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The influence of improved glycaemic control with chlorpropamide on microvascular reactivity and nitric oxide synthase activity in diabetic rats

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Abstract

Hyperglycaemia is a primary cause of vascular complications in diabetes. A hallmark of these vascular complications is endothelial cell dysfunction, which is partly due to reduced production of nitric oxide. The aim of this study was to verify the influence of improved glycaemic control with chlorpropamide on microvascular reactivity, endothelial nitric oxide synthase (e-NOS) expression, and NOS activity in neonatal streptozotocin-induced diabetic rats (n-STZ). Diabetes was induced by STZ injection into neonates Wistar rats. n-STZ diabetic rats were treated with chlorpropamide (200 mg kg^{-1}, 15 days, by gavage). The changes in mesenteric arteriolar and venular diameters were determined in anaesthetized control and n-STZ diabetic rats, before and after topical application of acetylcholine, bradykinin and sodium nitroprusside (SNP). We also assessed e-NOS expression (using polymerase chain reaction after reverse transcription of mRNAs into cDNAs) and NOS activity (conversion of L-arginine to citrulline) in the mesenteric vascular bed of chlorpropamide-treated n-STZ, vehicle-treated n-STZ, and control rats. In n-STZ, chlorpropamide treatment reduced high glycaemic levels, improved glucose tolerance and homoeostatic model assessment (HOMA-beta), and restored NOS activity. Impaired vasodilator responses of arterioles and venules to acetylcholine, bradykinin and SNP were partially corrected by chlorpropamide treatment in n-STZ. We concluded that improved metabolic control and restored NOS activity might be collaborating with improved microvascular reactivity found in chlorpropamide-treated n-STZ.

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Introduction

Sulphonylureas were developed as early as the 1950s and are widely prescribed for type 2 diabetes mellitus treatment, despite early observations suggesting a possible deleterious effect of these drugs (Meinert et al 1970; Melander et al 1990; Leibowitz et al 1996). However, the question is still open. In fact, in the early 1970s, the University Group Diabetes Program suggested that use of sulphonylureas might increase cardiovascular mortality, but a more recent UK Prospective Diabetes Study (UKPDS 1998) showed no evidence that intensive treatment with sulphonylureas had any specific adverse effect on cardiovascular disease in type 2 diabetes mellitus (Meinert et al 1970; UKPDS 1998). Sulphonylureas stimulate insulin secretion by blocking β-cell ATP-sensitive K^+ (K_{ATP}) channels (Sturgess et al 1985). K_{ATP} channels are also abundantly present in cardiac muscle cells, vascular smooth muscle cells and coronary and peripheral arteries where they play a key role in the protective mechanisms that are triggered during ischaemia (Noma 1983; Standen et al 1989). Diabetes elicits morphological and functional alterations on microcirculation and macrocirculation (Bohlen & Niggl 1979) and hyperglycaemia is a primary cause of these diabetic vascular complications. Since sulphonylureas might interact not only with K_{ATP} channels in pancreatic β-cells but also with those in the vascular system, and high glucose levels account for vascular dysfunction, it is important to know whether chlorpropamide treatment could affect the microvascular reactivity in diabetes.

Studies investigating endothelial dysfunction in animal models of diabetes have yielded conflicting results. Both impaired (Sakamoto et al 1998) and preserved endothelium-dependent (Bohlen & Lash 1995) responses have been reported. Hyperglycaemia is a

primary cause of diabetic vascular complications. Dysfunction is characterized by reduced nitric oxide-dependent phenomena, including vasodilatation. Studies have shown that hyperglycaemia impairs nitric oxide production (Ding et al 2000; Guo et al 2000). In addition, responses to endothelium-independent vasodilators, such as sodium nitroprusside, have been found to be impaired in some (Caballero et al 1999; Woodman et al 2002) but not in all (Goodfellow et al 1996; Avogaro et al 1997) studies. Impaired endothelium-dependent and endothelium-independent relaxation has been reported in different types of blood vessels and in different animal models of diabetes (De Vriese et al 2000). Moreover, experimental research has been mainly focused on large conduit arteries, such as aorta, and in adult chemical-induced diabetic rats.

The n-streptozotocin (STZ) model was first described by Portha et al (1974) and Weir et al (1981). It is an experimental diabetes model that presents pancreatic β-cell destruction followed by partial β-cell regeneration and glucose intolerance (Movassat et al 1997). Other authors confirmed these findings and showed that n-STZ in adulthood displays some of the typical characteristics of diabetes (Leahy & Weir 1985; Iwase et al 1986; Grill et al 1987; Welsh & Hellerstrom 1990; Hemmings & Spafford 2000). n-STZ has several characteristics as described above and it is considered to be a suitable experimental diabetes model (Arulmozhi et al 2004).

The aim of this study was to verify the influence of improved glycaemic control with chlorpropamide on microvascular reactivity, eNOS expression and NOS activity in n-STZ diabetic rats. We chose chlorpropamide because it is broadly used in type 2 diabetes mellitus treatment and it is not clear whether this drug might interact systematically with the microcirculation in diabetes.

Materials and Methods

Drugs and reagents

Chloral hydrate, acetylcholine chloride, bradykinin triacetate, sodium nitroprusside and STZ were purchased from Sigma-Aldrich (St Louis, MO); sodium citrate, D(+)-glucose, sodium bicarbonate, potassium chloride and calcium chloride.$2H_2O$ were purchased from Merck; sodium chloride was purchased from Synth (SP, Brazil); sodium pentobarbital (Hypnol) was from Cristalia (SP, Brazil); chlorpropamide (Diabinese) was from Pfizer (Brazil).

Induction of diabetes

Diabetes mellitus was induced in male newborn (2-day-old, 8–10 g) Wistar rats with bolus injection of STZ (160 mg kg^{-1}, i.p.) dissolved in citrate buffer (10 mM, pH 4.5). Control rats were sham injected with the same volume of citrate buffer. The n-STZ and matching controls were housed according to institutional guidelines (room temperature 22 ± 0.5°C, 12-h light–dark cycle, 60% humidity, freely accessible standard rat chow and water). The experimental protocols were approved and performed in accordance with the guidelines of the Institute of Biomedical Sciences Committee.

Chlorpropamide treatment

n-STZ diabetic rats received either a daily dose of chlorpropamide (200 mg kg^{-1}) or the same volume of the vehicle (water) by the same route and for the same period as the rats of the corresponding chlorpropamide-treated group for 15 days, by gavage, eight weeks after streptozotocin treatment.

Characterization of the n-STZ

The n-STZ were considered diabetic for study and used in experiments if they presented urinary glucose greater than 500 mg dL^{-1}. For a better characterization of the n-STZ, ten weeks after the injection of STZ or citrate buffer, 9 control, 7 vehicle-treated n-STZ and 7 chlorpropamide-treated n-STZ were placed in a metabolic cage for 24 h to evaluate food and water consumption and urine volume. Glycosuria was semi-quantitatively assessed in urine with the aid of reagent strips (Diastix; Bayer, SP, Brazil). In a separate set of experiments, an intravenous glucose tolerance test was performed in 8 control, 6 vehicle-treated n-STZ and 8 chlorpropamide-treated n-STZ rats. After 12 h of food restriction, the rats were anaesthetized with sodium pentobarbital (40.0 mg kg^{-1}, i.p.). After laparatomy, sequential blood samples were collected from the abdominal aorta immediately before glucose challenge (0.5 g glucose/kg, i.v.) and 5, 15, 30 and 60 min thereafter. After centrifugation at 40°C, 20 μL of plasma were immediately used for glucose determination using a kit (LabTest Diagnóstica, Brazil). We also performed the oral glucose tolerance test in 15 control, 10 vehicle-treated n-STZ and 10 chlorpropamide-treated n-STZ. After 6 h of food restriction (0700–1300 h) blood samples were collected from the cut tip of tail of the rats immediately before and 30 min after glucose overload (0.5 g glucose/kg body weight, by gavage). Blood glucose concentrations were estimated with the aid of a blood glucose monitor (Roche, SP, Brazil). Beta-cell function was assessed in 3 rats per group using the homoeostasis model assessment (HOMA-beta) (Matthews et al 1985, Pickavance et al 2005) in all groups. After 12 h of food restriction (2000–0800 h) blood samples were collected by decapitation. Blood glucose concentrations were estimated using a kit (LabTest Diagnóstica, Brazil) and insulin concentrations were assessed using a rat insulin enzyme immunoassay kit (Rat Insulin EIA kit; SPIbio, France). The following formula was used: β-cell function (%) = 20 × plasma insulin (mIU L^{-1})/[plasma glucose (mmol L^{-1}) × 3.5].

Intravital microscopy

The rats were anaesthetized with a subcutaneous injection of chloral hydrate (450–500 mg kg^{-1}). The mesentery was exteriorized and arranged for microscopic observation according to Zweifach & Lipowsky (1948) with slight modifications (Fortes et al 1983, 1984). In brief, the rats were kept on a special board, heated at 37°C, which included a transparent plate on which the tissue to be transilluminated was placed. The mesentery was kept moist and warm by irrigating the tissue with warmed (37°C) Ringer–Locke's solution, pH 7.2–7.4, containing 1% gelatin. The composition of the solution was (in mmol L^{-1}): 154.0 NaCl, 5.6 KCl, 2.0 $CaCl{\cdot}2H_2O$, 6.0

$NaHCO_3$ and 5.5 glucose. A 500-line television camera (JVC, Tokyo, Japan) was combined with a tri-ocular microscope to facilitated observation of the enlarged image (3400×) on the video screen. An image-splitting micrometer was adjusted to the phototube of the microscope (Carl Zeiss, Jena, Germany), shearing the optical image into two separate images, one displaced with respect to the other. By rotating the image splitter in the phototube, the shearing was maintained at a right-angle to the long axis of the vessel. The displacement of one image from the other permitted measurement of the vessel diameter (Baez 1969). A terminal arteriole and the venule paired with it were selected for study, and any change in vessel diameter was estimated after topical application of bradykinin (53.2 pmol), SNP (58.0 nmol) or acetylcholine (24 nmol). The doses of vasodilator agents were selected based on previous work from our laboratory and were the lowest doses that caused maximum vasodilatation (Fortes et al 1989). A given section of the vascular bed was tested only once and no more than two drugs were used on a single rat. The drugs, dissolved in Ringer–Locke's solution, were added to the preparation in a standard volume of 0.015 mL and were removed by washing with warm Ringer–Locke's solution.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Arterioles and venules from mesenteric bed were dissected and immediately frozen in liquid nitrogen, and stored at −70°C. The mesenteric arterial tissue that was subjected to vasoreactivity studies in intravital microscopy was not the same we used for RT-PCR. Total RNA was isolated from the mesenteries using TRizol Reagent (Invitrogen Co.) according to the manufacturer's instruction. cDNA was generated from 2 μg total RNA using SuperScript II (Invitrogen Co.). Products of the RT reaction were subjected to PCR amplification using TaqDNA polymerase (Invitrogen Co.) and specific oligonucleotide primers for eNOS (size, base pairs 165; cycles, 30; annealing temperature, 65°C; forward, GCCAGGAGGGACTGCAGTAC; reverse, GCGGGGAAGTGATGTCCAGG), and GAPDH (size, base pairs 406; cycles, 28; annealing temperature, 65°C; forward, GGTGCTGAGTATGTCGTGGA; reverse, TTCAGCTCTGGGATGACCTT). The conditions for PCR were as follows: initial denaturation at 94°C for 5 min was carried out, followed by 30 cycles of 94°C for 1 min; annealing temperature of 65°C for 1 min and 72°C for 1 min. The PCR was terminated with a final extension step at 72°C for 10 min. Three pooled RNA samples were routinely sham reverse transcribed (i.e. reverse transcriptase omitted) to ensure the absence of products other than those from the reverse transcribed mRNAs. PCR products were electrophoretically resolved by 1% agarose gel and visualized with ethidium bromide. The band intensity was measured using a software package (Kodak Digital Science).

Determination of NOS activity

NOS activity was measured by the biochemical conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline according to Franco et al (2004). The mesenteric arterial tissue that was subjected to vasoreactivity studies in intravital microscopy was not the same tissue that we used for NOS activity studies. Briefly, arterioles and venules from the mesenteric arteriolar bed were dissected and immediately homogenized in ice-cold buffer containing (in mmol L^{-1}): 20 HEPES, 0.32 sucrose, 1.0 DTT, 0.1 EDTA, 1.0 pepstatin and 1.0 PMFS in the presence of 10 gmL^{-1} leupeptin. The incubation (37°C for 60 min) was performed in buffer containing 4 μM FAD/FMN, 10 μg mL^{-1} calmodulin, 1.25 mM Ca^{2+}, 1 mM NADPH, 120 nM L-arginine and 50 nM [^{3}H]arginine (specific activity: 45.2 Ci $mmol^{-1}$; NEN Life Science Products, Inc, Boston, MA) in the presence of 10 μM BH4. Cation-exchange resin (Dowex 50WX8-400 equilibrated with 50 mM HEPES, pH 5.5) was added to the reaction mixture to remove the excess of L-[^{3}H]arginine. The supernatants were collected in vials with scintillation liquid and the radioactivity was quantified.

Statistical analysis

Data are given as mean ± s.e.m. One-way analysis of variance followed by Tukey–Kramer multiple comparisons test was used when more than two means were compared. The minimum acceptable level of the significance was $P < 0.05$.

Results

General characteristics of the rats

Two weeks after chlorpropamide treatment, the body weight and the body weight gain of n-STZ groups were significantly less than control rats (Table 1). Water intake and urine volume were increased in n-STZ groups compared with the respective control (Table 1). There was no difference in the absolute food intake between n-STZ and control rats (Table 1). Glucose intolerance was observed in n-STZ (Figure 1). Blood (Figure 1, Table 1), but not urine (Table 1) glucose concentrations were reduced in chlorpropamide-treated n-STZ. Although chlorpropamide treatment did not correct metabolic alterations, such as reduced body weight gain, increased water intake, urine volume and urine glucose levels observed in n-STZ (Table 1), it improved glucose tolerance (Figure 1) and β-cell function, tested by HOMA-beta (Table 1).

Microvascular reactivity in-situ

At resting conditions, there was no difference in the initial diameters (micrometres) of comparable type of arterioles and venules: n-STZ (arteriole 24.40 ± 0.86, n = 12; venule 34.26 ± 1.44, n = 14), controls (arteriole 22.52 ± 0.60, n = 12; venule 34.93 ± 1.78, n = 14), and chlorpropamide-treated n-STZ (arteriole 25.62 ± 0.98, n = 12; venule 31.47 ± 0.99, n = 14).

Impaired responses of arterioles (Figure 2A) and venules (Figure 2B) to all vasoactive agents tested were observed in n-STZ. Chlorpropamide treatment improved the impaired responses to all vasoactive agents tested (Figure 2).

NOS mRNA expression

We did not find significant changes in eNOS expression when the n-STZ groups were compared with the control group (Figure 3).

Table 1 General characteristics of the rats

	HOMA-beta index	AUC glucose (mg dL^{-1} min^{-1})	Glycosuria (mg dL^{-1})	Glucose (mg dL^{-1})	Glucose OGTT (mg dL^{-1})	Water intake (mL/24 h)	Urine volume (mL/24 h)	Food intake (g/24 h)	Body weight (g)	Body weight gain (g)
C	0.27 ± 0.06 (3)	8702 ± 1511 (8)	Absent (7)	94.6 ± 1.9 (15)	105.4 ± 2.8 (15)	47 ± 1.7 (9)	7 ± 1 (6)	33 ± 1 (9)	306 ± 7 (12)	49 ± 2 (7)
D	0.13 ± 0.2* (3)	12915 ± 426* (6)	≥ 1000 (8)	129.0 ± 9.3* (10)	202.5 ± 11.0** (10)	96 ± 9** (7)	50 ± 7* (6)	36 ± 3 (6)	211 ± 5*** (18)	27 ± 2*** (7)
Ch	0.34 ± 0.3 (3)	10457 ± 427 (8)	≥ 1000 (9)	100.4 ± 4.9 (10)	153.0 ± 7.1* (10)	102 ± 14*** (7)	52 ± 11* (5)	36 ± 2 (7)	217 ± 7*** (14)	35 ± 3** (9)

Results are expressed as means ± s.e.m. of control (C), n-STZ (D) and n-STZ treated with chlorpropamide (Ch) rats. Brackets indicate number of rats used per group. AUC = area under curve obtained from intravenous glucose tolerance test. Glucose = plasma glucose levels after 6 hours of food restriction. Glucose OGTT = plasma glucose levels after 30 min of glucose overload (0.5 g kg^{-1}, by gavage). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with C.

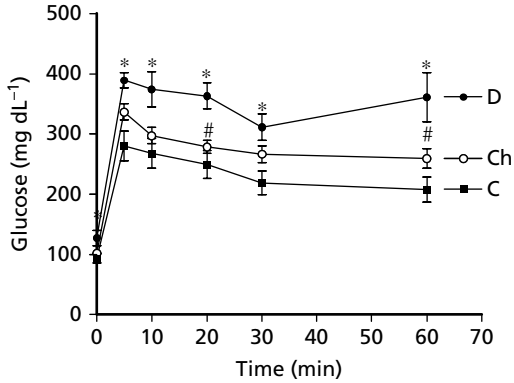

Figure 1 Plasma glucose levels in control rats (C), n-STZ (D) and n-STZ treated with chlorpropamide (Ch) during intravenous glucose tolerance test (0.5 g kg^{-1}, by gavage), ten weeks after administration of STZ. The results are expressed as mean ± s.e.m. The number of rats per group is ≥ 6. *$P < 0.05$ compared with C, #$P < 0.05$ compared with D.

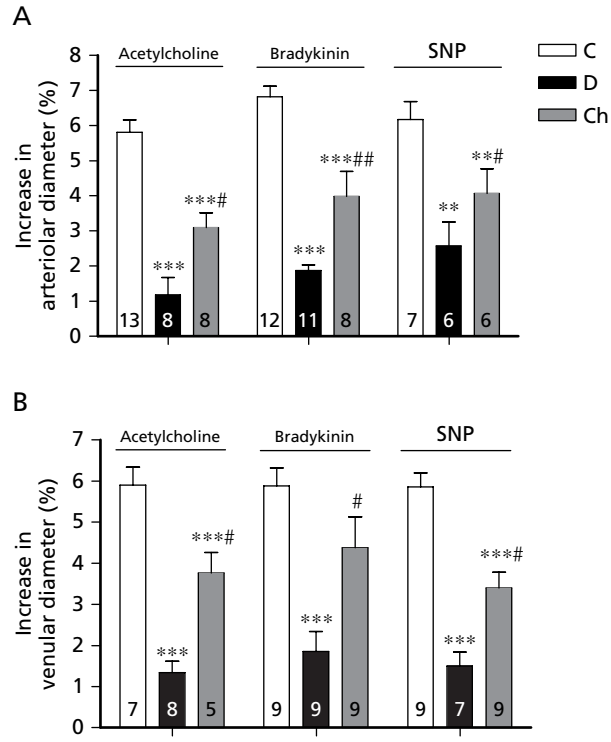

Figure 2 Bar graphs showing percentage of the increase in arteriole (A) and venule (B) diameter induced by vasoactive agents in control rats (C), n-STZ (D) and n-STZ treated with chlorpropamide (Ch). Data expressed as mean ± s.e.m. The number of rats per group is shown inside columns. SNP = sodium nitroprusside. ***$P < 0.001$, **$P < 0.01$ compared with C; ##$P < 0.01$, #$P < 0.05$ compared with D.

NOS activity

As illustrated in Figure 3, the total NOS activity was reduced in the mesenteric bed in the n-STZ group in comparison with the control group. Chlorpropamide treatment restored NOS activity.

Discussion

This report shows that impaired mesenteric microvascular responses to acetylcholine and bradykinin, endothelial-dependent vasodilators and SNP, an endothelial-independent vasodilator, found in n-STZ diabetic rats, was improved by chlorpropamide treatment concomitantly with improvement in glucose tolerance and HOMA-beta index. Moreover, chlorpropamide treatment restored NOS activity in the mesenteric vascular bed of n-STZ diabetic rats.

The administration of streptozotocin in Wistar rats produced experimental diabetes, characterized by glucose intolerance, hyperglycaemia, glycosuria, polyuria and polydipsia, in agreement with other studies (Grill et al 1987; Cuman et al 2001; Sartoretto et al 2005). Although chlorpropamide treatment improved glucose tolerance and HOMA-beta index in n-STZ diabetic rats, it did not affect urine glucose levels, water intake, urine volume or body weight gain in these rats. This is not an unexpected finding since it is rather difficult to attain a tight metabolic control in experimental diabetes models with oral anti-hyperglycaemic drugs.

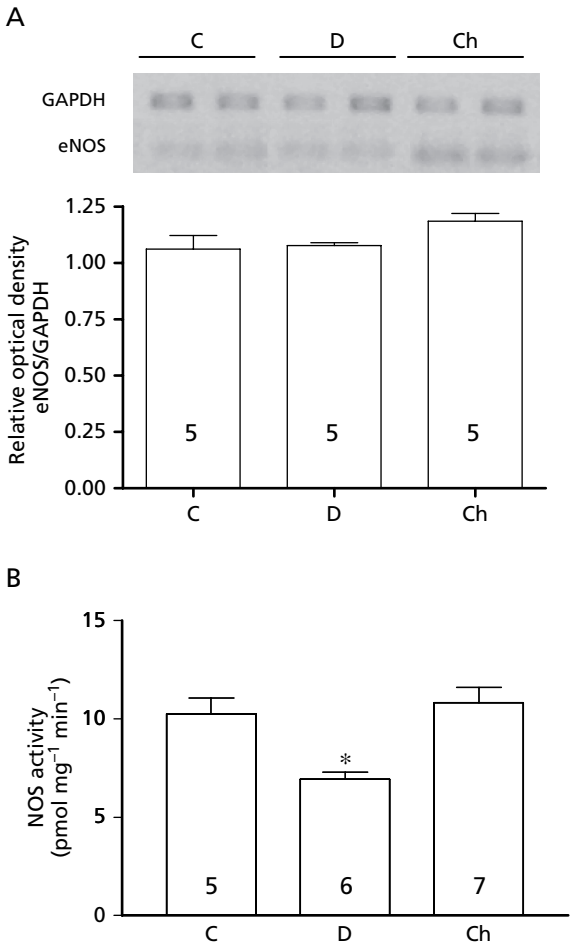

Figure 3 A. Representative RT-PCR products of 2 mg cDNA extracted from mesenteries of control rats (C), n-STZ diabetic (D) and n-STZ treated with chlorpropamide (Ch). The bar graphs show the relative optical density values of eNOS bands obtained from the different experiments. Values were normalized by the corresponding RT-PCR products for GAPDH, used as the internal control. B. Bar graphs show the total NOS activity (pmol$mg^{-1}min^{-1}$). Values are expressed as mean ± s.e.m. The number of rats per group is shown inside the columns. $*P < 0.05$ compared with C and D.

Studies on resistance vessels in-vivo allow for vascular reactivity assessment under physiological flow conditions and in the presence of diabetic extracellular fluid composition. Using an in-vivo preparation, we have already demonstrated that responses to endothelial-dependent and endothelium-independent vasodilators were reduced in n-STZ rats and that metformin treatment restored this alteration (Sartoretto et al 2005). Metformin, an antidiabetic agent that increases insulin sensitivity, recovered the reduced vasodilator responses without affecting glycaemic levels. On the other hand, chlorpropamide, which improved glucose tolerance by stimulating insulin secretion, partially recovered the reduced vasodilator responses. The mechanisms by which these two antidiabetic drugs affect the vascular dysfunction might differ. Moreover, a positive relationship between improvement in metabolic alterations and vascular dysfunction need not necessarily be associated in n-STZ diabetic rats.

Reduced expression of eNOS might be involved in the vascular dysfunction in diabetes. However, there are conflicting data in the literature about the expression of different isoforms of NOS (endothelial, neuronal and inducible) in diabetic rats. There are studies showing decrease (Yagihashi et al 1996; Yu et al 1999), increase (Serino et al 1998; Bardell & McLeod 2001; Cosenzi et al 2002) and unaltered (Felaco et al 2001; Sartoretto et al 2005) expression of different isoforms of NOS mRNA in different tissues of different diabetes models. This could be due to the fact that NOS regulation is tissue-specific and depends on metabolic alterations caused by diabetes in these tissues. The type of diabetes, the duration after STZ injection (Oyadomari et al 2001) and the age of animals could also contribute to the differences observed. In our study, we did not observe difference in mRNA eNOS levels among control, untreated and chlorpropamide-treated n-STZ diabetic rats. However, a decreased eNOS activity was observed in n-STZ diabetic rats. Chlorpropamide treatment restored the reduced activity in the mesenteric vascular bed of treated n-STZ diabetic compared with untreated rats. One possible explanation for this could be the improvement in glucose tolerance, as suggested by intravenous glucose test. Since treatment with high glucose levels prevented fluid shear stress-induced NO release (Wessells et al 2006) and chronic exposure to elevated glucose reduced total nitrite production, levels of eNOS mRNA and eNOS protein in human aortic endothelial cells (Srinivasan et al 2004), by improving glucose tolerance chlorpropamide would increase the NO system. This mechanism might be involved in the improved effect of chlorpropamide on the impaired endothelial-dependent vasodilatation.

Restoration of NOS activity, however, might not be the sole factor involved in the effect of chlorpropamide on the altered vasodilatation, since SNP, which acts via direct stimulation of vascular smooth muscle cells independently of intact endothelium, had its effect improved by chlorpropamide. It is known that NO, which is released by endothelial-dependent vasodilators, such as acetylcholine and bradykinin, and SNP share a final common pathway to produce vasodilatation, stimulating guanylate cyclase and increasing cGMP levels. We cannot rule out that improved glucose tolerance might be affecting smooth muscle cells to work properly. However, a direct effect of chlorpropamide on the smooth muscle layer, ameliorating the vascular reactivity, might be a plausible hypothesis to explain the correction of the impaired vasodilatation found in n-STZ diabetes.

Conclusion

In summary, we concluded that improved glucose tolerance and HOMA-beta index concomitantly with restored NOS activity by chlorpropamide might be collaborating to improve the reduced microvascular response found in n-STZ diabetic rats. Moreover, improvement of the reduced smooth muscle microvascular response to NO (tested as SNP) might also contribute to the ameliorating effect of chlorpropamide.

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