. Body weight and blood glucose

These data are represented in Table 1. Thirty days after STZ administration, body weights were lower in diabetic than in control rats ($P < 0.0001$). All 14 rats given citrate buffer remained normoglycemic throughout the 4-week study. Blood glucose levels were significantly higher in the diabetic compared with the control rats ($P < 0.0001$).

3.2. Baseline hemodynamic status

Table 1 also shows the systolic, diastolic, and mean arterial pressure at rest, which were markedly reduced in

Table 1
Characterization of diabetic and control groups

	Control (<i>n</i> =14)	STZ (<i>n</i> =14)
Body wt (g)	265 \pm 5	197 \pm 6*
Blood glucose level (mg/dL)	122 \pm 3	439 \pm 22*
Diastolic AP (mm Hg)	95 \pm 3	80 \pm 2*
Systolic AP (mm Hg)	139 \pm 3	116 \pm 3*
Mean AP (mm Hg)	114 \pm 3	98 \pm 2*
Heart rate (bpm)	331 \pm 9	299 \pm 11*

Baseline metabolic and cardiovascular characteristics of the animals studied. Values are means \pm S.E.M.

* Significant differences between groups ($P < 0.05$).

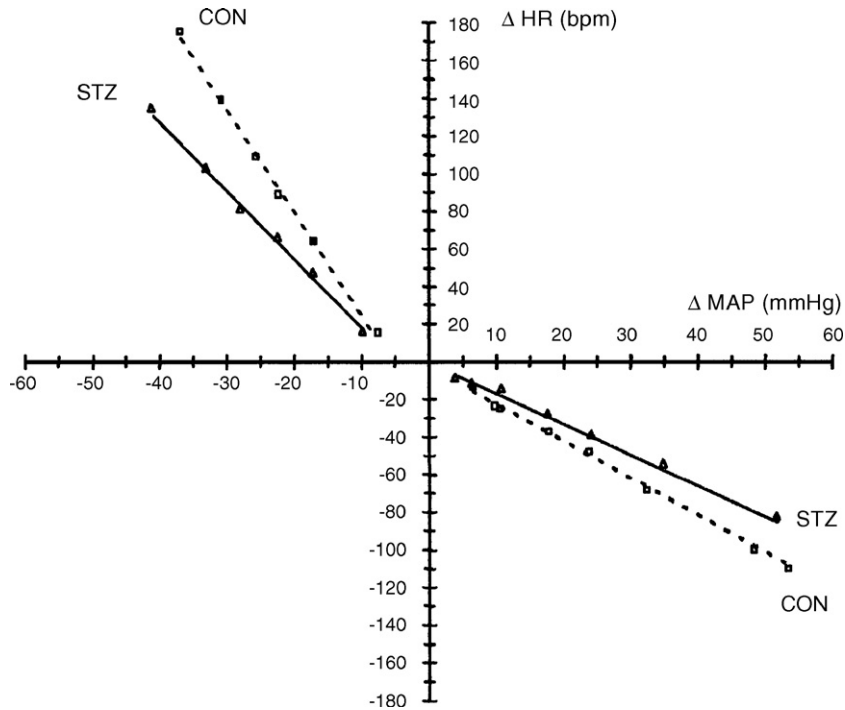


Fig. 1. Regression lines showing the effects of STZ-induced diabetes on bradycardic (lower right) and tachycardic (upper left) responses to pressor changes induced by increasing doses of phenylephrine and sodium nitroprusside, respectively. STZ-diabetic rats presented an impairment of tachycardic responses, while reflex bradycardia was unaltered. The slope of the lines obtained by linear regression was -3.40 vs. -2.70 bpm/mm Hg ($P < 0.05$, unpaired Student's *t*-test) for control (CON) and diabetic (STZ) rats, respectively after a decrease in MAP.

STZ-diabetic rats, $P < 0.0001$. Also, resting heart rate was significantly lower in the diabetic vs control group, $P < 0.0001$.

3.3. Baroreflex and chemoreflex sensitivity

The reflex tachycardia elicited by sodium nitroprusside-induced hypotension was significantly reduced by diabetes, as indicated by the slope of the regression line plotting changes in HR with changes in MAP ($b: -3.4 \pm 0.3$ and -2.7 ± 0.2 bpm/mm Hg, in control and diabetics, respectively, $P < 0.05$). The reflex bradycardia elicited by phenylephrine-

induced hypertensive stimulus was similar between groups ($b: -1.6 \pm 0.1$ and -1.4 ± 0.07 bpm/mm Hg, in control and diabetics, respectively, $P = 0.177$). These data are shown in Fig. 1.

Fig. 2 shows the dose-dependent bradycardic and pressor responses to intravenous injection of KCN produced in both control and diabetic rats. Bradycardia evoked by the chemoreflex activation was reduced in diabetic rats at all KCN doses employed (Fig. 2A). The pressor responses induced by chemoreflex stimulation (Fig. 2B) were also markedly decreased in the diabetic group at all KCN doses employed.

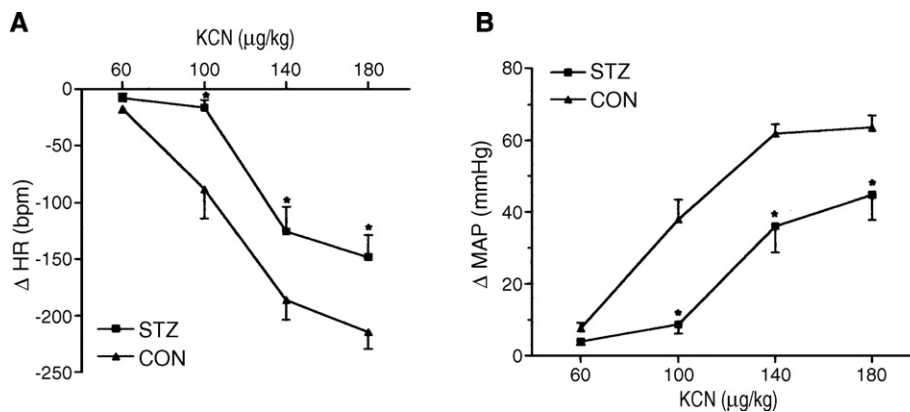


Fig. 2. Line graphs showing the effects of streptozotocin-induced diabetes on heart rate (HR) responses (A) and mean arterial pressure (MAP) responses (B) of control (filled triangles, $n = 14$) and diabetic (filled squares, $n = 14$) rats to increasing doses of KCN. Data are reported as means \pm S.E.M. * $P < 0.05$ compared with controls (ANOVA, post hoc Student Newman-Keuls).

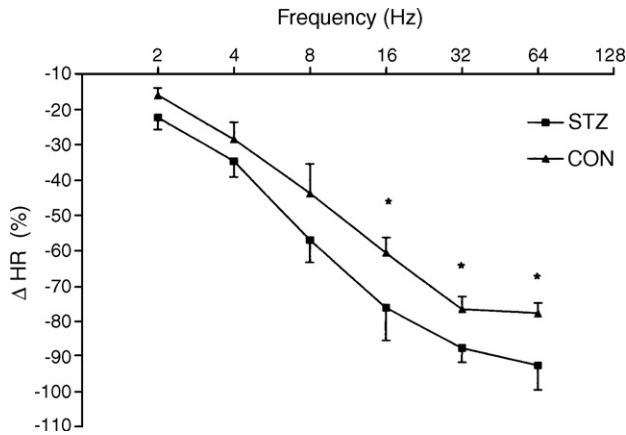


Fig. 3. Heart rate (HR) response to right vagal nerve electrical stimulation on 2, 4, 8, 16, 32, and 64Hz of control (filled triangles, $n=14$) and diabetic (filled squares, $n=14$) rats. * $P<0.05$ compared with controls (ANOVA, post hoc Student–Newmann–Keuls).

3.4. Vagal nerve stimulation and bradycardic responses to methacholine

Fig. 3 shows the HR response to right vagal nerve electrical stimulation on 2, 4, 8, 16, 32, and 64Hz. The responses to 16, 32, and 64Hz were 15%, 11%, and 14% higher in diabetics compared with that in control rats. Typical arterial pressure records in diabetic (Fig. 4A) and control (Fig. 4B) rats after methacholine injections are also shown. Fig. 4C shows that the HR response to increasing doses of methacholine was significantly increased in diabetic (-130 ± 24 , -172 ± 18 , -206 ± 15 bpm) compared with HR

response in the control group (-48 ± 15 , -116 ± 12 , -151 ± 18 bpm), $P<0.05$ for all comparisons (Student's t -test).

3.5. Muscarinic binding assays

The number of [3 H]NMS binding sites (B_{max}) calculated from the results of three experiments was higher in atria from diabetic rats (267.4 ± 11 vs 193.5 ± 22 fmol/mg/prot, $P<0.05$), as compared with controls. However, the affinity expressed by mean KD values was not different between groups (1.4 ± 0.8 vs 3.9 ± 0.8 nM/l, in control and diabetics, respectively, $P>0.05$).

4. Discussion

The results obtained in the present study show that short-term STZ-induced diabetes (30 days) in rats produces enhancement of HR responsiveness to cardiac vagal electrical stimulation and to methacholine stimulation. The diabetic rats presented increased number of atrial muscarinic receptors, with no change in their affinity. Also, as we reported previously (Dall'Agò et al., 1997, 2002; Maeda et al., 1995; Schaan et al., 2004), this animal model has (1) a reduction in systolic, diastolic, and mean arterial pressure, as well as resting bradycardia, (2) maintenance of baroreflex-mediated bradycardia in spite of impaired reflex tachycardia, and (3) impaired characteristic cardiovascular responses (bradycardia and hypertension) evoked by chemoreflex stimulation. Together, these abnormalities suggest that the reduced parasympathetic activity observed in this animal model is mainly

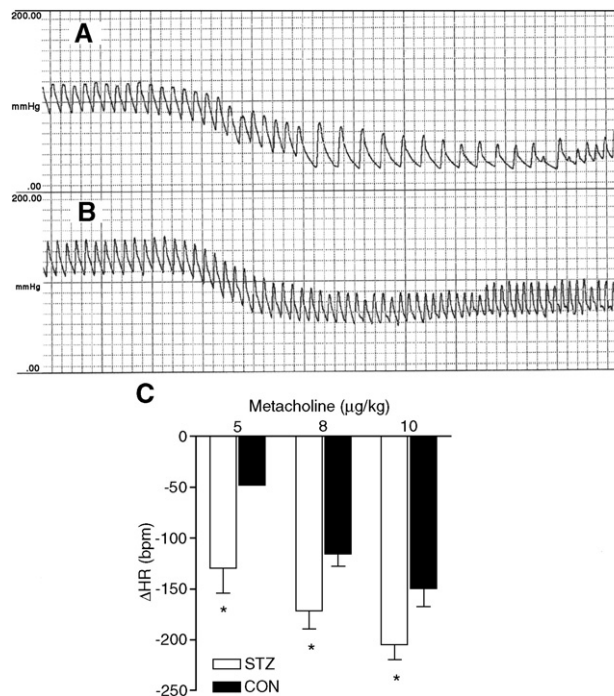


Fig. 4. Left: Typical arterial pressure records in diabetic (A) and control (B) rats after methacholine injections. Right: Heart rate (HR) response (C) to increasing doses of methacholine (5, 8, and 10 μg/kg) of control (filled bars, $n=14$) and diabetic (open bars, $n=14$) rats. * $P<0.05$ compared with controls (Student's t -test).

derived from a central nervous system derangement. We could not exclude the possibility that defects in reflex responses could lie on the afferent side, or on either efferent or afferent side to neuropathy.

The hypotension observed in the present study in diabetic rats confirms our previously published results (Dall'Ago et al., 1997, 2002; De Angelis et al., 2000a; Maeda et al., 1995; Schaan et al., 1997) and is in accordance with results reported by others (De Angelis et al., 2000b; Jackson and Carrier, 1983; Tomlinson et al., 1982). Some authors have described a so-called streptozotocin hypertension, which probably reflects discrepancies between the direct and indirect blood pressure measurements (Bunag et al., 1982; Kusaka et al., 1987). Resting bradycardia observed after STZ treatment has also been previously demonstrated (Bunag et al., 1982; Dall'Ago et al., 2002; Jackson and Carrier, 1983; Maeda et al., 1995). Using pharmacological blockade, De Angelis et al. (2002) demonstrated resting HR fell 5 days after STZ administration. This change was maintained for 3 months and seems to be related to a reduced intrinsic heart rate, suggesting that bradycardia in STZ-induced diabetic rats is associated with changes in the electrical activity of the sinoatrial node.

Baseline changes in MAP and HR could be related to changes in reflex mechanisms that control circulation. In fact, the reflex tachycardia response elicited by decreasing MAP was attenuated in the 30-day diabetic group. Previous reports from our laboratory have shown similar baroreflex control of HR impairment after 5 and 15 days of the STZ injection, suggesting that early alterations in reflex control were maintained during longer periods (1 month), as shown by the present study, or even later (De Angelis et al., 2002). Although bradycardic responses to increasing MAP were maintained in this experiment, reports show impairment in HR-reducing mechanisms. These differences seem to be related to methodological differences: undernutrition of the diabetic rats and prolonged diabetes duration (Chang and Lund, 1986) and experiments in anesthetized preparations (Van Buren et al., 1998).

The impairment of central parasympathetic pathway function with preservation of sympathetic control have been suggested as causal mechanisms involved in the attenuation of bradycardic responses in diabetic rabbits (McDowell et al., 1994). In this context, we observed in the present study a reduction in cardiac-vagal activation evoked by chemoreceptors in the diabetic group, as shown by the reduced bradycardia produced by KCN injection. The bradycardic responses evoked by KCN represent the cardiac-vagal activation and were not modulated by respiratory hyperventilation induced by chemoreflex activation. Because baroreflex- and chemoreflex-mediated responses are integrated by a common neural network in the central nervous system, we were not surprised that similar vagal efferent responses could be evidenced during both receptor stimuli. In the rat, the respiratory-dependent effects exert little influence in the final cardiovascular responses evoked by chemoreflex,

since bradycardia and hypertension, simultaneously to hyperventilation are observed in response to carotid body chemoreceptors activation (Franchini and Krieger, 1993). However, the magnitude of bradycardia and hypertension, as well as the specific response (tachycardia or bradycardia) could be changed if the secondary respiratory and behavioural responses are controlled by anesthetic agents. The use of anesthetized rats and the type and level of anesthesia can alter the final cardiovascular responses to chemoreflex stimulation, since defense areas activation during hypoxia may occur (Marshall, 1998).

The pressor responses produced by chemoreflex activation of vascular sympathetic pathway were also reduced in diabetic rats. In the present study and in previous ones from our group (Dall'Ago et al., 2002) and other authors (Jackson and Carrier, 1983), it has been demonstrated that the diabetic state induced not only an impairment in heart responses, but also a depressed vascular reactivity in this animal model. Jackson and Carrier (1983) induced blood pressure responses with norepinephrine and angiotensin II, and we used phenylephrine. These responses were depressed in the short-term diabetic rat in both studies; however, the baroreceptor reflexes in these rats were more sensitive to increases in blood pressure. Therefore, it appears that some type of nonspecific alteration occurs in the responsiveness of the cardiovascular system to the vasopressor agonists in the short-term diabetes in the rat.

Although the reduced reflex bradycardia elicited by baro- and chemoreceptor stimulation in diabetic rats has been attributed to the impairment in efferent parasympathetic function to the heart, there is no data to distinguish whether this dysfunction originates from central or peripheral nervous system derangements. The greater bradycardic responses obtained by vagal electrical stimulation or by methacholine injection in diabetic rats in our study suggest that the efferent pathway is actually more effective in diabetic than in normal rats. We (Dall'Ago et al., 2002; Maeda et al., 1995) and others (McDowell et al., 1994) have previously reported on the enhancement of efferent parasympathetic function. The responses to vagal nerve stimulation and methacholine injection are necessarily performed under general anesthesia (thiopental, 35 mg/kg, i.v.); the evaluation of a control group allowed us to exclude anesthesia-induced changes as the determinant of the responses obtained.

These findings, associated with the increased density of muscarinic receptors in the heart, that we also observed, suggest an up-regulation of these receptors to compensate for a reduction in central parasympathetic activity. This is the first study showing a complete analysis of the parasympathetic efferent to the heart in experimental diabetes. An increase in the density of cardiac muscarinic receptors matches the observed cholinergic supersensitivity in the diabetic atria. Muscarinic receptor populations are regulated by the degree of effective neurotransmission. These receptor populations are decreased by chronic exposure to agonists or by inhibition of acetylcholinesterase (Gazit et al., 1979),

whereas they are increased by exposure to antagonists (Wise et al., 1980). However, atrial muscarinic receptor density in diabetes has been variably reported to decrease (Carrier et al., 1984; Kofo-Abayomi and Lucas, 1987), not change (Wegner et al., 1987) or to increase in humans (Richardson et al., 2004) and in rats (Liu et al., 2005). These 2 last studies specifically evaluated the muscarinic receptor protein and mRNA densities in the heart, techniques that add to our results. Liu et al also showed that the up-regulation of these receptors could be normalized by inhibiting the sorbitol pathway with an aldose reductase inhibitor (Liu et al., 2005). Also, muscarinic receptor density was increased in the urinary bladder (Tong et al., 2002) and ileum (Coulson et al., 2004) of diabetic rats. It is well accepted that in the atria of mammals the most important muscarinic acetylcholine receptor is of the M₂ subtype, however it cannot be excluded the possible presence of the M₁ or M₃ subtypes (Krejci and Tucek, 2002; Wang et al., 2001, 2004). In the present study a nonselective antagonist was used in the binding assays and this matter was not addressed. Future studies are necessary to investigate the possible expression of muscarinic acetylcholine receptors others than the M₂ subtype in diabetic rats' atria.

5. Conclusion

STZ-induced experimental diabetes promotes hypotension accompanied by resting bradycardia, impaired baro- and chemoreflex control of circulation, enhancement of HR responsiveness to cardiac vagal stimulation, and an increased density of muscarinic receptors in the heart. These latter findings allowed us to hypothesize that the mechanisms involved in the impaired HR reflex changes depend on a reduced central parasympathetic tonus generation. This vagal impairment is associated with an up-regulation of muscarinic heart function, although sympathetic activity changes should also be considered. Further studies are necessary to test these latter hypotheses.

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