Histopathological nerve and skeletal muscle changes in rats subjected to persistent insulin-induced hypoglycemia

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Abstract: New insulin analogues with a longer duration of action and a flatter pharmacodynamic profile are developed to improve convenience and safety for diabetic patients. During the nonclinical development of such analogues, safety studies must be conducted in nondiabetic rats, which consequently are rendered chronically hypoglycemic. A rat comparator model using human insulin would be valuable, as it would enable differentiation between effects related to either persistent insulin-induced hypoglycemia (IIH) or a new analogue per se. Such a model could alleviate the need for an in-study-comparator and thereby reduce the number of animals used during development. Thus, the aims of the present study were i) to develop a preclinical animal model of persistent hypoglycemia in rats using human insulin infusion for four weeks and ii) to investigate histopathological changes in sciatic nerves and quadriceps femoris muscle tissue, as little is known about the response to persistent hypoglycemia in these tissues. Histopathologic changes in insulin-infused animals included axonal degeneration and myofibre degeneration. To our knowledge, this is the first study to show that persistent IIH provokes peripheral nerve and skeletal myofiber degeneration within the same animals. This suggests that the model can serve as a nonclinical comparator model during development of long-acting insulin analogues. (DOI: 10.1293/tox.2015-0041; J Toxicol Pathol 2016; 29: 17–30)

Key words: human insulin, hypoglycemia, animal models, peripheral nerve, skeletal muscle, rat

Introduction

The number of people affected by diabetes is estimated to rise to approximately 366 million globally by 2030, and insulin therapy represents a cornerstone in the treatment of type 1 and advanced type 2 diabetes. In practice, the high dosing frequency needed with conventional basal insulins to achieve target blood glucose levels renders compliance to an adequate treatment regimen challenging for many patients. Thus, development of new basal insulin analogues with a longer duration of action as well as flatter time versus action profiles would allow reduction of the dosing frequency and diminish the risk of hypoglycemia, increasing convenience and safety and thus, quality of life for many patients.

According to regulatory guidelines, toxicological evaluations of new insulin analogues during nonclinical drug development should be conducted in healthy nondiabetic animals. The rat is often chosen as the most appropriate rodent species for toxicological evaluation of insulin analogues owing to its pharmacological responsiveness to insulin and history as a preferred rodent species in insulin safety studies. In repeated dose toxicity studies, rats will usually be administrated doses approaching the maximal tolerable dose in order to achieve the highest exposure possible. Consequently, nondiabetic rats are rendered chronically hypoglycemic in studies with long-acting insulin analogues. Surprisingly little is known about the adaptive effect of the endocrine system as well as relevant target organs to the resulting persistent hypoglycemia. This means that differentiation between adaptive effects to hypoglycemia and toxicological effects of a new insulin analogue per se may be challenging in these studies. Therefore, guidelines state that the use of human insulin as a reference compound should always be considered with toxicological studies of new insulin analogues. This is usually accomplished by including an in-study-comparator group receiving human insulin once or twice daily. However, for long-acting insulin analogues this is not an adequate comparator as due to the short half-life of human insulin the blood glucose...
lowering effect will persist for less than 7 h and not during the entire dosing interval as in the case of long-acting analogues. Therefore, development of a general comparator model demonstrating effects of persistent insulin-induced hypoglycemia (IIH) for use as a reference would be of great value in the safety assessment of new long-acting insulin analogues. Furthermore, such a model may reduce the need for an in-study-comparator group and may thereby reduce the number of animals included in toxicity studies. Peripheral nerve and skeletal muscle are one of the tissues to be examined histopathologically in repeated dose toxicity studies. While the effect of IIH on the central nervous system (CNS) has been studied rather extensively, little is known about the response of the peripheral nervous system (PNS) to IIH. The PNS seems to be vulnerable to low blood glucose levels, with hypoglycemia causing a distal degenerative axonopathy in peripheral nerves. The PNS relies on glucose as its primary source of energy, a dependency which probably contributes to its susceptibility towards IIH-induced injury. Changes caused by acute IIH (<1 week of dosing) and chronic IIH (≥2 weeks of dosing) using, for example, porcine or bovine insulin or induction of insulinomas in mice and rats, causing persistent hypoglycemia for 12 h to several months included axonal degeneration in peripheral nerves. Furthermore, based on a few insulinoma cases in the rat and dog, it appears that changes in skeletal muscle are also associated with hypoglycemia; these are primarily located in hind-limb muscles, and include atrophy of both type 1 and type 2 fibers. The pathogenesis of this PNS injury as well as a potential association between skeletal muscle and nerve changes has so far not been shown. In addition, there is a lack of chronic studies using human insulin and/or inducing persistent hypoglycemia in nondiabetic rats to investigate histopathologic changes in peripheral nerves as well as skeletal muscle.

Consequently, the first objective of the present study was to develop a nonclinical animal model for induction of persistent hypoglycemia for four weeks in healthy Sprague-Dawley rats using human insulin. Due to the short half-life of human insulin in the rat (14 and 23 min for intravenous [i.v.] and subcutaneous [s.c.] administration, respectively), continuous infusion was used in order to mimic the drug exposure of a long-acting insulin analogue. The second objective was to investigate the histopathological effects of persistent IIH on peripheral nerves and skeletal muscle tissue. Based on the effects seen by others using bovine or porcine insulin in mice and rats or insulinoma-bearing rats, we hypothesized that four weeks of persistent hypoglycemia instigated by human insulin would cause axonal degeneration in peripheral nerves and skeletal muscle changes, such as atrophy and/or degeneration of myofibers, in hind-limb muscles.

Materials and Methods

Animals
Male and female Sprague-Dawley (Crl:CD (SD)) rats (n=18/sex) approximately 6 to 7 weeks of age were supplied by Charles River (UK) Limited. Upon arrival, animals were randomly allocated to cages (up to four animals/cage), each sex kept separately, and housed in solid bottom polycarbonate cages with a stainless steel mesh lid and sawdust litter. The animals were acclimatized to local environmental conditions (19–23°C, 40–70% humidity, 12 h light-dark cycle) for 7 days before surgery (catheter placement) was performed. They were offered a commercial diet (Rat and Mouse No. 1 Maintenance Diet, Special Diet Services, Essex, UK) and water ad libitum. Body weight was monitored daily for at least three days post surgery or until the animals regained their presurgery body weights; thereafter body weight and food consumption were monitored weekly. Post surgery animals were housed individually. All procedures involving live animals were performed under the Project Licence authorized by the United Kingdom Secretary of State and according to EC Commission Directive 2004/10, OECD Principles and Good Laboratory Practice, and SI 1999/3106 as amended by SI 2004/94/2004 and Huntingdon Life Sciences and Novo Nordisk A/S company policy on the care and use of laboratory animals.

Surgical procedure and study design

Prophylactic antibiotic cover (enrofloxacin, 5 mg/kg s.c.) and analgesics (meloxicam, 1 mg/kg s.c.) were administered prior to induction of general anaesthesia (isoflurane). Using aseptic techniques, a vascular catheter was inserted into the caudal vena cava through the right femoral vein and tunneled s.c. from the site of venous access to the nape of the neck, where it was exteriorized, connected to a vascular access port, and protected by a harness. The catheter was filled with heparinized saline and kept clean using a septum cover. Appropriate postoperative analgesia and antibiotic treatment (as above) were provided for three days postoperatively. All animals had at least seven days of post-surgery recovery prior to commencement of infusion. During the recovery period, animals were randomized into three different groups (CTRL, HI-Low, and HI-High groups, n = 6/sex per group, Table 1) stratified for body weight and sex. Delivery of compound and vehicle was achieved by external infusion pumps. Approximately 24 h before the start of infusion, saline was infused (0.5 ml/h) to ensure catheter patency. At the start of infusion (Day 1) animals were approximately 8–9 weeks old and weighed 310–364 g (males) and 194–242 g (females). Animals received 29 full days of infusion with either buffered human insulin stock solution or buffered vehicle, respectively, diluted in dilution medium (see Supplemental material). The aim was to approach the maximum tolerable dose and to achieve similar blood glucose levels in males and females; thus males received higher insulin doses than females, as they have lower insulin sensitivity compared with females. A group receiving a lower dose was included to investigate the dose-response relationship of any potential histopathologic changes. Doses were chosen based on results from previous studies in rats with insulin aspart (unpublished data), which has similar pharmacokinetic parameters to human insulin in the rat. Flow rates were adjusted weekly based on individual body...
weights to keep doses constant in nmol/kg/day over the infusion period. Syringes containing infusion formulations were changed daily. One sample of each final formulation prepared for administration in Weeks 1 and 3 of infusion for all groups was analyzed to confirm correct preparation of the test substance.

Blood samples for plasma glucose and plasma human insulin quantification were obtained in isofurane anaesthesia from the sublingual vein of 2 animals/sex/group at each of the following time points: predose (time point zero), 0.25, 0.5, 6, and 24 h after commencement of infusion on Day 1. Blood samples were collected again on Days 15 (plasma glucose only) and 29 at each of the following time points: 0.25, 0.5, 6, and 24 h. Furthermore, additional blood samples for plasma glucose quantification only were obtained at 0.5 h on Day 8. Each animal was sampled a maximum of twice within a 24 hour time period. Additionally, whole blood glucose spot sampling from the tail vein was carried out every other day except Day 15.

After completion of the last scheduled blood sampling, animals were euthanized by carbon dioxide asphyxiation on Day 30 (i.e. after 29 full days of infusion), with subsequent exsanguination. Infusion was ongoing until euthanasia. As histopathologic examination in nonclinical toxicity studies is usually performed on the sciatic nerve and skeletal muscle from the proximal hind limb (quadriceps femoris muscle). Furthermore, the quadriceps femoris muscle contains a mixture of both Type 1 and Type 2 myofibers (in contrast, biceps femoris muscle consists of 99–100% Type 2 fibers). The two fiber types differ in susceptibility to specific pathophysiologic signals; thus, choosing a muscle containing both types should increase the chance of finding any histopathologic changes in skeletal muscle. Additionally, tissue samples from the catheter exteriorization site (interscapulary region), the femoral vein access site, and the vena cava, were obtained. Samples were fixed in 10% neutral buffered formalin and processed, and transverse and longitudinal sections of a nominal thickness of 4–5 μm were stained with H&E and evaluated microscopically. Lesions were graded on a five-step grading scale (minimal, slight, moderate, marked, severe). Sciatic nerve changes were graded on longitudinal sections according to following criteria: minimal (grade 1), 2–7 axons in section affected; slight (grade 2), >7 axons to ≤25% of axons in section affected; moderate (grade 3), >25–50% axons in section affected; marked (grade 4), >50–80% of axons in section affected; and severe (grade 5), >80–100% of axons in section affected. Quadriceps femoris muscle degenerative changes in myofibers were graded on transverse sections according to following criteria: minimal (grade 1), 2–10 single myofibers in section affected; slight (grade 2), >10 single myofibers to ≤25% of tissue in section affected; moderate (grade 3), 25–50% of myofibers in section affected; marked (grade 4), >50–80% of myofibers in section affected; and severe (grade 5), >80–100% of myofibres in section affected. Infiltration of inflammatory cells (leukocytes) interstitially in quadriceps femoris muscle tissue was graded on transverse sections according to the following criteria: minimal (grade 1), 2–4 foci in section; slight (grade 2), >4 foci to ≤25% of tissue in section affected; moderate (grade 3), 25–50% of the tissue in section affected; marked (grade 4), >50–80% of the tissue in section affected; and severe (grade 5), >80–100% of the tissue in section affected.

### Insulin formulations and infusion system

The test substance used was recombinant human insulin formulated in a phosphate buffered vehicle (HI-Low and HI-High groups) and buffered vehicle (CTRL groups). Recombinant human insulin stock solutions in phosphate buffered vehicle (600 nmol/ml) as well as buffered vehicle were supplied by Novo Nordisk A/S (Maaloev, Denmark). Recombinant human insulin stock solution and buffered vehicle were diluted with dilution medium to obtain the formulations for infusion. The compositions of buffered human insulin stock formulation, buffered vehicle, and dilution medium are listed in Supplemental material. Furthermore, stability of human insulin formulations in the infusion system was tested in vitro prior to in vivo use (see Supplemental material).

For insulin infusion, external infusion pumps (Harvard Apparatus Pump 11 Plus Syringe Pump, Harvard Apparatus, Holliston, MA, USA) with Plastipak syringes (no. 300613 and 301231, BD, Oxford, UK), and a vascular access harness (cat. no. VAH95AB, Intech Laboratories Inc., Plymouth Meeting, PA, USA) connected to a custom VAH tether kit (KHL 02, Intech Laboratories Inc., Plymouth Meeting, PA, USA) were used.

### Quantification of glucose in plasma and whole blood

For plasma glucose evaluation, samples were centrifuged (3000 RPM, 1,400–1,942 g for 15 minutes at 2–8°C) and plasma collected and stored at 2–8°C prior to analysis. The plasma glucose concentration was determined by the hexokinase method (Roche Modular P system, Roche Diagnostics, Indianapolis, IN, USA). Spot glucose measurements were carried out by a snap blood glucose monitoring device (Accu-Chek Aviva, Cat/Typ 05911974002, Roche).

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**Table 1. Study Design of in vivo Study (weeks 1–4)**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Flow rate (ml/kg/hour)</th>
<th>Concentration of HI (nmol/ml)</th>
<th>Dose (nmol/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL</td>
<td>6</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HI-Low</td>
<td>6</td>
<td>1.4</td>
<td>2.4</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>HI-High</td>
<td>6</td>
<td>1.4</td>
<td>3.0/2.7</td>
<td>100/90 ± 2</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL</td>
<td>6</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HI-Low</td>
<td>6</td>
<td>1.4</td>
<td>1.8</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>HI-High</td>
<td>6</td>
<td>1.4</td>
<td>2.4</td>
<td>80 ± 2</td>
</tr>
</tbody>
</table>

*From Day 10, the dose for the HI-High group males was lowered from 100 to 90 nmol/kg/day.*
Quantification of human insulin in plasma

Human insulin was quantified in plasma using a luminescent oxygen channelling immunoassay (LOCI) as described by others. The LOCI was optimized with regard to sample material and assay volume and validated according to European Medicines Agency (EMA) and Food and Drug Administration (FDA) guidelines prior to use. Europium-coated acceptor beads (cat. no. 1624891) and streptavidin-coated donor beads (cat. no. 676002L) were supplied by Perkin Elmer, California, USA. A monoclonal antibody specific for human insulin (HUI-018, Novo Nordisk A/S, Bagsværd, Denmark) was conjugated to the acceptor beads and biotin-labelled monoclonal antibody specific for human insulin (OXI-005, Novo Nordisk A/S, Bagsværd, Denmark) was added to make up the immuno-sandwich in the presence of human insulin. The properties of the antibodies have been described elsewhere. The assay response was assumed to represent the steady state concentration (C_{ss}) due to constant infusion. In accordance with the sparse sampling design of the study, composite mean profiles of plasma human insulin concentration versus time data (n = 2/time-point/sex) were generated in the various dose groups. On Day 1, the plasma human insulin concentration at 6 hours represented the steady state is theoretically obtained after 5-7 half-lives; the half-life of human insulin in rats has been shown to be approximately 14 min after i.v. administration. On Day 29, all measurements were assumed to represent the C_{ss} due to constant infusion. In addition, individual AUC_{0-24h} values were calculated from the individual mean C_{ss} values on Day 29 as AUC_{0-24h} = C_{ss} × 24 h. Mean plasma concentrations for each animal and day, as well as for each dose group, were calculated to yield individual and group mean C_{ss} values. The calculations were performed by non-compartmental analysis in Phoenix™ WinNonlin® version 6.2, build 62.0.495 (Pharsight®, St. Louis, MO, USA).

Formulation analysis

Determination of total peptide content in insulin formulations was carried out by reversed-phase high-performance liquid chromatography (RP-HPLC) using a short C8 column, TFA/acetonitrile eluents, and detection at 280 nm. Samples were analyzed as single determinations. Results are given as percent recovery of total peptide content in insulin formulations compared with nominal concentrations.

Toxicokinetic analysis

In accordance with the sparse sampling design of the study, composite mean profiles of plasma human insulin concentration versus time data (n = 2/time-point/sex) were generated in the various dose groups. On Day 1, the plasma human insulin concentration at 6 hours represented the steady state is theoretically obtained after 5-7 half-lives; the half-life of human insulin in rats has been shown to be approximately 14 min after i.v. administration. On Day 29, all measurements were assumed to represent the C_{ss} due to constant infusion. In addition, individual AUC_{0-24h} values were calculated from the individual mean C_{ss} values on Day 29 as AUC_{0-24h} = C_{ss} × 24 h. Mean plasma concentrations for each animal and day, as well as for each dose group, were calculated to yield individual and group mean C_{ss} values. The calculations were performed by non-compartmental analysis in Phoenix™ WinNonlin® version 6.2, build 62.0.495 (Pharsight®, St. Louis, MO, USA).

Statistical analysis

Data were analyzed applying a two-way analysis of variance (ANOVA) with group and sex as fixed variables followed by a one-way ANOVA with a Tukey multiple comparisons post hoc test for each sex separately in case of in-

Results

Clinical signs and mortality

Animals generally behaved normally without any signs of discomfort or stress resulting from the catheter and/or harness and without any clinical signs of locomotor system impairment. We attempted to approach maximum tolerable dose. Clinical signs of hypoglycemia (such as underactivity, piloerection, reduced body temperature, or convulsions) were observed in six animals from insulin-infused groups: three from the HI-Low group and three from the HI-High group. There were a total of 11 premature deaths (Table 2). Of the animals with clinical signs of hypoglycemia, four were sacrificed prematurely due to severe hypoglycemia, and one animal was later found dead. In HI-High group males, one animal was found dead on Day 6 and another was sacrificed due to severe hypoglycemic symptoms on Day 8. Thus the dose of 100 nmol/kg/day seemed to exceed the maximum tolerable dose. Therefore, the dose was reduced in this group to 90 nmol/kg/day from Day 10. Four animals in total were euthanized prematurely due to issues related to the infusion harness or catheter.

Table 2. Unscheduled Deaths: Numbers of Animals (Day of Mortality). Unless Otherwise Noted, Animals were Euthanized for Welfare Reasons

<table>
<thead>
<tr>
<th>Group</th>
<th>Related to hypoglycemia</th>
<th>Related to infusion harness/catheter</th>
<th>Total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL</td>
<td>0</td>
<td>3 (5, 28, 29)</td>
<td>3/6</td>
</tr>
<tr>
<td>HI-Low</td>
<td>1 (27)</td>
<td>0</td>
<td>1/6</td>
</tr>
<tr>
<td>HI-High</td>
<td>4 (6b, 8, 16b, 29b)</td>
<td>0</td>
<td>4/6</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL</td>
<td>0</td>
<td>0</td>
<td>0/6</td>
</tr>
<tr>
<td>HI-Low</td>
<td>1 (13)</td>
<td>1 (19)</td>
<td>2/6</td>
</tr>
<tr>
<td>HI-High</td>
<td>1 (29)</td>
<td>0</td>
<td>1/6</td>
</tr>
</tbody>
</table>

a For example, disconnection of the catheter causing irretrievable loss of catheter patency. b Found dead, presumably due to an unobserved severe hypoglycemic episode (based on the dose level, lack of pathologic findings at necropsy, and lowered in vivo whole blood glucose levels). c Found dead following clinical signs of hypoglycemia despite glucose treatment.

Formulation analysis

Recovery values of total peptide content in human insulin stock formulations from Week 1 and Week 3 and in the human insulin formulations used for infusion sampled at Week 1 and Week 3 were all between 96 and 108% (data not shown), confirming correct formulation. Values for samples
from CTRL group formulations were all below the LLOQ (i.e., 1 nmol/ml).

**Whole blood and plasma glucose concentrations**

In general, individual glucose values in males in insulin-infused groups were lower than those in the CTRL group throughout the four weeks, whereas values from females in insulin-infused groups showed the same tendency, but with sporadic hyperglycemic spikes (Fig. 2). Throughout the study, mean whole blood glucose concentrations were generally statistically significantly lower in insulin-infused groups compared with the CTRL group (data not shown). Mean plasma glucose concentrations decreased within the first 24 hours after the start of infusion in the insulin-infused groups and were lower than in CTRL groups as measured after 15 and 29 days of infusion, except for a few time points (Fig. 3). When measured at a single time point after 8 days of infusion, all insulin-infused groups showed similar plasma glucose levels, independent of dose and sex (Fig. 4).

**Plasma human insulin concentrations**

The human insulin plasma concentration was below the LLOQ (<25 pmol/L) in all Day 1 predose samples from insulin-infused groups and all samples from the CTRL group (Fig. 5). On Day 1, all samples from insulin-infused groups except three contained measurable human insulin concentrations after the start of infusion (concentrations <LLOQ in one HI-Low group male on Day 1 at 0.5 h and two HI-High group females on Day 1 at 0.25 and 0.5 h, respectively). This was most likely due to the infusion solution not reaching the blood stream yet at these early time points after the start of infusion due to the lag time between start of infusion and the infused solution reaching the tip of the vein catheter. On Day 29, all samples from insulin-infused groups contained measurable human insulin concentrations, except one sample (concentration <LLOQ in one HI-Low group male at 6 h). The reason for this was unknown.
Individual and group mean $C_{ss}$ values on Day 1 and Day 29 are depicted in Fig. 6. The group mean $AUC_{0-24\,h}$ Day 29 were 39,729 and 52,074 h*pmol/l for HI-Low and HI-High males, respectively, and 146,528 and 36,750 h*pmol/l for HI-Low and HI-High females, respectively. Due to the inter-individual variability for both $C_{ss}$ and $AUC_{0-24\,h}$, any evaluation of dose dependency was precluded. The reason for this variability was unknown. The mean versus dose lines for the $C_{ss}$ values were essentially horizontal, indicating approximately equivalent mean exposures across the dose groups (Fig. 6). With regard to time dependency, the $C_{ss}$ values on Day 1 (6 h time point only) and Day 29 were in a comparable range; however, the comparison should be viewed with caution, as the $C_{ss}$ on Day 1 represents fewer animals and data points than the $C_{ss}$ on Day 29.

Histopathology

Histopathological examination of sciatic nerves revealed minimal to moderate axonal degeneration in the majority of animals from insulin-infused groups, but in none of the animals from the CTRL group (Table 3A). Axonal degeneration was characterized by fragmentation of axons and by accumulation of axonal debris and/or phagocytic macro-
phages in dilated spaces of myelin sheaths ("digestive chambers") (Fig. 7). In terminally sacrificed animals, the incidence in both HI-Low and HI-High group males was 100%, while the incidences were 50% and 100% in the HI-Low and HI-High female groups, respectively. When looking at the combined data including the prematurely sacrificed animals, the incidence was similar to those in terminally sacrificed animals, although with a slightly lower incidence in group HI-High males (67%). No dose-response was evident in males. Females showed a higher incidence and severity of changes in the HI-High group versus the HI-Low group, suggesting that a dose-response might be present. Generally, insulin-infused males had a similar incidence and severity of axonal degeneration in sciatic nerves compared with insulin-infused females.

In the quadriceps femoris muscle, focal or multifocal myofiber degeneration confined to single myofibrils was observed in several animals from insulin-infused groups, but in none of the CTRL group animals (Table 3B, Fig. 8). The incidence was 50%, or less and the severity was minimal, regardless of the severity of axonal degeneration. When looking at the combined data, the incidence was similar in HI-Low and HI-High group males (40% versus 50%) and slightly higher in HI-High females versus HI-Low group females (33% versus 17%). Males had a slightly higher incidence compared with females. No clear dose-response relationship was evident in insulin-infused males or females. In terminally sacrificed animals, myofiber degeneration was only seen in animals with concurrent axonal degeneration in the sciatic nerves. Including prematurely sacrificed animals, axonal degeneration was present in sciatic nerves from all animals with myofiber degeneration except two.

Both of these animals were HI-High males, which had focal myofibre degeneration but no axonal degeneration. One was found dead on Day 6, and one was sacrificed on Day 8 due to hypoglycemic symptoms (Table 2). Minimal inflammatory cell infiltration was seen in skeletal muscle in all three groups in males but only in the HI-High group females (Table 3C).

The minimal inflammatory cell infiltration in skeletal muscle in all groups including the CTRL group (Table 3C) did not seem to correlate with muscle degeneration, as cell infiltration was seen in animals without myofiber degeneration and vice versa. However, in terminally sacrificed females, a higher incidence was seen in the HI-High group (80% versus 0% in the CTRL and HI-Low groups), and a tendency for a dose response was seen in terminally sacrificed males, but this was based on very few animals. For this reason, it cannot be completely ruled out that cell infiltration was related to infusion with human insulin.

At the catheter exteriorization site in the interscapular region, fibrogranulomatous inflammation in the cutis/subcutis and focal epidermal hyperplasia were present in animals from all groups. In the femoral vein access site and vena cava, changes included inflammatory cell infiltration in the vein wall, suture granulomas, proliferation of vein intimae, thrombus formation, necrosis, and hemorrhages. The incidence and severity of changes were similar between groups, including the CTRL group (data not shown).

Discussion

In the present study, i.v. infusion of human insulin for 4 weeks by an external infusion system was successfully employed in healthy growing SD rats. Thus, the basis of a general nonclinical comparator model of persistent IIH using human insulin mimicking the pharmacokinetic and pharmacodynamic response of a long-acting insulin analogue was established. The model was used to investigate histopathological effects on sciatic nerve and skeletal muscle tissue.

In nonclinical repeated dose studies, potential treatment-related effects on peripheral nerves and skeletal muscle are assessed by examination of formalin-fixed, paraffin-embedded H&E-stained longitudinal sections of nerve and on transverse and longitudinal sections of muscle. Therefore, this approach was also taken in this study. The axonal degeneration seen in the present study after infusion of human insulin was in agreement with previous reports of IIH for two weeks inducing axonal degeneration in the sciatic nerve in mice and rats infused with bovine insulin. Furthermore, the axonal degeneration seen here had the characteristics of the Wallerian-like axonal degeneration found by others in hypoglycemic diabetic rats. The nerve findings were present in the majority of animals from insulin-infused groups (83% of males and 73% females, total incidence for the HI-Low and HI-High groups combined). Blood glucose levels were 2–4 mM in insulin-infused groups in the present study, which is in line with the blood glucose levels seen in
other rodent studies with IIH for two weeks inducing peripheral nerve changes\textsuperscript{11, 13, 14}. Two HI-high dose males and one HI-low dose female did not display axonal degeneration in sciatic nerves; however, these animals died prematurely after approximately one-two weeks of insulin infusion (Table 2), and most likely, the duration of IIH was not sufficient to induce nerve changes in these animals. Furthermore, three terminally sacrificed females from the HI-Low group did not display any nerve or muscle changes (Table 3). Yasaki and Dyck observed axonal degeneration after only 12 hours of hypoglycemia in rats of similar age dosed with porcine insulin; however, this was after severe hypoglycemia of 1.4 mM\textsuperscript{17}. In addition, one to two episodes of hypoglycemia for approximately 2 days each with blood glucose levels <2.5 mM and 6 days of persistent hypoglycemia with blood glucose levels <3.0 mM have also been shown to cause axonal degeneration in rats\textsuperscript{11, 18, 19}. This suggests that the duration of hypoglycaemia leading to nerve damage is longer for less
Table 3. A) Summary of Histopathological Findings in A) the Peripheral Nerve (Sciatic Nerve) and B) Skeletal Muscle (Quadriceps Femoris). Myofiber Degeneration and Inflammatory Cell Infiltration in Skeletal Muscle were Focally Distributed Unless Otherwise Stated

<table>
<thead>
<tr>
<th>Sex Group</th>
<th>Terminally sacrificed</th>
<th>Prematurely sacrificed</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTRL Males</td>
<td>HI-L Males</td>
<td>HI-H Males</td>
</tr>
<tr>
<td>A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axonal degeneration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade (proportion in section affected)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal, grade 1 (2-7 axons affected)</td>
<td>0 2 1 0 2 2 0 0 1 0 0 1 0 2 2 0 2 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slight, grade 2 (&gt;7 axons to ≤25% of axons)</td>
<td>0 3 1 0 0 1 0 1 1 0 0 0 0 4 2 0 0 1</td>
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<td></td>
</tr>
<tr>
<td>Moderate, grade 3 (&gt;25-50% of axons)</td>
<td>0 0 0 0 0 2 0 0 0 0 0 0 0 0 0 0 0 2</td>
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<tr>
<td>Total/No. of animals examined</td>
<td>0/3 5/5 2/2 0/6 2/4 5/5 0/2</td>
<td>1/1 2/4 0/0 0/1</td>
<td>6/6 4/6 0/6 2/5</td>
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<tr>
<td>Incidence</td>
<td>0% 100% 100% 0% 50% 100% 0% 100% 50% 0% 0% 100% 0% 100% 67% 0% 40% 100%</td>
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<tr>
<td>Of these &gt; grade 1</td>
<td>0% 60% 50% 0% 0% 60% 0% 100% 50% 0% 0% 0% 0% 67% 50% 0% 0% 50%</td>
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<tr>
<td>B)</td>
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<tr>
<td>Myofiber degeneration</td>
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<tr>
<td>Grade (proportion in section affected)</td>
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<tr>
<td>Minimal, grade 1 (2-10 single myofibers)</td>
<td>0 1 1 0 1 1</td>
<td>0 1 2 0 0 1 0 2</td>
<td>3 0 1 2</td>
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<tr>
<td>Total/No. of animals examined</td>
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<td>1/1 2/4 0/0 0/2</td>
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<tr>
<td>Incidence</td>
<td>0% 25% 50% 0% 25% 20% 0% 100% 50% 0% 0% 100% 0% 40% 50% 0% 17% 33%</td>
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<td>C)</td>
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<tr>
<td>Inflammatory cell infiltration</td>
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<tr>
<td>Grade (proportion in section affected)</td>
<td></td>
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<td></td>
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<tr>
<td>Minimal, grade 1 (2-4 foci)</td>
<td>1 3 2 0 0 4 1 1 1 0 0 0 2 4</td>
<td>3 0 0 4</td>
<td></td>
</tr>
<tr>
<td>Total/No. of animals examined</td>
<td>1/3 3/4</td>
<td>2/2 0/6 0/4 4/5 1/3 1/1 1/4 0/0 0/2 0/1 2/6 4/5 3/6 0/6 0/6 4/6</td>
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<tr>
<td>Incidence</td>
<td>33% 75% 100% 0% 0% 80% 33% 100% 25% 0% 0% 0% 33% 80% 50% 0% 0% 67%</td>
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CTRL = control group, HI-L = low dose human insulin group, HI-H = high dose human insulin group. a) An error occurred in sampling sciatic tissue from one animal in this group. b) An error occurred in sampling skeletal muscle tissue from one animal in this group. c) Multifocally distributed in both animals. d) The distribution was multifocal in one of these animals.
Fig. 7. Histopathological lesions in the sciatic nerve. Bars indicate 100 μm. A and B) Sciatic nerve from a control animal. C and D) Sciatic nerve from a HI-High group animal. The insert illustrates an area with axonal degeneration.

Fig. 8. Histopathological lesions in skeletal muscle tissue. Bars indicate 100 μm. A and B) Quadriceps femoris muscle tissue from a control animal. C and D) Quadriceps femoris muscle tissue from a HI-High group animal. The insert illustrates a degenerating myofiber.
severe hypoglycemia, e.g., when blood glucose levels are around 2–4 mM, as in the present study. This is supported by findings in other studies, which suggested that the duration and severity of hypoglycemia may be related to the severity of the neuropathy. As animals found dead or sacrificed prematurely before Day 14 did not display any nerve changes and a HI-High male group sacrificed on Day 16 did display axonal degeneration, the results of the present study suggest that a duration of hypoglycemia with blood glucose levels of 2–4 mM exceeding two weeks will cause axonal damage (subject to individual variation). Additionally, it is not known if nerve changes seen following dosing with, for example, porcine or bovine insulin are comparable to those seen following dosing with human insulin. However, as the effects are most likely caused by the pharmacodynamic effects of the insulins rather than a direct effect of the insulin itself, it seems probable that the effects are comparable, which are the results of the present study support.

The dose-response relationship should also be considered when evaluating the incidence of nerve changes. In males, no dose-response was seen, which reflects the lack of dose dependency of the plasma human insulin concentration in a steady state (Fig. 5 and 6) or of the plasma glucose concentration on Day 8 (Fig. 4). In females, an apparent dose response was seen in the incidences of nerve changes, as the incidences were slightly higher and severity increased in the HI-high compared with the HI-low group (Table 3). However, this apparent difference cannot be explained by the plasma human insulin or glucose levels, as the levels were similar to those in males (Fig. 4 and 5) and the mean steady-state plasma insulin concentration on Day 29 was actually slightly lower in the HI-High group females than the HI-low group females (Fig. 6). Therefore, it might instead be coincidental and attributable to individual variation and the limited number of animals in the study.

Myofiber degeneration as part of IIH-induced peripheral nerve changes has not been previously described in rats. Therefore, the histopathological investigation also included skeletal muscle tissue. Examination of skeletal muscle tissue from insulinoma cases in the rat and dog has shown myofiber atrophy and possibly degeneration in hind-limb skeletal muscle tissue. Examination of skeletal muscle tissue from the quadriceps femoris muscle was expected, as insulin treatment in rats has been shown to cause hyperphagia and, consequently, secondary increased body weight, supporting the validity of the model. The increase in food consumption (and resulting increase in body weight) is presumably due to the pharmacodynamic effect of insulin, the hypoglycemia, rather than a direct effect of the insulin itself.

Development of clinical signs of hypoglycemia in some of the animals was anticipated, as the pharmacodynamic response to insulin dosing typically is subject to high interindividual variation in nondiabetic SD rats (unpublished data), which has also been noted by others in diabetic rats. The increase in food consumption (and resulting increase in body weight) is presumably due to the pharmacodynamic effect of insulin, the hypoglycemia, rather than a direct effect of the insulin itself.

The increased food consumption and body weights seen in insulin-infused groups were expected, as insulin treatment in rats has been shown to cause hyperphagia and, consequently, secondary increased body weight, supporting the validity of the model. The increase in food consumption (and resulting increase in body weight) is presumably due to the pharmacodynamic effect of insulin, the hypoglycemia, rather than a direct effect of the insulin itself.

Development of clinical signs of hypoglycemia in some of the animals was anticipated, as the pharmacodynamic response to insulin dosing typically is subject to high interindividual variation in nondiabetic SD rats (unpublished data), which has also been noted by others in diabetic rats. Six animals displayed clinical signs of hypoglycemia, supporting that the maximum tolerable dose was reached. The present study will further support dose setting in future larger-scale and longer-term studies with this model, minimizing the number of animals with development of severe hypoglycemic symptoms, thus improving animal welfare and reducing the number of animals needed in each group. Despite the apparent lack of a dose response of insulin exposure or blood glucose levels, four out of the five males that died prematurely due to hypoglycemic symptoms belonged to the HI-High group (Table 2). This might be incidental; however, another explanation could be that any dose response in insulin exposure is masked by the high interindividual variation.
whole blood spot glucose concentrations in insulin-infused females unexpectedly showed intermittent sporadic hyperglycemic spikes (Fig. 2). The cause of these hyperglycemic spikes is not known. A very simple explanation could be the transient disruption of infusion in these animals. The females received lower infusion rates, and female rats are known to have smaller vessel diameters than males due to their lower body weights, suggesting a higher risk of compromised infusion compared with males. It is well known that endogenous insulin production is usually suppressed in chronic insulin-treated rats; hence, sudden cessation of insulin infusion would render the animals hyperglycemic. This is in line with the plasma insulin and glucose levels in a HI-Low group male, which had a plasma human insulin concentration below the LLOQ at the 6 h time point on Day 29 and a plasma glucose concentration of 24.17 mM. The hyperglycemic spikes may alternatively represent hormone-induced insulin resistance in the female rats: thus, reproductive cycle-related changes in ovarian steroids levels may be a possible explanation, as progesterone and/or oestrogen have been shown to cause transient insulin resistance in female rats. Normally, animals would compensate for this insulin resistance by upregulating insulin production/release. However, as endogenous insulin production is suppressed, infusion of exogenous insulin may be insufficient to compensate for the insulin resistance, resulting in transient hyperglycemia. This would explain the transient nature of the spikes, as the reproductive cycle of the female rat is only 4–5 days. However, only one animal had recurrent spikes (Days 7 and 13), suggesting a more random occurrence unrelated to the reproductive cycle. Irrespective of the cause, a maximum of one hyperglycemic spike was seen per animal, with the exception of a single female with two spikes. No apparent correlation was seen between incidence or severity of axonal degeneration, and the spikes were deemed to have had little if any impact on the overall hypoglycemic profile and thus, the histopathologic changes seen in the present study.

Using an external infusion system for continuous intravenous dosing of human insulin to produce persistent hypoglycemia in rats has, to our knowledge, not been tested previously for a period of four weeks. A major advantage of this infusion system is that the dose of insulin can easily be adjusted on an individual basis. It allows relatively unrestrained movements of the animal, and none of the animals showed clinical evidence of limb weakness, suggesting that collateral circulation was adequate for leg perfusion. Histopathological changes related to the indwelling catheter in the vena cava caudalis, femoral vein access site, and the exteriorization site in the interscapular area were inconspicuous and comparable to findings by others using this infusion system in SD rats. A disadvantage of this infusion system might be the relatively large infusion volume potentially causing hemodilution and urine dilution as well as increased urine volumes. However, the infusion rate used in this study (1.4 ml/kg/h) was well below the possible maximal value considered “good practice” in the rat, which is 4 ml/kg/h. Increasing the infusion rate slightly in future studies to reduce the risk of clotting of the catheter (e.g., to 2.0 ml/kg/h) should be considered. Nevertheless, the present rat model has proven to be useful for investigations of the effects of chronic hypoglycemia induced by human insulin for up to four weeks and most likely also for longer durations.

In conclusion, a novel experimental model for inducing persistent hypoglycemia for four weeks by i.v. infusion of human insulin was presented in the healthy growing SD rat. The model provides a scientifically acceptable rodent model to be used as a general comparator in the nonclinical evaluation of possible toxicological effects of new basal long-acting insulin analogues. Importantly, this may reduce the need for an in-study comparator, thus reducing the number of animals needed for development of long-acting insulin analogues. The induced persistent hypoglycemia with blood glucose levels generally between 2–4 mM over 4 weeks in both male and female rats resulted in peripheral nerve and skeletal muscle changes including axonal degeneration in the majority of animals in the insulin-infused groups and myofiber degeneration in up to half of animals in the insulin-infused groups. Multifocal or focal myofiber degeneration was predominantly seen in animals with axonal degeneration, supporting the hypothesis that muscle changes might be secondary to nerve changes. As it has now been shown that the present animal model of IIH produces histopathological changes in sciatic nerve and skeletal muscle tissue, the results and model presented here form the basis of future larger-scale follow-up studies. These should include more animals as well as a more detailed histopathological examination of the myofiber changes and their distribution including a comparison to changes typically seen in denervated skeletal muscle tissue in order to verify this hypothesis. Such studies could further clarify the sequence of events in the development and distribution of these nerve and skeletal muscle changes. To our knowledge, this is the first study to demonstrate experimentally that persistent hypoglycemia in rats induced by human insulin results in axonal degeneration in peripheral nerves with accompanying myofiber degeneration in skeletal muscle.

Disclosure of Potential Conflicts of Interest: The authors have no conflicts of interest to declare.
Acknowledgments: The authors thank Alan Broadmeadow for his contributions to the present study and results. VFH Jensen and J Lykkesfeldt are supported by the LifePharm Centre for In Vivo Pharmacology of the University of Copenhagen. *In vitro* and *in vivo* studies were funded by Novo Nordisk A/S.

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