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Insulin resistance occurs in parallel with sensory neuropathy in streptozotocin-induced diabetes in rats: differential response to early vs late insulin supplementation

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ABSTRACT

We investigated whether progressive sensory neuropathy was accompanied by changes in whole-body insulin sensitivity (WBIS) in rats made diabetic by streptozotocin (STZ). The effects of early and late insulin supplementation were also studied. The STZ-treated rats failed to gain weight and exhibited stable hyperglycemia and low plasma insulin levels with a decrease in nerve conduction velocity (NCV) measured in A and C fibers of the saphenous nerve. A decreased sensory neuropeptide (SNP) release such as that of substance P, somatostatin, and calcitonin gene–related peptide determined from organ fluid of tracheal preparations subjected to electrical field stimulation also occurred in diabetic animals. These features were accompanied by a decrease in WBIS measured by hyperinsulinemic-euglycemic glucose clamping and a decrease in insulin-stimulated glucose uptake in cardiac and gastrocnemius muscle. When insulin supplementation with slow-release implants (2 IU/d) was started 4 weeks after STZ injection, blood glucose level normalized. Both insulin sensitivity and sensory nerve function reflected in either NCV or SNP release completely recovered by the 12th post-STZ week. When the insulin implants were applied from the eighth post-STZ week, both WBIS and glucose uptake remained significantly decreased, with a seriously impaired NCV and SNP release with strong hyperglycemia. Late insulin supplementation, however, even by using double implantation from the 10th post-STZ week, was unable to restore blood glucose, WBIS, NCV, and SNP release by the 12th week. Insulin resistance occurs in parallel with sensory neuropathy in STZ-diabetic rats. Both can be improved by early but not late insulin supplementation.

Authors' contributions: Zoltan Szilvassy: study idea, design, scientific advisory ; Joseph Nemeth: radioimmunoassay measurements; Peter Kovacs, György Paragh: scientific advisory, sensory neuropeptide release study, endogenous glucose production measurements, peripheral glucose uptake measurements; Reka Sari: nerve conduction velocity measurements, statistics; Laszlo Vígh: scientific advisory, biochemical measurements; Barna Peitl: animal experiments, insulin sensitivity determination, tissue glucose flux measurement, statistics, scientific advisory.

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1. Introduction

It has long been recognized that sensory nerves not only transmit sensory information but also act upon the local environment by releasing several biologically active mediators (for reviews, see Holzer[1] and Szolcsanyi [2]). It is widely accepted that these mediators underpin the local effector function of sensory nerves [2]. Some sensory neurotransmitters, principally neuropeptides, however, in addition to mediating local effects, may enter the circulation and initiate effects at remote sites [3]. For example, somatostatin of sensory neural origin has been shown to play a major role in the development of a systemic anti-inflammatory effect promoted by local neurogenic inflammation [3]. Subsequently, Porszasz et al [4] demonstrated that stimulation of the anterior hepatic plexus fibers either electrically or by treatment with perineurial capsaicin, an agent acting on receptors exclusively expressed in sensory fibers [5], attained an increase in circulating somatostatin level with an increase in whole-body insulin sensitivity (WBIS). This latter observation suggested that somatostatin as a sensory neuropeptide (SNP) was involved in insulin sensitization pathways controlled by autonomic and sensory fibers in the anterior hepatic plexus.

That sensory neural function is important in the regulation of WBIS was recognized more than a decade ago. Koopmans et al [6] found that sensory deafferentation by capsaicin resulted in an increase in insulin sensitivity in conscious rats. Similar results were found in adult guinea pigs with systemic capsaicin desensitization [7]. Therefore, it was suggested that systemic attenuation of sensory effector function yields insulin sensitization, possibly due to decreased systemic availability of the SNP calcitonin gene–related peptide (CGRP), a physiological antagonist of insulin [8,9]. Nevertheless, in type 1 diabetes mellitus, a disease known to be underlain by decreased insulin release/production, definite insulin resistance, rather than insulin sensitization, occurs with marked sensory neuropathy [10,11]. It is therefore unclear as to whether a deficiency in sensory effector function that is characteristic of insulin-deficient diabetes contributes to or counteracts the development of insulin resistance seen in this disease.

The present work was therefore undertaken to investigate the parallelism between insulin sensitivity and sensory neural dysfunction in experimental diabetes induced by streptozotocin (STZ), a model of insulin-deficient diabetes, in rats.

2. Methods

2.1. Ethics

All treatments and diets were formally approved by the University of Debrecen Animal Ethics Committee. The experiments presented conform to European Community guiding principles for the care and use of laboratory animals. The experimental protocols applied had been approved by the ethical boards of the University of Debrecen, Hungary (08/2007 DE MÁB and 16/2007 DEMÁB).

2.2. Experimental groups and study design

The study was carried out with 48 male Wistar rats weighing 200 to 210 g. They were housed in an animal room (12-hour light/dark periods a day, temperature of 22°C-25°C, humidity of 50%-70%) with 4 animals per pen and fed commercial laboratory chow and tap water ad libitum. The animals were randomly divided into 2 experimental groups. The control animals were treated with the solvent for STZ, whereas the rats in the second group were treated with 50 mg/kg STZ intravenously (Zanosar; Upjohn, Kalamazoo, MI) to make them diabetic. After 4 weeks, the STZ-treated animals were further randomized into 2 additional groups, one of which comprised animals that were supplied with continuous-delivery (approximately 2 IU/d) subcutaneous insulin implants (Linplant; Linshin Canada Inc, Toronto, Canada). This group was referred to as the *early insulin-supplemented group*. The implants were placed at the back of the neck under thiopental (Thiopental Sandoz; Sandoz, Kundl, Austria) anesthesia (45 mg/kg intraperitoneally). The other subgroup of rats received matching placebo implants (diabetic or nonsupplemented group). Eight weeks after STZ injection, the animals in the nonsupplemented group were further randomized. Half of the rats were given an active insulin implant instead of their placebo implants (late insulin-supplemented group); the rest of the rats wore matching placebo implants. Because the rats in the late insulin-supplemented group revealed hyperglycemia (see later), half of the animals of this subgroup received a second insulin implant at the 10th week (Fig. 1).

2.3. Study end points

From both control and the STZ-treated animals (including the diabetic and the insulin-supplemented subgroups of animals), the following measurements were done: hyperinsulinemic and euglycemic clamp experiments supplemented with insulin-induced suppression of hepatic glucose production and peripheral glucose uptake by cardiac and gastrocnemius muscle were performed to determine peripheral glucose disposal and tissue response to insulin, respectively. Nerve conduction velocity (NVC) studies were executed to assess the degree of diabetic neuropathy, whereas SNP release studies were carried out to estimate changes in sensory effector nerve function over the course of long-lasting experimental insulin-deficient diabetes with or without exogenous insulin. Because previous studies revealed that STZ diabetes was always accompanied by hypersomatostatinemia [12,13], plasma somatostatin level was determined simultaneously with plasma insulin and blood glucose level. To elucidate the role of hypersomatostatinemia in adjustment of WBIS in STZ diabetes, subgroups of 6-6 rats were devoted to study the effect of cysteamine, a somatostatin-depleting agent [14,15], in hyperinsulinemic-euglycemic clamp experiments.

2.4. NVC studies

This series of experiments was carried out to verify/exclude sensory neuropathy involving unmyelinated slow conducting "C" fibers. Left saphenous NVC was determined in animals from both healthy and STZ-diabetic groups as described

Fig. 1 – The schematic diagram of the study protocol. Initially, 2 groups were formed; the "Healthy" animals remained untreated, whereas the "STZ" group was treated with 50 mg/kg STZ intravenously. The "STZ + Linplant 4-12 weeks" subgroup was formed from the STZ group. These STZ-treated animals were subjected to insulin implantation 4 weeks after the STZ treatment. The "STZ + Linplant 8-12 weeks" subgroup was also formed from the STZ group, and these STZ-diabetic rats were subjected to insulin implantation 8 weeks after the STZ treatment. The "STZ + Linplant 10-12 weeks" subgroup was formed from the "STZ + Linplant 8-12 weeks" group and was given the insulin implant 2 weeks after the first insulin implant.

[12,16,17]. In brief, the saphenous nerve was prepared cleaned of fat and adhering connective tissues in artificially ventilated animals anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). Afterward, strains of square-wave (500 microseconds) constant voltage stimuli were applied through pairs of platinum electrodes (Experimetria Ltd, London, UK) placed as high as possible. Another pair of electrodes was applied 2 cm distal to the stimulating electrodes for recording the summation action potentials evoked by the proximal stimulation. The time lags between stimulation and the appearance of corresponding "A" and "C" signals were determined for calculation of average conduction velocity. The interelectrode distance was divided by the time interval between the end of the stimulatory impulse and the appearance of the corresponding "A" and "C" signals [18]. The STZ-induced "C" signal delay was used for characterization of diabetic sensory neuropathy.

2.5. *Neurotransmitter release studies*

These methods have been described in detail elsewhere [16]. In brief, after the animals were killed by exsanguination, the lower third of the tracheae with the main bronchi were removed and cleaned of fat and adhering connective tissues. They were prepared for perfusion in a temperature (37°C)- and pH (7.2)-controlled, oxygenized Krebs solution over 60 minutes. Electrical field stimulation (FS; 40 V, 0.1 millisecond, 10 Hz for 120 seconds) was applied to elicit neurotransmitter release. The CGRP, substance P, and somatostatin concentrations were determined from 200-μL samples of organ fluid of the preparations by means of radioimmunoassay methods developed in our laboratories as described previously [16,19]. These studies were carried out to characterize the deficient sensory effector function with the progression of diabetic neuropathy. The tracheal preparations were chosen because, in this tissue, the sensory nerve endings locate superficially enough to enable release of neuropeptides into the organ fluid in response to various challenges. Because these neuropeptides mediate a significant portion of the widespread effector function of sensory fibers, we think that the data obtained with this method provide an analytical approach to determining changes in sensory effector function in experimental diabetes [13,16].

2.6. *Determination of plasma somatostatin, insulin, blood glucose, free fatty acids, and triglyceride concentrations*

Arterial blood samples (3 mL per rat) were taken into ice-cold tubes containing EDTA (6 mg) and Trasylol (1000 IU). The samples were then centrifuged at 4°C (2000 rpm for 10 minutes). The somatostatin content of 1 mL plasma was extracted by addition of 3 vol of absolute ethanol. After precipitation and a second centrifugation with the same parameters, the supernatants were aspirated and subsequently evaporated under nitrogen as described [20]. Plasma somatostatin and insulin immunoreactivity was determined by means of radioimmunoassay [21]; blood glucose level was determined by glucose peroxidase method; and plasma triglyceride and free fatty acid (FFA) were determined by enzyme colorimetry for the glycerol phosphate oxidase and the acyl coenzyme synthetase enzymes, respectively.

2.7. *Hyperinsulinemic-euglycemic glucose clamping*

Hyperinsulinemic-euglycemic glucose clamping (HEGC) experiments were performed in anesthetized, spontaneously breathing animals. The food-deprived rats (food withdrawal 12 hours preceding the experiments) were anesthetized with intraperitoneal sodium thiopental (50 mg/kg Thiopental Sandoz), and the trachea was cannulated. Throughout the experimental period, the rectal temperature of anesthetized rats was maintained at 37.0°C to 37.5°C using heatable operating table. Human regular insulin (NOVO Nordisk, Copenhagen, Denmark) was infused at a constant rate of 20 mU/(kg min) over 120 minutes via a cannula inserted into one of the jugular veins. This insulin infusion rate yielded plasma insulin immunoreactivity of 185 ± 5 μU/mL in the steady state (see below). Blood samples (0.05 mL) for blood glucose and for plasma peptide determination were taken from the carotid artery. Blood glucose level was determined every 10 minutes, and euglycemia (5.5 ± 0.5 mmol/L) was maintained by a variable rate of glucose infusion (20%) via a

second venous cannula introduced into one of the femoral veins. When blood glucose had stabilized for at least 20 minutes, we defined this condition as steady state. This generally occurred within 40 minutes from commencement of the insulin infusion. In the steady state, additional blood samples (0.3 mL) were taken for plasma peptide determination 3 times at 10-minute intervals. The glucose infusion rate (GIR; expressed in milligrams per kilogram per minute) during steady state was used to characterize insulin sensitivity [4,22,23].

2.8. Tissue glucose flux

The details of these determinations were described previously [7]. In brief, a continuous infusion of high-performance liquid chromatography–purified [3-^{3}H]glucose (12-μCi bolus, followed by 10 μCi/min; DuPont-NEN, Boston, MA, USA) was commenced 60 minutes before the start of the HEGC. Subsequently, 12 μCi [3-^{3}H] glucose was added to the glucose infusion to maintain specific activity within 25% of the baseline. Blood samples were taken simultaneously with those for plasma insulin immunoreactivity determination. For determination of insulin-stimulated glucose uptake by the gastrocnemius and cardiac muscle, tissue samples were estimated using the administration of an intravenous bolus of 2-deoxy-D [L-^{14}C] glucose (NEN Life Science Products Inc., Boston, MA, USA), a nonmetabolizable glucose analogue, and by determining the tissue content of 2-deoxyglucose-6-phosphate. A 10-μCi bolus of 2-deoxy-D [L-^{14}C] glucose was given in the 10th minute of the steady state of glucose clamping. For determination of tissue [L-^{14}C] 2-deoxyglucose-6-phosphate, the heart and gastrocnemius muscles were removed from each animal within 3 minutes after the experiments. The tissue samples were homogenized and centrifuged.

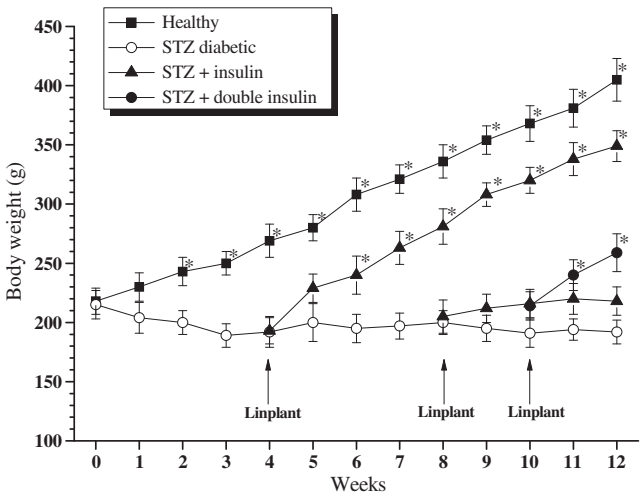


Fig. 2 – The change in body weight during the 12 weeks of the experiment in rat assigned to different treatment interventions. The STZ treatment resulted in a lag in body weight gain as compared with the healthy rats; but after an adequate insulin supplementation, the animals started to regain weight. Arrows indicate the time when the insulin implantation was carried out. *Statistically significant difference ($P < .05$) from the untreated, STZ-diabetic group.

The supernatants were run on an ion exchange column to separate deoxyglucose from deoxyglucose-6-phosphate.

2.9. Drugs and chemicals

Beyond the isotopes used, all drugs and chemicals have been purchased from Sigma (St Louis, MO).

2.10. Statistics

The results expressed as means ± SD were analyzed with one-way analysis of variance followed by a modified t test for repeated measures according to the Bonferroni method [24]. Changes were considered significant at $P \leq .05$.

3. Results

3.1. Effects of insulin supplementation on body weight in STZ-induced diabetes

As shown by the results in Fig. 2, the healthy animals grew steadily over the 12-week observation period with an average weight gain of 172 ± 18 g. The STZ-treated animals without insulin supplementation exhibited a transient marginal weight loss. Essentially, the body weight of the STZ-treated animals without application of exogenous insulin remained the same throughout the 12-week period. Insulin supplementation from the slow-release implants (approximately 2 IU/d) over the period of 4 to 12 weeks caused a significant increase in body weight to a level approaching that seen in healthy animals. When the insulin implants were applied only from the eighth week after STZ treatment, no increase in body weight was seen. Nevertheless, when an additional implant was applied in a subgroup of animals in this latter group from the 10th week, body weight tended to increase again with a degree of significance seen at the end of either the 11th or the 12th week.

3.2. Effects of insulin supplementation on blood glucose and plasma insulin, somatostatin, FFA, and triglyceride levels in STZ-induced diabetes

It is seen from the data in Table 1 that fasting blood glucose levels in healthy animals and in those 4, 8, and 12 weeks after STZ injection were 4.9 ± 0.6, 22.6 ± 4.2, 18.2 ± 3.9, and 15 ± 2.8 mmol/L, respectively. Insulin supplementation with the slow-release implants applied 4 weeks after STZ treatment was found to normalize blood glucose level measured either 4 or 8 weeks later. However, in diabetic animals with an initial 8-week post-STZ period without insulin supplementation, application of the slow-release implants from the eighth week attained a much less pronounced hypoglycemic effect than in those wearing insulin implants with an effect from the fourth week. This is reflected in a significantly higher fasting blood glucose level seen in STZ-treated rats with implants during 8 to 12 weeks than in those with implants during 4 to 12 weeks. Fasting blood glucose level measured at the end of the 12th week was significantly higher in the subgroup of rats with double insulin supplementation (one implant from the eighth post-STZ week, completed with an additional one from

Table 1 – Fasting blood glucose, fasting plasma insulin, and fasting plasma somatostatin level during the experimental period

	0 wk	4 wk	8 wk		12 wk			
	Healthy	STZ	STZ	STZ + L4	STZ	STZ + L4	STZ + L8	STZ + L10
FBG (mmol/L)	4.9 ± 0.6	22.6 ± 4.2*	18.2 ± 3.9*	5.7 ± 0.8†	15.0 ± 2.8*	7.4 ± 1.2*‡	12.9 ± 3.1*	9.0 ± 2.2*‡
FPI (mU/mL)	12.0 ± 3.3	2.1 ± 0.4*	2.9 ± 0.4*	16.2 ± 3.9†	6.4 ± 1.5*	17.7 ± 4.1‡	18.6 ± 4.4‡	26.3 ± 4.9*‡
FPS (pmol/L)	5.5 ± 0.9	19.7 ± 2.8*	22.1 ± 3.0*	7.1 ± 1.6†	18.7 ± 2.6*	7.4 ± 2.0‡	16.0 ± 2.4*	11.0 ± 1.5*‡

FBG indicates fasting blood glucose; FPI, fasting plasma insulin; FPS, fasting plasma somatostatin; Healthy, solvent-treated nondiabetic; STZ, STZ-treated diabetic; STZ + L4, STZ-treated diabetic with insulin implant from the fourth week; STZ + L8, STZ-treated diabetic with insulin implant from the eighth week; STZ + L10, STZ-treated diabetic with additional insulin implant from the 10th week.
‡ Statistically significant difference ($P < .05$) from the rats treated with STZ 12 weeks ago.
* Significant difference ($P < .05$) from the healthy group.
† Statistically significant difference ($P < .05$) between the STZ diabetic and STZ + L4 group.

the 10th week) (Table 1.). Similar results were seen with plasma FFA and triglyceride levels. The data in Table 2 show that early insulin supplementation nearly normalized plasma FFA and triglyceride elevation in diabetic animals. Insulin supplementation commenced at the eighth week or later failed to normalized either FFA or triglyceride increase.

A dramatic fall in fasting plasma insulin immunoreactivity was seen in response to STZ treatment. Insulin supplementation normalized plasma insulin immunoreactivity in each group. The plasma insulin level seen in STZ-treated animals receiving an implant at the eighth post-STZ week and another one at the 10th week was significantly higher than that measured in healthy animals.

3.3. *Nerve conduction velocity*

It is shown in Fig. 3 that the STZ-diabetic state was associated with a decrease in NVC in fast-conducting myelinated (A fibers in Fig. 3A) and slow-conducting unmyelinated (C fibers in Fig. 3B) fibers. At a stimulation intensity suprathreshold for A (5 V, 5 Hz) or for C (3 V, 0.5 Hz) fibers, NVC significantly decreased in diabetic rats irrespective of whether determinations were done 8 or 12 weeks after the STZ injection. In the insulin-supplemented animals, when the exogenous insulin delivery systems were applied from the fourth post-STZ week, NVC for either A or C fibers did not differ from that determined in the control group. Nevertheless, when the insulin implants were applied as late as 8 weeks after STZ administration, the delay in NVC measured in either fiber was marked by the end of the 12th post-STZ week.

Table 2 – Free fatty acid and triglycerides level after various treatment schedules with or without insulin supplementation with implantable insulin (Linplant)

Intervention	FFA (pmol/mL)	Triglyceride (mg/dL)
Control	754 ± 96.6	101 ± 29.2
STZ diabetes 4 wk	1155 ± 105.7*	212 ± 41.4*
STZ diabetes 8 wk	1495 ± 152.8*	336 ± 54.7*
STZ diabetes 8 wk with Linplant from the 4th wk	842 ± 100.3	116 ± 32.2
STZ diabetes 12 wk	1965 ± 211.9*	411 ± 52.7*
STZ diabetes 12 wk with Linplant from the 4th wk	811 ± 104.4	109 ± 28.8
STZ diabetes 12 wk with Linplant from the 8th wk	1655 ± 162.8*	362 ± 49.9*
STZ diabetes 12 wk with 2 Linplants	1522 ± 171*	329 ± 52.4*

The data are means ± SD obtained with 8 animals per group.
* Significantly different from control at $P < .05$.

3.4. *SNP release*

The FS-induced release of somatostatin, CGRP, and substance P was significantly attenuated in tracheal preparations from STZ-treated rats than in those from healthy animals (Fig. 4A-C). Insulin supplementation started in the fourth post-STZ week yielded complete restoration of FS-induced SNP release by the eighth week with a near-complete recovery by the 12th week. However, a significant reduction of FS-induced release of the neuropeptides studied was seen when insulin supplementation started at the eighth week either with or without additional insulin implants from the 10th post-STZ week.

3.5. *Changes in WBIS in STZ-diabetic rats*

Whole-body insulin sensitivity determined by HEGC decreased with time elapsed after STZ treatment. Significant changes were observed 8 and 12 weeks after STZ injection. Insulin supplementation with a single slow-release implant on the fourth post-STZ week completely restored baseline insulin sensitivity measured either on the 8th or the 12th week. When the Linplant device was implanted 8 weeks after STZ treatment, the insulin sensitivity determined at the end of the 12th post-STZ week did not significantly differ from that measured in the diabetic animals without exogenous insulin. The insulin-resistant state was not modified even by application of an additional Linplant device on the 10th week. Only a tendency to an improvement of insulin sensitivity was seen in this latter case (Fig. 5).

3.6. *The effect of STZ diabetes on percentage suppression of endogenous glucose production at HEGC*

The intravenous insulin plus glucose infusion administered during the steady state of the clamp procedure as shown in Fig. 6 induced complete inhibition of endogenous glucose production both in healthy rats and in those with a 4-week history of STZ diabetes. The insulin-induced suppression of

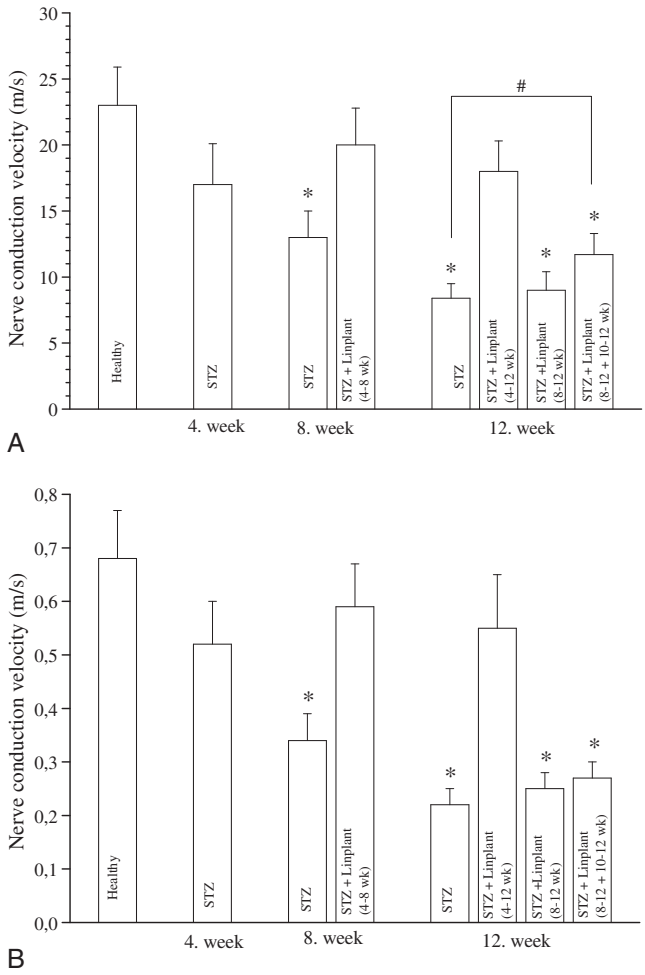
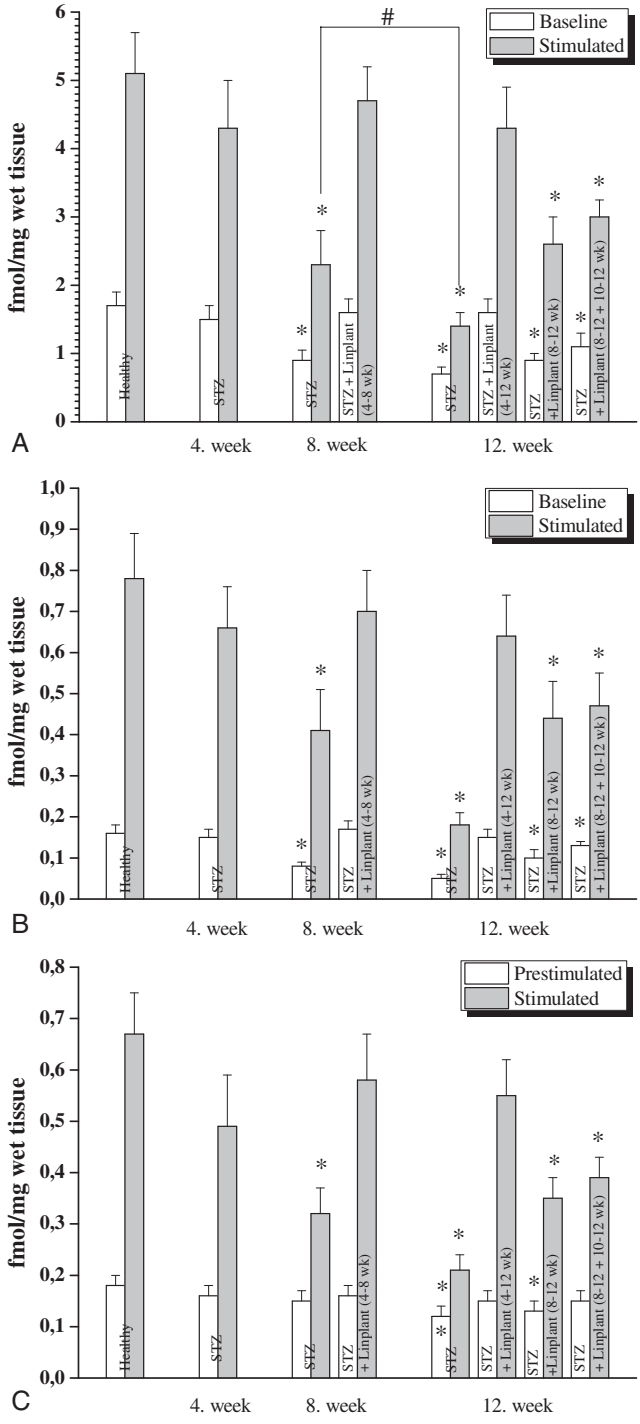


Fig. 3 – A, This figure shows the change in NVC of the myelinated A fibers in response to STZ treatment as well as to insulin supplementation by insulin implant(s). *Statistically significant difference ($P < .05$) from the healthy group. #Statistically significant difference ($P < .05$) from the 12th-week STZ diabetic group. B, This figure shows the change in NVC of the small, unmyelinated C fibers in response to STZ treatment as well as to insulin supplementation by insulin implant(s). *Statistically significant difference ($P < .05$) from the healthy group.

Fig. 4 – A, The change in Substance P release from the rat trachea before and after field stimulation in healthy and STZ diabetic rats supplemented with or without subcutaneous insulin. *Statistically significant difference ($P < .05$) from the corresponding healthy group. #Statistically significant difference ($P < .05$) between the interconnected groups. B, The change in CGRP release from the rat trachea before and after FS in healthy and STZ diabetic rats supplemented with or without subcutaneous insulin. *Statistically significant difference ($P < .05$) from the corresponding healthy group. C, The change in somatostatin release from the rat trachea before and after FS in healthy and STZ diabetic rats supplemented with or without subcutaneous insulin. *Statistically significant difference ($P < .05$) from the corresponding healthy group.

glucose production was decreased to 78% ± 11% and 53% ± 9% in rats with STZ diabetes at 8 and 12 weeks, respectively. Application of the slow-release Linplant applied 4 weeks after STZ treatment restored the endogenous (hepatic) glucose production inhibitory effect of the insulin + glucose infusion determined at either the 8th or 12th post-STZ week. When the Linplant was used only after the eighth week, the inhibitory effect of insulin infusion was significantly decreased irrespective of whether 1 or 2 implants were applied later.



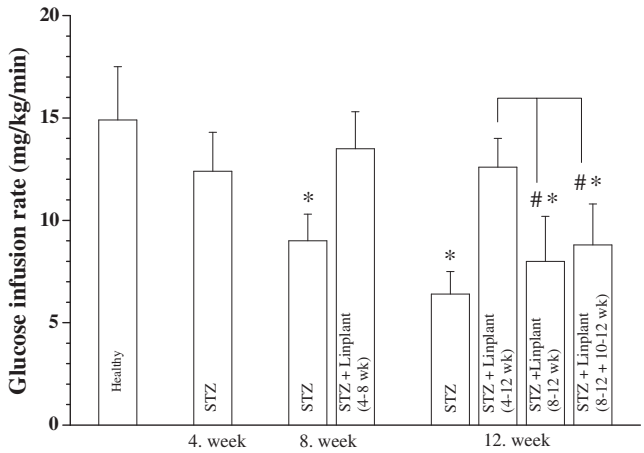


Fig. 5 – The time-dependent deterioration in insulin sensitivity in response to STZ treatment as well as the beneficial effect of insulin supplementation on the STZ diabetes-induced insulin resistance in rats. *Statistically significant difference ($P < .05$) from the healthy group. #Statistically significant difference ($P < .05$) between the interconnected groups.

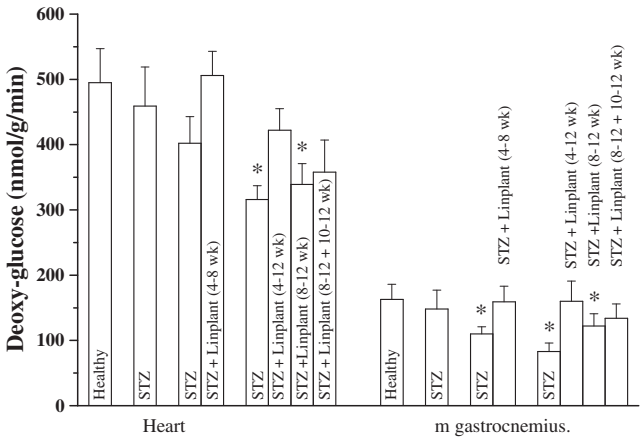


Fig. 7 – How the STZ treatment with or without insulin supplementation influenced the peripheral glucose uptake in cardiac muscle and in striped muscle (m. gastrocnemius). *Statistically significant difference ($P < .05$) from the corresponding healthy group.

3.7. *Effect of STZ diabetes and insulin supplementation on cardiac and skeletal muscle 2-deoxy-D [L-^{14}C] glucose uptake*

Cardiac and gastrocnemius muscle 2-deoxy-D [L-^{14}C] glucose uptake was significantly decreased in animals 8 or 12 weeks after treatment with STZ. Insulin supplementation was able to reverse the decreased uptake of the glucose analogue when it was commenced on the fourth week after STZ injection. Later insulin supplementation was without effect (Fig. 7).

3.8. *The possible role of hypersomatostatinemia on WBIS in STZ-induced diabetes*

Plasma somatostatin values as measured by means of radioimmunoassay in healthy animals and in 4-, 8-, and 12-week STZ-diabetic animals were 5.5 ± 0.9, 19.7 ± 2.8, 22.1 ± 3.0, and 18.7 ± 2.6 pmol/L ($P < .05$ for each), respectively. In insulin-supplemented diabetic rats, when the application of the slow-release implant had taken place on the fourth week, the plasma somatostatin level measured at 8 and 12 weeks after STZ administration returned to 7.1 ± 1.6 and 7.4 ± 2.0 pmol/L, respectively. When the implants were used from the eighth week, an increase in plasma somatostatin level was observed at the 12th post-STZ week with 16 ± 2.4 and 11 ± 1.5 pmol/L ($P < .05$ from corresponding normal values for each) at single and double implant application, respectively (Table 1).

Subcutaneous injection of 200 mg/kg cysteamine, a somatostatin-depleting agent, made plasma somatostatin level undetectable in either healthy or diabetic animals (data not shown), with a slight but significant decrease in GIR (from 9.0 ± 1.3 to 8.2 ± 0.9 mg/[kg min] and from 6.4 ± 1.1 to 5.8 ± 0.9 mg/[kg min], respectively) during the steady state of the HEGC in 8- and 12-week STZ-diabetic rats.

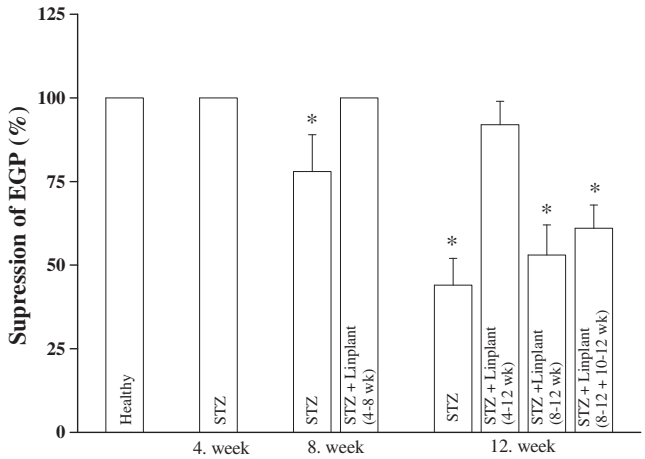


Fig. 6 – The effect of STZ-induced diabetes as well as insulin supplementation on endogenous glucose production in rats. *Statistically significant difference ($P < .05$) from the healthy group.

4. Discussion

These results show that STZ diabetes of 4 to 12 weeks' duration results in an insulin-resistant state, the severity of which increases with the duration of the disease and exhibits strong parallelism with the development of sensory neuropathy. The results also show that early and adequate restoration of euglycemia by means of insulin supplementation prevents the development of both neuropathy and insulin resistance in STZ diabetes, whereas late insulin supplementation can neither control hyperglycemia nor successfully treat neuropathy. The previous observation that STZ diabetes yields hypersomatostatinemia [13,16] is also confirmed by the results presented; however, here we first demonstrate that somatostatin depletion and the concomitant sensory neuropathy aggravate the insulin-resistant state in a model of insulin-deficient diabetes.

To the best of our knowledge, this report is the first to describe an accurate follow-up of changes in WBIS during the

time course of STZ diabetes with and without insulin supplementation of various strategies. Alternatively, no studies have been performed to investigate the effect of various insulin supplementation schedules on diabetic neuropathy characterized by NVC and the release of 3 SNPs such as somatostatin, substance P, and CGRP over such a long period of STZ diabetes in rats. Moreover, no results were found in the literature as to whether the progression of diabetic neuropathy parallels a progression of insulin resistance.

The STZ-treated rats exhibited characteristics of type 1 diabetes mellitus in that they failed to gain weight, they suffered from hyperglycemia, and direct determination of fasting plasma insulin levels showed a substantial insulin deficiency [25]. Moreover, the 8- and the 12-week diabetic states were associated with substantial sensory neuropathy, which was reversible by early insulin supplementation. By definition, diabetic neuropathy is a demonstrable disorder, either clinically evident or subclinical, that occurs in the setting of diabetes mellitus without other cause of peripheral neuropathy [26]. As an experimental approach, STZ-induced diabetes has been extensively used to study the pathogenesis and consequences of diabetic neuropathy (for review, see Brain [27]). The STZ-induced diabetic rat is an easily attained and reliable experimental model animal to study diabetic neuropathy, similar to that seen in patient with type 1 diabetes mellitus, which is characterized by detrimental changes in sensory, autonomic, and motor nerve functions [28,29]. As far as the pathogenic mechanism of sensory neuropathy associated with type 1 diabetes mellitus is concerned, a defective axonal transport including that of SNPs is believed to be a major critical initiating factor in degenerative distal neuropathies leading to severe microcirculatory changes in both diabetic patients and experimental animals [30] (for review, see Fedele and Giugliano [20] and Bennett et al [31]). As the local effector function of peripheral sensory nerves is known to be underlain by the ability of these nerves to release neuropeptides in response to various stimuli (for review, see Brain [27] and Szolcsanyi [2]), it is not surprising that STZ diabetes, a state characterized by a deficient SNP content and release, has a profound influence on neuropeptide release-dependent phenomena such as neurogenic inflammation [12] or bronchial asthma [13]. Depletion of CGRP and substance P content has been shown to occur from sensory nerves of STZ-diabetic rats [32]. Direct measurement of CGRP, substance P, and somatostatin released in response to a highly standardized challenge evidenced a parallel attenuation of the release of these SNPs from isolated trachea of the rat with a preexisting duration of the insulin-deficient state over the 12-week experimental period as seen in the present work. However, the reason for recruitment of these neuropeptide measurements into methods used in this study (besides NVC studies) was to assess the degree of sensory neuropathy with special regard to sensory effector function during the time course of STZ diabetes. Probably one exception, at least in part, is the mystery of the role of somatostatin in diabetes. The release of somatostatin of neural origin decreased in parallel with that of CGRP and substance P from tracheal preparations in response to FS with the progression of diabetic neuropathy. However, the plasma somatostatin level was moderately elevated, similar to that previously observed in experiments with STZ diabetic animals [12,16]. Moreover, neural somatostatin was found to be one of the mediators of postprandial insulin sensitization [4]. Accordingly, somatostatin depletion by cysteamine was found to worsen insulin sensitivity in the present work. Therefore, it is likely that hypersomatostatinemia associated with STZ diabetes serves as a compensatory insulin-sensitizing mechanism.

Regarding neural regulation of insulin sensitivity, a crucial point in diabetic state, our previous results verified the importance of sensory fibers [4,33], especially the effector function of sensory nerves [34]. Subsequently, it has been shown that when either sensory nerves were specifically defunctionalized by capsaicin desensitization [4] or a deficiency in sensory effector function was observed as a complication of treatment with cisplatin [7], profound changes in insulin sensitivity occurred [6,7]. Moreover, selective functional deterioration of sensory fibers in the anterior hepatic plexus per se can induce diabetes preceded by insulin resistance in rabbits [35], resembling the correlation between neuropathy and type 2 diabetes mellitus. As shown by the results presented, as a more profound diabetic neuropathy occurred in STZ-treated rats characterized by results from both NVC and SNP release studies, a more pronounced insulin-resistant state was seen reflected in both GIR data and tissue responses to insulin irrespective of whether hepatic glucose production or muscle glucose uptake was investigated. Of course, parallelism cannot be presented as causality; therefore, based on results of the current work it cannot be estimated whether sensory neuropathy is a major determinant of insulin resistance in STZ-treated rats. If this were true, in advanced diabetes, treatment of diabetic neuropathy would be successful for the treatment of insulin sensitivity as well.

The overall prevalence of neuropathy is 66% for type 1 diabetes mellitus and 59% for type 2 diabetes mellitus. By far, the most common form of neuropathy is a length-dependent diabetic sensorimotor neuropathy with a lifetime prevalence of approximately 55% for type 1 diabetes mellitus and 45% for type 2 diabetes mellitus (for review, see Said [36]). Thus, there is no doubt that metabolically uncontrolled diabetes, nearly irrespective of the type of diabetes, leads to neuropathy, which speculatively but not exclusively in turn may aggravate the diabetic state by reducing tissue insulin sensitivity. The pathogenesis of diabetic neuropathy remains unclear, although insulin resistance, oxidative stress, mitochondrial dysfunction, abnormal glucose metabolism, advanced glycation end products, neurotrophic factors, and protein kinase C activation all may play a role. However, strict glycemic control remains the only available treatment option, although other treatments are in development [37,38]. This widespread clinical observation is strongly supported by our experimental data because the adequate glucose control by early insulin supplementation was effective in preventing both insulin resistance and sensory neuropathy. We conclude/hypothesize that the developing neuropathy has a role in the insulin resistance observed, but the role of other factors in the development of insulin resistance associated with STZ-induced diabetes could not be ruled out. Such a mechanism could be the leptin deficiency, which usually occurs in STZ-

induced type 1 diabetes mellitus [39]; but the detailed investigation of this complex glucoregulatory mechanism is far beyond the primary scope of the present study. On the other hand, our results also show that an inadequate glucose control with late insulin supplementation was not effective against either insulin resistance or neuropathy, despite using much higher insulin doses achieving higher plasma insulin immunoreactivity in case of early supplementation. One possible explanation for the failure of late insulin supplementation to reverse either neuropathy or insulin resistance is that the applied additional insulin implants were unable to counteract detrimental effects evoked by the long-lasting inadequate glycemic control and the concomitant gluco- and lipotoxicity. The clinical relevance of these observations is that early and strict glycemic control is a prerequisite for successful diabetes management, and long-lasting uncontrolled diabetes could lead to irreversible molecular and/or cellular damage that makes antidiabetic therapy difficult.

4.1. *Strengths and weaknesses*

We suggest that sensory neuropathy is of crucial importance in the development of insulin resistance in our experimental model of insulin-deficient diabetes. Here we demonstrated the existence of sensory neuropathy by means of 2 different methods, that is, the NVC as well as neuropeptide release studies. Moreover, WBIS was determined by HEGC method, an approach considered to be the "criterion standard" method for determination of WBIS as proposed by DeFronzo et al [40]. This method is proposed instead of calculation of the insulin sensitivity index (eg, homeostasis model assessment index) based on the fasting plasma insulin and blood glucose level because the insulin insufficiency in STZ diabetes makes any conclusions being drawn very limited. This, together with data obtained by the endogenous glucose production and peripheral glucose uptake studies, provides a detailed insight into the composition of the state of insulin resistance (ie hepatic, peripheral, or both).

On the other hand, the data obtained from HEGC investigations and endogenous glucose production studies fairly correlate with WBIS when insulin resistance either peripheral or hepatic does not exist. If so, determination of whole-body glucose uptake (instead of GIR) and data regarding the net effect of hepatic glucoregulation (glycogen synthesis, glycogenolysis) could disclose more information on the nature of insulin resistance. In our experiments, the results of glucose suppression and 2-deoxy-D [L-^{14}C] glucose uptake studies indicate that the hepatic as well as peripheral insulin action is impaired; nevertheless, whole-body glucose uptake along with data on glycogen synthesis and glycolysis in addition to the present results would have provided a more precise information on metabolic changes in our experimental animals. Another possible weakness of this study is the lack of mechanistic data on some other important parameters of STZ diabetes known to play a role in the disease process in this model. Among these, possibly one of the most important is the involvement of leptin in metabolic alterations accompanying insulin-deficient diabetes. Severe leptin deficiency is a well-documented consequence of insulin-deficient diabetes that occurs after destruction of insulin-secreting Langerhans β-cells, the key event in STZ diabetes [41,42]. In human type 1 diabetes mellitus, the development of insulin resistance [43] is quite similar to that described in STZ diabetes in rats [44]. As plasma levels of leptins have been shown to normalize when insulin is adequately supplemented in STZ diabetes, some of the beneficial effects of insulin at least in part have been suggested to be attained by leptin [45]. This is strongly supported by the fact that an increase in leptin levels either by exogenous leptin [46] or by indirect maneuvers such as adenoviral gene therapy [47] decreases hypoglycemia despite persistent hypoinsulinemia in STZ diabetes. Nevertheless, in the framework of the current study, a detailed analysis of the role of leptins in the context of neuropathy associated with STZ diabetes seemed to exceed the possibilities in the present work.

5. Summary

Taken together, the results present for the first time that insulin resistance develops in insulin-deficient state in parallel with the progression of diabetic sensory neuropathy. The latter involves a decline in sensory effector function that has been shown to regulate several pathways determining endogenous insulin-sensitizing mechanisms [4,6]. Therefore, any therapeutic interventions that ameliorate diabetic neuropathy may, in theory, improve insulin sensitivity that underlies other complications of diabetes such as cardiomyopathy and peripheral vascular resistance. We think that the results presented can contribute to novel therapeutic approaches for the treatment of diabetes and its complications.

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Conflict of Interest

None.

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