Upregulation of hypothalamic nitric oxide synthase gene expression in streptozotocin-induced diabetic rats

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Summary Plasma arginine vasopressin (AVP) is known to be elevated in patients with uncontrolled insulin-dependent diabetes mellitus who have plasma hyperosmolality with hyperglycaemia. Although osmotic stimuli cause an increase in nitric oxide synthase (NOS) activity as well as synthesis of AVP and oxytocin in the paraventricular (PVN) and supraoptic nuclei (SON), it is not known whether NOS activity in the hypothalamus changes in the diabetic patients who have plasma hyperosmolality with hyperglycaemia caused by insulin deficiency. Expression of the neuronal (n) NOS gene in the PVN and SON in streptozotocin (STZ)-induced diabetic rats was investigated by using in situ hybridization histochemistry and NADPH-diaphorase histochemical staining. Four weeks after intraperitoneal (i.p.) administration of STZ, male Wistar rats developed hyperglycaemia and plasma hyperosmolality. The expression of nNOS gene and NADPH-diaphorase staining in the PVN and SON remarkably increased in STZ-induced

diabetic rats compared to control rats. Three weeks after administration of STZ, the diabetic rats were subcutaneously treated with insulin for 1 week, which resulted in significant suppression of the induction of nNOS, AVP and oxytocin gene expression in the PVN and SON. Furthermore, the induction of nNOS gene expression in the PVN and SON was suppressed in STZ-induced diabetic rats treated with phlorizin and diet to normalize hyperglycaemia without insulin treatment. These results suggest that upregulation of nNOS gene expression as well as AVP and oxytocin gene expression in the PVN and SON in STZ-induced diabetic rats may be associated with hyperglycaemia and plasma hyperosmolality. [Diabetologia (1998) 41: 640–648]

Keywords Histochemistry, hyperosmolality, mRNA, nitric oxide, oxytocin, paraventricular nucleus, supraoptic nucleus, vasopressin.

The peripheral administration of streptozotocin (STZ) causes marked hyperglycaemia, plasma hyperosmolality, hypoinsulinaemia, hyperphagia and poly-

dipsia. In STZ-induced diabetic animals, insulin treatment reverses these symptoms. Several studies have demonstrated that insulin deficiency induced diabetes causes alteration of hypothalamic transmitters and the gene expressions of neuropeptides [1–7]. These studies suggest that the alteration of monoamine and neuropeptides in the hypothalamus may play a role in the multiple behavioural and hormonal abnormalities in STZ-induced diabetic rats.

Recent studies have demonstrated that nitric oxide (NO) may be associated with various functions of the central nervous system, including feeding [8–12] and drinking behaviour [13, 14]. It has been demonstrated that both systemic administration of a competitive antagonist of NO synthase (NOS) and

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Abbreviations: AVP, arginine vasopressin; NOS, nitric oxide synthase; PVN, paraventricular nucleus; SON, supraoptic nucleus; STZ, streptozotocin; nNOS, neuronal NOS; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PBS, phosphate buffered saline; NPY, neuropeptide Y.

central administration of an NOS inhibitor inhibited food intake in food deprived mice and rats [8–10], and moreover systemic administration of an NOS inhibitor inhibited food intake in chickens [11] and obese Zucker rats [12]. Central administration of *L*-arginine, the precursor amino acid of NO, inhibited drinking in water-deprived rats [13, 14]. Several studies have demonstrated that expression of neuronal NO synthase (nNOS) gene in the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON) is increased by osmotic stimuli such as dehydration [15, 16] and salt loading [17, 18]. The magnocellular neurosecretory cells in the PVN and SON synthesize arginine vasopressin (AVP) and oxytocin and release them into the systemic circulation from their axon terminals located in the posterior pituitary. Some studies have demonstrated that hypothalamic NO inhibits release of AVP [17, 19, 20] and central NO tonically inhibits basal release of AVP and oxytocin [21, 22]. Thus, NO may play an important role in the modulation of secretion of AVP and oxytocin in the hypothalamo-neurohypophyseal system [17, 19, 23].

Plasma AVP is known to be elevated in patients with uncontrolled insulin-dependent diabetes who have plasma hyperosmolality with hyperglycaemia [24]. The synthesis of hypothalamic AVP and oxytocin in diabetic rats was also seen to be increased [6]. Although NOS activity as well as synthesis of AVP and oxytocin in the hypothalamus is increased by plasma hyperosmolality under physiological conditions, it remains unknown whether NOS activity is increased or not in the diabetic rats that have plasma hyperosmolality with hyperglycaemia caused by insulin deficiency.

In the present study, the expression of nNOS gene in the PVN and SON was examined using *in situ* hybridization histochemistry in STZ-induced diabetic rats. Nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry was used to confirm the change in NOS enzyme activity in the PVN and SON. Furthermore, the effects of insulin treatment of diabetic rats on nNOS gene expression in the PVN and SON were examined. In addition, phlorizin (a continuous inhibitor of renal tubular reabsorption of glucose)-treatment and diet were used to normalize the plasma glucose level in STZ-induced diabetic rats. The expression of AVP and oxytocin genes in the PVN and SON were simultaneously examined as inner controls because they were expected to be upregulated in the diabetic rats.

Materials and methods

Animals. Adult male Wistar rats, weighing between 80 and 150 g, were used in all experiments from the start. They were housed three per plastic cage in an air-conditioned room (23–25 °C) under a 12-h light (07.00–19.00 hours) / 12-h dark (19.00–07.00 hours) cycle.

Experimental procedure. A solution (1 ml/kg) of 0.9% NaCl containing STZ (80 mg/ml) was administered once intraperitoneally (i.p.) to rats fasted for 24 h ($n = 20$). The same volume of 0.9% NaCl was administered i.p. to control rats ($n = 6$). These solutions were freshly dissolved and shielded from the light. At 2 days and 3 weeks after i.p. administration of STZ, the plasma concentration of glucose was measured using a gluco-sensor (Glutest E; Sanwa Co. Ltd., Nagoya, Japan). Rats that developed hyperglycaemia (> 16.6 mmol/l) were chosen as typical diabetic rats. These diabetic rats were divided into two groups: one group was given insulin (10–12 IU/rat, Humulin N; Eli Lilly, Kobe, Japan) s.c. once a day at 18.00 hours for 1 week and the other group was given 0.9% NaCl s.c. at the same time. The units of insulin administered were decided by a preliminary study that was monitoring the daily plasma glucose concentration. All rats had *ad libitum* access to tap water. STZ-treated animals and the corresponding controls were always handled in the same way and were killed by decapitation on the same day, 4 weeks after STZ administration. Blood and brain tissue samples were collected and analysed simultaneously. All procedures were in accordance with the Japanese Physiological Society's guidelines for animal care. This experiment was repeated twice independently. The first experiments were undertaken for the analysis of nNOS and AVP transcripts. The second experiments were undertaken for the analysis of nNOS and oxytocin transcripts. In addition, to examine the effects of insulin on expression of the nNOS gene in the PVN and SON in normal rats, insulin (0.4 IU/rat) was administered s.c. at 09.00 hours and rats were decapitated the next day at 09.00 hours. These rats were allowed free access to water but deprived of food for 1 day.

Furthermore, to normalize the glucose levels in STZ-induced diabetic rats, phlorizin (Sigma, St. Louis, Mo., USA) ($0.4 \text{ g} \cdot \text{kg body weight}^{-1} \cdot \text{day}^{-1}$); made up as a 25% solution in propylene glycol, was administered s.c. every 8 h for 12 days in STZ-induced diabetic rats, according to the published protocol [25]. In Group I phlorizin ($0.4 \text{ g} \cdot \text{kg body weight}^{-1} \cdot \text{day}^{-1}$) was s.c. administered every 8 h for 12 days in normal rats ($n = 6$). In these phlorizin-treated rats food was restricted (approximately 25 g of pelleted food/rat) with free access to water for one and one half days before killing. Control rats usually ate a similar volume of the pelleted food for 1 day. In Group II food was restricted (approximately 25 g pelleted food/rat) for one and one half days before killing in normal rats ($n = 6$). In Group III were STZ-induced diabetic rats ($n = 7$). In Group IV phlorizin ($0.4 \text{ g} \cdot \text{kg body weight}^{-1} \cdot \text{day}^{-1}$) was administered s.c. every 8 h for 12 days, and food was restricted (approximately 25 g pelleted food/rat) with free access to water for one and one half days before killing in STZ-induced diabetic rats ($n = 5$). All groups (control and Group I–IV) were decapitated at 09.00–10.30 hours of the same day.

Measurement of glucose, sodium, total protein and osmolality in plasma. Plasma concentrations of glucose, sodium and total protein (TP) were measured by conventional methods. The osmolality of plasma was measured using an Osmotic Pressure AUTO & STAT (OM-6030; Kyoto Daiichi Pure Chemicals Co. Ltd., Kyoto, Japan).

In situ hybridization histochemistry for nNOS, AVP and OXT mRNA. In situ hybridization histochemistry was performed on frozen 12 μm -thick coronal brain sections cut by cryostat at -20°C , thawed and mounted onto gelatin/chrome alum-coated slides that were kept at -80°C until used. The PVN and SON were localized by referring to the atlas 'The rat brain in stereotaxic coordinates' (2nd ed.) by Paxinos and Watson. The PVN and SON of four sections, eight sites per rat were

used to measure the density of autoradiography. The slides were warmed to room temperature and allowed to dry for 10 min, then fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 5 min, washed twice in PBS, and incubated in 0.9% NaCl containing 0.25% acetic anhydride (vol/vol) and 0.1 mol/l triethanolamine at room temperature for 10 min. The sections were then dehydrated through 70% (1 min), 80% (1 min), 95% (2 min) and 100% (1 min) ethanol and delipidated in 100% chloroform for 5 min. The slides were then partially rehydrated in 100% (1 min) followed by 95% (1 min) ethanol and allowed to dry briefly in air. Hybridization was performed at 37°C overnight in 45 µl of buffer consisting of 50% formamide and 4 × SSC (1 × SSC = 150 mmol/l NaCl, 15 mmol/l sodium citrate) containing 500 µg/ml sheared salmon sperm DNA (Sigma, St. Louis, Mo., USA), 250 µg/ml baker's yeast total RNA (Boehringer Mannheim GmbH, Mannheim, Germany), 1 × Denhardt's solution and 10% dextran sulfate (500 000 MWt, Sigma), under a Nescofilm (Bando Chemical IMD Ltd., Osaka, Japan) coverslip. The probes used were ³⁵S 3' end-labelled deoxyoligonucleotide complementary to transcripts coding for nNOS (complementary to bases 796–840 of rat nNOS nucleotides), AVP (complementary to bases 1843–1868 of rat AVP nucleotides) and oxytocin (complementary to bases 912–941 of rat oxytocin nucleotides). A total of 1 × 10⁶ cpm/slide for nNOS transcripts was used. Total of 3 × 10⁵ cpm/slide for AVP and oxytocin transcripts was used. After hybridization, the sections were washed for 1 h in four changes of 1 × SSC at 55°C and for a further 1 h in two changes of 1 × SSC at room temperature. All independent experimental sections were treated simultaneously to minimize the effects of variation in hybridization and wash stringency. Hybridized sections of the PVN and SON were apposed to autoradiography film (Hyperfilm, Amersham, Bucks, UK) for 7–14 days for nNOS transcripts and 8 h for AVP and oxytocin transcripts. The resulting images were analysed by computerized densitometry using an MCID imaging analyzer (Imaging Research Inc., Ontario, Canada). The mean optical density of autoradiographs was measured by comparison with simultaneously exposed [¹⁴C] micro-scale (Amersham, Bucks, UK). Slides hybridized to the nNOS probe were dipped in nuclear emulsion (K-5; Ilford, Cheshire, UK) and further exposed for 28 days.

NADPH-diaphorase histochemistry was performed as follows; STZ-induced diabetic rats ($n = 3$) and control rats ($n = 3$) were deeply anaesthetized by i.p. administration of pentobarbital sodium (50 mg/kg body weight). They were perfused transcardially with 0.9% NaCl containing heparin (1000 U/l) followed by 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4). The brain was removed and postfixed with 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 10–12 h at 4°C. Tissue samples were then cryoprotected in 10% sucrose in 0.1 mol/l phosphate buffer (pH 7.4) for 24 h at 4°C. Serial sections, 50 µm, were cut using a microtome. The sections were rinsed twice with 0.1 mol/l phosphate buffer (pH 7.4) and washed in 0.1 mol/l Tris buffer (pH 7.6) containing 0.3% Triton X-100. The free-floating sections were then incubated in 0.1 mol/l Tris buffer (pH 7.6) containing 0.3% Triton X-100, 0.2 mg/ml nitroblue tetrazolium and 1 mg/ml NADPH for 40 min at 37°C. To stop the reaction, the sections were transferred to 10% formaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) and rinsed twice with 0.1 mol/l phosphate buffer (pH 7.4). The sections were mounted onto slides coated with gelatin, air dried, dehydrated in 100% ethanol, cleared with xylene and covered with a coverslip.

Statistical analysis. All data are presented as mean ± SEM. The changes in plasma glucose, osmolality and electrolytes after

Table 1. Plasma glucose, sodium, total protein and osmolality in plasma after 4 weeks of streptozotocin and insulin treatment

	Control rats	Diabetic rats	Insulin treatment
Plasma glucose (mmol/l)	7.9 ± 0.2	29.4 ± 1.6 ^a	8.9 ± 1.3
Na (mEq/l)	141.0 ± 0.7	132.0 ± 0.8 ^a	140.0 ± 0.6
Total protein (g/dl)	5.8 ± 0.1	5.6 ± 0.1	6.0 ± 0.1
Plasma Osm (mOsm/kg H ₂ O)	294 ± 1.7	318 ± 2.9 ^a	302 ± 2.0

Values are mean ± SEM; $n = 6$ rats per group. ^a $p < 0.01$, vs control

STZ-treatment were statistically analysed using one-way fractional ANOVA followed by Bonferroni-type adjustment for multiple comparisons. The data obtained from in situ hybridization histochemistry were also analysed using one-way fractional ANOVA followed by Bonferroni-type adjustment for multiple comparisons. Statistical significance was established at the less than $p < 0.05$ level.

Results

Effects of STZ-induced diabetes and insulin treatment on nNOS and AVP gene expression. Body weights increased from 149 ± 1.4 to 246 ± 8.6 g in diabetic rats ($n = 6$), from 150 ± 3.0 to 300 ± 13 g in diabetic rats treated with insulin ($n = 6$) and from 146 ± 2.1 to 373 ± 9.0 g in control rats ($n = 6$). The plasma concentrations of glucose, sodium, total protein and osmolality are shown in Table 1. Plasma glucose and osmolality were significantly increased in diabetic rats. Plasma concentration of sodium was significantly decreased in diabetic rats. Total protein in plasma did not change in diabetic and insulin-treated rats. Treatment with insulin for 1 week induced a reduction in plasma concentrations of glucose, sodium and osmolality within the normal range in diabetic rats. In situ hybridization histochemistry revealed that diabetes for 4 weeks induced a significant increase in nNOS transcripts in the PVN (175 ± 8.5% of controls) and SON (201 ± 12% of controls) ($n = 6$ in each case, $p < 0.01$) (Fig. 1 A and B). This increase in expression of the nNOS gene in the PVN and SON in diabetic rats was reversed by insulin treatment to 110 ± 5.2% of control in the PVN and 119 ± 8.1% of control in the SON ($n = 6$ in each case) (Fig. 1 A and B). The absolute values of the control levels of nNOS gene expression were 3284 kBq/g in the PVN and 4446 kBq/g in the SON. Microscopic examination revealed that the number of cells expressing the nNOS gene and associated signals increased in the PVN and SON of diabetic rats (Fig. 2). Cells that expressed the nNOS gene were primarily observed in the magnocellular and the medial parvocellular parts of the PVN and throughout the SON. In situ hybridization histochemistry also re-

Fig. 1. A–F. Effects of STZ-treatment on nNOS (A, B), AVP (C, D) and oxytocin (E, F) transcript prevalence in the PVN and SON. Values represent mean $\pm$ SEM, $n = 5$ –7 rats; * $p < 0.01$ compared to control (one-way fractional ANOVA followed by Bonferroni-type adjustment for multiple comparisons). Panels 'A–D' were obtained from the same groups. Panels 'E' and 'F' were obtained from different experimental groups

vealed that diabetes for 4 weeks induced a significant increase in AVP transcripts in the PVN ($200 \pm 9.3\%$ of control) and SON ($178 \pm 11\%$ of control) ($n = 6$ in each case, $p < 0.01$) (Fig. 1C and D). This increase in expression of the AVP gene in the PVN and SON in diabetic rats was also reversed by insulin treatment to $108 \pm 16\%$ of control in the PVN and $123 \pm 11\%$ of control in the SON ($n = 6$ in each case) (Fig. 1C and D). The absolute values of the control levels of AVP gene expression were 741 682 kBq/g in the PVN and 712 378 kBq/g in the SON. Representative autoradiography hybridized to the specific probe complementary to AVP mRNA in the magnocellular parts of the PVN and SON of control, diabetic and insulin-treated diabetic rats are shown in Figure 3 A–a–c and B–a–c.

Eight rats did not develop hyperglycaemia after i.p. administration of STZ. Two of them were treated with insulin for 1 week. They were similarly examined simultaneously with the diabetic and control rats. Their results obtained from six non-diabetic rats were not significantly different from those of the control rats. Although the two rats treated with insulin became hypoglycaemic (2.8 and 3.4 mmol/l in plasma), expression of the nNOS gene did not decrease (115% of the control in the PVN and 124% of the control in the SON).

Effects of STZ-induced diabetes and insulin-treatment on nNOS and oxytocin gene expression. The body weights changed from 84 ± 2.6 to 236 ± 12 g in diabetic rats ($n = 6$) and from 80 ± 1.7 to 298 ± 9.4 g in control rats ($n = 6$) 4 weeks after i.p. administration of STZ or saline. The plasma concentration of glucose was significantly higher in diabetic rats and than in control rats at the end of 4 weeks after i.p. administration of STZ or 0.9% NaCl. The plasma concentrations of glucose, sodium and osmolality were similar to the results obtained from the first experiment (Table 1). Plasma glucose and osmolality were significantly increased in diabetic rats. Plasma concentration of sodium was significantly decreased in diabetic rats. Treatment with insulin for 1 week induced a reduction in plasma concentrations of glucose, sodium and osmolality within the normal range in diabetic rats. In situ hybridization histochemistry again revealed that diabetes for 4 weeks induced a significant increase in nNOS transcripts in the PVN ($157 \pm 12\%$ of control) and SON ($191 \pm 8.9\%$ of control) ($n = 6$; control rats, $n = 7$; diabetic rats, $p < 0.01$). This increase in expression of the nNOS gene in the PVN and SON in diabetic rats was reversed by insulin treatment to $101 \pm 3.6\%$ of control in the PVN and $131 \pm 14\%$ of control in the SON ($n = 5$; insulin-treated diabetic rats). The absolute values of the control level of nNOS gene expression were 15 707 kBq/g in the PVN and 13 351 kBq/g in the SON. In situ hybridization histochemistry also revealed that diabetes for 4 weeks induced a significant increase in oxytocin transcripts in the PVN ($170 \pm 13\%$ of control) and SON ($198 \pm 15\%$ of control) ($n = 6$ in control rats and $n = 7$ in diabetic rats, $p < 0.01$) (Fig. 1E and F). This increase in expression of the oxytocin gene in the PVN and SON in diabetic rats was reversed by insulin treatment to $130 \pm 11\%$ of control in the PVN and $143 \pm 16\%$ of control in the SON ($n = 5$ in insulin-treated diabetic rats) (Fig. 1E and F). The absolute measurements of control level of oxytocin gene expression were 517 050 kBq/g in the PVN and 351 470 kBq/g in the SON. Representative autoradiography hybridized to the specific probe complementary to oxytocin mRNA in the magnocellular parts of the PVN and SON of control, diabetic and insulin-treated diabetic rats are shown in Figure 3A–d–f and B–d–f.

Fig. 2. A–F. Dark field photomicrographs of emulsion-dipped tissue hybridized to a ^{35}S -labelled oligodeoxynucleotide probe complementary to nNOS mRNA in the PVN (A–C) and SON (D–F). A and D show sections from control rats; B and E from STZ-induced diabetic rats; C and F from diabetic rats with insulin treatment. Scale bars are 100 μm OC, Optic chiasma

In this experiment eight rats did not develop hyperglycaemia after i.p. administration of STZ. They were similarly examined simultaneously with the diabetic and control rats. Their results were not significantly different from those of the control rats.

Effects of insulin treatment on nNOS gene expression in normal rats. The plasma concentrations of glucose in control ($n = 6$), fasted for 24 h ($n = 6$) and insulin-treated and fasted rats ($n = 4$) were 5.6 ± 0.2 , 3.3 ± 0.2 and 3.7 ± 0.2 mmol/l, respectively. Expression of the nNOS gene in the PVN and SON in insulin-treated and fasted rats was significantly increased to $150 \pm 4.6\%$ of control in the PVN and $155 \pm 16\%$ of control in the SON, respectively. The expression of nNOS gene in the PVN and SON in fasted rats without insulin treatment were similar to that in control.

Effects of phlorizin-treatment on nNOS gene expression in STZ-induced diabetic rats. The body weights changed from 122 ± 1.5 to 264 ± 6.1 g in control ($n = 6$), from 127 ± 1.4 to 246 ± 6.1 g in Group I ($n = 6$), from 124 ± 2.0 to 241 ± 3.9 g in Group II ($n = 6$), from 123 ± 3.1 to 160 ± 9.0 g in Group III ($n = 7$) and from 122 ± 1.4 to 152 ± 8.9 g in Group IV ($n = 5$). The plasma concentrations of glucose, sodium and osmolality are shown in Table 2. The plasma concentrations of glucose and sodium are normalized in Group IV. Plasma osmolality was decreased in Group IV, but significantly higher than that in control. The expression of nNOS gene in the PVN and SON did not change significantly in control, Group I and Group II (Fig. 4). The increase of nNOS gene in the PVN and SON in Group III was reversed by phlorizin treatment and diet from $178 \pm 5.9\%$ to $109 \pm 6.9\%$ of control in the PVN and from $225 \pm 6.3\%$ to $139 \pm 5.5\%$ of control in the SON (Fig. 4). The absolute values of the control levels of nNOS gene expression were 1169 kBq/g in the PVN and 8785 kBq/g in the SON.

Effects of STZ-induced diabetes on NADPH-diaphorase staining. As shown in Figure 5, NADPH-diaphorase staining revealed that the cell bodies and

Fig. 3. A, B. Representative computer-generated colour images of autoradiograms of tissue hybridized to a ^{35}S -labelled oligodeoxynucleotide probe complementary to AVP mRNA (Aa-c and Ba-c) and oxytocin mRNA (Ad-f and Bd-f) in the PVN (A) and SON (B). 'Red' is most intense signal and 'dark blue' is least intense signal. The density of autoradiographs was measured by comparison with simultaneously exposed ^{14}C micro-scale. Panels 'Aa,d' and 'Ba,d' are sections from control rats; panels 'Ab,e' and 'Bb,e' from diabetic rats; panels 'Ac,f' and 'Bc,f' from diabetic rats with insulin treatment. The cartoons illustrate the limits of autoradiographs. Scale bars are 1 mm

fibres in the PVN and SON were more densely stained in diabetic rats compared to control rats. NADPH-diaphorase staining appeared to be located in the dorsal part more than the ventral part of the SON. In the PVN, the distribution of NADPH-diaphorase stained cell was observed in the posterior magnocellular and the medial parvocellular regions. This increase in NADPH-diaphorase staining supports the notion that NOS activity in the PVN and SON increases in diabetic rats.

All experiments were undertaken independently. The results obtained from five independent experiments were consistent.

Discussion

The present study has provided the first evidence of significant increases in the expression of nNOS gene as well as the AVP and oxytocin genes in the PVN and SON in STZ-induced diabetic rats. The upregulation of the nNOS gene in the PVN and SON in diabetic rats was suppressed by insulin treatment, indicating that non-specific effects of STZ were excluded. The upregulation of the nNOS gene in the PVN and SON in diabetic rats was also suppressed by normalizing the hyperglycaemia in diabetic rats treated with phlorizin and diet.

In diabetic rats, we observed a significant increase in plasma osmolality compared with control rats. Hyperosmolality caused by dehydration and chronic salt loading increases in the expression of the nNOS gene in the PVN and SON [15–18]. Although hypernatraemia may upregulate nNOS gene expression in the PVN and SON in diabetic rats, the plasma concentration of sodium significantly decreased in these rats. The reduction in plasma sodium may be the result of the increased excretion of sodium in the urine of the diabetic rats. As total protein in plasma did not change in the diabetic and insulin-treated diabetic rats, hypovolaemia may not have occurred in those rats. Therefore, osmotic stimulation may explain the upregulation of nNOS gene expression in the PVN and SON observed in the STZ-induced diabetic rats. Recent studies have demonstrated that NO in the hypothalamus may modulate secretion of AVP [17, 21,

Fig. 4. A, B. Effect of phlorizin treatment and diet on nNOS transcript prevalence in the PVN and SON. Values represent mean $\pm$ SEM, $n = 5-7$ rats; * $p < 0.01$ compared to control (one-way fractional ANOVA followed by Benferroni-type adjustment for multiple comparisons). **A** and **B** were obtained from the same groups. Group I: phlorizin (0.4 g/kg body weight/day) was administered s.c. every 8 h for 12 days in normal rats ($n = 6$), Group II: food was restricted for one and one half days before the decapitation in normal rats ($n = 6$), Group III: STZ-induced diabetic rats ($n = 7$), Group IV: phlorizin (0.4 g/kg body weight/day) was administered s.c. every 8 h for 12 days, and food was restricted for one and one half days before the decapitation in STZ-induced diabetic rats ($n = 5$)

22] and oxytocin [21–23]. Some anatomical studies have demonstrated that NADPH-diaphorase activity co-exists with AVP- and oxytocin immunoreactivity [26, 27]. They also showed that the co-existence of NADPH-diaphorase staining and oxytocin immunoreactivity is more predominant than that of NADPH-diaphorase staining and AVP immunoreactivity. In the present study, the distribution of NADPH-diaphorase staining in the PVN and SON is consistent with previous studies [26, 27]. The synthesis of hypothalamic AVP and oxytocin increases in diabetic rats [6] as confirmed by our result that the expression of AVP and oxytocin genes in the PVN and SON is upregulated in diabetic rats.

The sensitivity of the AVP response to osmotic stimuli has been shown to be significantly reduced in

diabetic patients [28] and STZ-induced diabetic rats [29]. One possible reason is that NO suppresses the excitatory input to the PVN and SON and causes a reduction in sensitivity to osmotic stimuli in the diabetic state, because NO in the PVN and SON tonically inhibit secretion of AVP and oxytocin [21, 22]. Furthermore, diabetes is known to induce changes in the noradrenaline and adrenaline concentrations of the various brain regions, including the hypothalamus [1, 2]. These changes may also be influenced by excessive NO in the PVN and SON. These possibilities require further examination.

Chiodera et al. [20] demonstrated that the increase in plasma AVP and oxytocin response to insulin-induced hypoglycaemia in men is enhanced by NOS inhibitor, N, G-nitro-L-arginine methyl ester (L-NAME). They suggested that NO may have an inhibitory role in the control of AVP and oxytocin responses to hypoglycaemia. Although marked hyperglycaemia was observed in the diabetic rats, whether NO has a physiological role in the control of AVP and oxytocin responses to hyperglycaemia remains unknown. As phlorizin, a continuous inhibitor of renal tubular reabsorption of glucose, is a useful drug with which to distinguish between the roles of hyperglycaemia and hypoinsulinaemia in the regulation of nNOS gene expression in the PVN and SON, we made phlorizin-treated rats according to the previously published protocol [25]. The phlorizin-treated rats with diet showed the reduction of nNOS gene in the PVN and SON by normalizing the hyperglyca-

Table 2. Plasma glucose, sodium and osmolality in plasma after 2 weeks after administration of streptozotocin and phlorizin treatment

	Control	Group I	Group II	Group III	Group IV
Plasma glucose (mmol/l)	8.3 $\pm$ 0.3	8.5 $\pm$ 0.3	7.4 $\pm$ 2.8	29.7 $\pm$ 1.9 ^b	8.2 $\pm$ 0.9
Na (mEq/l)	141 $\pm$ 1.0	142 $\pm$ 0.4	142 $\pm$ 0.7	128 $\pm$ 1.5 ^b	142 $\pm$ 1.2
Plasma Osm (mOsm/kg H ₂ O)	297 $\pm$ 2.6	292 $\pm$ 3.7	295 $\pm$ 2.8	315 $\pm$ 2.1 ^b	307 $\pm$ 2.2 ^a

Group I: phlorizin (0.4 g/kg body weight/day) administered s.c. every 8 h for 12 days in normal rats ($n = 6$), Group II: food was restricted for one and one half days before the decapitation in normal rats ($n = 6$), Group III: STZ-induced diabetic rats ($n = 7$), Group IV: phlorizin (0.4 g/kg body weight/day)

administered s.c. every 8 h for 12 days, and food was restricted for one and one half days before the decapitation in STZ-induced diabetic rats ($n = 5$). Values are the mean $\pm$ SEM; $n = 5-7$ rats per group. ^a $p < 0.05$, ^b $p < 0.01$ vs control

Fig. 5. A–D. Representative NADPH-diaphorase staining in the SON and PVN. **A** and **B** are sections from control rats; **C** and **D** from diabetic rats. Density of NADPH-diaphorase staining increased in the SON (**C**) and PVN (**D**) of diabetic rats compared with the SON (**A**) and PVN (**B**) of controls. Scale bars are 100 μ m

emia. The plasma osmolality in these rats decreased, but was significantly higher than control levels. Therefore, hyperglycaemia may be more dominant than plasma hyperosmolality in causing the upregulation of nNOS gene expression in the PVN and SON in the diabetic rats. In addition, we found that expression of the nNOS gene in the PVN and SON did not decrease in STZ-administered non-diabetic rats treated with insulin and significantly increased in normal rats treated with insulin. Therefore, insulin treatment itself probably does not decrease expression of the nNOS gene in the PVN and SON.

Diabetes causes behavioural abnormalities such as hyperphagia and polydipsia. Several studies have demonstrated that diabetes induces changes in the expression of genes coding for feeding-related peptides such as neuropeptide Y (NPY), cholecystokinin and corticotropin-releasing hormone in the hypothalamus [3]. The hypothalamic contents of NPY [4, 5] and prodynorphin-derived peptides in the hypothalamus

[7] have been shown to increase in diabetic rats. As it has been demonstrated that NO may modulate hypothalamic NPY-induced feeding [8], the central serotonergic system related to feeding [10], an excess of NO may be involved in the central mechanism of hyperphagia in diabetes. In addition, several previous studies have demonstrated that feeding and metabolic change such as food deprivation [15, 30] and hyper- or hypothyroidism [31] caused a significant change in the expression of nNOS gene in the hypothalamus, suggesting that NO may be associated with feeding control and metabolism.

In conclusion, we have demonstrated that expression of the nNOS gene as well as AVP and oxytocin genes in the PVN and SON increases in diabetic rats, suggesting that hyperglycaemia and plasma hyperosmolality may cause upregulation of these genes in the hypothalamus.

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