



Original Research

Impaired hepatic counterregulatory response to insulin-induced hypoglycemia in hepatic denervated pigs

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ABSTRACT

Objective: The liver reacts to hypoglycemia by increasing its glucose output. This response is assumed to depend both on glucose sensing at the liver and the brain, as well as efferent impulses from the brain to the liver. We tested the importance of this signaling pathway by studying the hepatic response to insulin-induced hypoglycemia in hepatic complete denervated pigs.

Materials/methods: Two weeks prior to the metabolic study, 36-kg pigs underwent either total hepatic denervation (DN; n = 12) or sham operation (sham; n = 12). On the metabolic study day, measurements were performed at baseline conditions and during a hypoglycemic hyperinsulinemic (5 mU/kg/min) clamp. Endogenous insulin and glucagon secretions were inhibited by somatostatin, and glucagon was replaced at baseline levels. Endogenous glucose production (EGP) and glucose utilization (Rd) were determined by [³-³H] glucose infusion.

Results: Baseline plasma concentrations of glucose, insulin, EGP and Rd did not differ significantly between the two groups of animals. During insulin infusion, the plasma glucose concentration was clamped at ~3 mmol/L in both groups of animals resulting in an increase in plasma concentrations of epinephrine and norepinephrine in sham pigs (both P < 0.05), while this effect was abolished in DN pigs. While insulin action (P = 0.09) and glucose utilization (P = 0.44) were similar, EGP was markedly decreased in the DN pigs (P < 0.05).

Conclusion: The findings indicate a blunted hepatic counterregulatory response to hypoglycemia following complete hepatic denervation. This implies that intact neural impulses to and from the liver are necessary to maintain the increase in EGP that protects the organism against hypoglycemia.

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Introduction

Plasma glucose concentration is 4–5.5 mmol/L following an overnight fast and rarely exceeds 7–8 mmol/L after eating [1–5]. An important mechanism for the prevention of hypoglycemia is the ability of the liver to increase hepatic glucose production [6]. This protective response depends on combined neuronal and humoral factors, but the role of the individual factors is a subject of debate.

It is well established that efferent signals from glucose sensing receptors in the central nervous system to the liver play a key role [7], but evidence also points toward the existence of glucose sensors located in the periportal region of the liver [8,9] with afferent impulses stimulating centers in the central nervous system [8,10]. Both ways act by sympathoadrenal stimulation of hepatic glucose production, i.e. via increased activity of the sympathetic nervous system that acts on the adrenal medulla of the kidneys to release epinephrine and norepinephrine [11,12].

The importance of hepatic innervation on hepatic glucose metabolism has been examined in a variety of animal experiments. Pagliassotti et al. demonstrated that orally ingested glucose in hepatic denervated dogs results in glucose intolerance [13]. Similarly, studies by Adkins-Marshall et al. concluded that intact nerve supply is vital

Abbreviations: DN, denervated pigs; EGP, endogenous glucose production; Rd, rate of glucose utilization.

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for the normal hepatic response to an intraportally delivered glucose load [14]. In a canine study by Donovan et al. [15] mild hypoglycemia was created using a peripheral insulin infusion. When hepatic euglycemia was maintained by glucose infusion into the portal vein, a decrement of about 40% in the response to hypoglycemia was observed. Likewise, experiments by Donovan et al. [16] and Hamilton-Wessler et al. [17] both demonstrated that the sympathoadrenal response to hypoglycemia is determined by the concentration of glucose in the portal vein, and experiments by Adachi et al. [18] suggested that afferent impulses conducted by the vagal nerve are conveyed to glucose sensing sites in the brain. A recent study by Ionut et al. demonstrated that portal vein denervation impairs hypoglycemic counterregulation due to a suppression of the sympathoadrenal response [19] and impaired glucose tolerance has been observed following portal vein denervation during GLP-1 receptor stimulation [20]. Overall, these experiments support that the portal vein is an important sensing site for hypoglycemia and of hormones such as GLP-1. In contrast, experiments by Jackson et al. [21] and Moore et al. [22] failed to find a metabolic effect of hepatic and portal vein denervation [21] and studies by Wasserman et al. [23] and Kjær et al. [24] both concluded that hepatic nerve supply is not required for the exercise induced stimulation of EGP in euglycemic liver transplant patients.

Taken together, evidence implies that hepatic impulses may play a role for hypoglycemic counterregulation, but the mechanism and the significance of these impulses remain controversial. Therefore, in the present study in anesthetized pigs, we wished to test the hypothesis that hepatic glucose production is reduced following complete hepatic denervation and that this impairs the ability of the liver to increase its glucose production during hypoglycemia.

Materials and methods

Animals

Twenty-four male Göttingen minipigs, 2 years old and with a mean body weight of 35.8 ± 3.9 kg (mean \pm SD) were used. Twelve pigs were randomly selected for hepatic denervation (DN) and twelve for sham operation (sham). The experiments were conducted according to institutional and national guidelines for animal welfare on a license granted by the Danish Ministry of Legal Affairs.

Surgical procedure

The surgical procedure was performed 2 weeks before the metabolic study and under halothane anesthesia. Denervation of the liver was achieved by surgically dividing the connective tissue components surrounding the hepatic veins from the region of the upper edge of the liver parenchyma to the inferior caval vein. The coronal ligaments were also divided resulting in a complete separation of the liver from the diaphragm. Subsequently, the gastroduodenal ligament was exposed and the connective tissue surrounding the common bile duct, the hepatic artery and the portal vein was removed. This procedure was meticulously performed from the level of the porta hepatis to the superior margin of the duodenal arch. The procedure was extended also to involve the connective tissue on the inferior surface of the liver leaving it completely exposed solely attached to the hepatic artery, the hepatic and portal veins and the common bile duct. To ensure the completeness of any residual neural tissue left on these anatomical structures, the procedure was completed by painting 90% phenol on the exposed surfaces of the hepatic and portal veins, on the hepatic artery and on the common bile duct (7). Ampicillin (Anhypen®) 1 g per day was given intramuscularly during the first 3 days after the operation. During the sham procedure the tissue along the hepato-duodenal and the

coronal ligaments was manipulated, but the innervation was not interrupted. Prior to the hepatic denervation and at the end of the metabolic study, liver tissue samples from two different liver lobes were taken for measurements of norepinephrine concentration and immune histology of hepatic nerves in order to validate the liver denervation procedures.

Experimental design

Two weeks prior to the denervation procedures and 3 days prior to the metabolic study the amount of food given to the pigs was increased from 400 g standard pig food per day (DLG, Aarhus, Denmark) to 600 g food plus 200 g sucrose per day to avoid glycogen depletion during the fasting period of 12 hours before the experiments. On the metabolic study day and after an overnight fast general anesthesia was introduced by an intravenous infusion of ketamine 4–8 mg/kg/hr and midazolam 0.4–0.8 mg/kg/min. Air containing 40% oxygen was given via an endotracheal tube by a Servo 900C® ventilator. Catheters were placed in a femoral vein for infusions and in a femoral artery for blood sampling and pressure measurements. When the catheters were in place, the pigs were allowed to stabilize for 1 hour before the first blood samples were drawn. Each study consisted of a 2-hour baseline period (–120 to 0 min) followed by a hyperinsulinemic hypoglycemic clamp (0–150 min) (Fig. 1). At 10.00 (–120 min) a primed-continuous infusion of [$3\text{-}^3\text{H}$] glucose (20 μCi prime, 0.20 $\mu\text{Ci}/\text{min}$ continuous) was commenced and continued until the end of the study. At 12.00 (0 min) an unprimed infusion of insulin (5 mU/kg/min), somatostatin (300 $\mu\text{g}/\text{hour}$) and glucagon (0.5 ng/kg/min) was started and the blood glucose concentration was allowed to fall to 2.8 mmol/L. When this level was reached we initiated an infusion of hot-GINF ([$3\text{-}^3\text{H}$] glucose added to glucose solution (50 mg/mL) to obtain a concentration of 0.2 $\mu\text{Ci}/\text{mL}$). The infusion rate was adjusted to maintain glucose concentrations at 3.0 mmol/L. Mean baseline values were calculated from measurements from 11.30 to 12.00 (–30 to 0 min) (Fig. 1) and mean steady-state values were calculated from 14.00 to 14.30 (120–150 min) during the hyperinsulinemic hypoglycemic clamp (Fig. 1).

Materials

[$3\text{-}^3\text{H}$] glucose was purchased from Dupont New-England Nuclear, Boston, Mass. USA. Human insulin (Actrapid®) and glucagon was purchased from Novo, Copenhagen, Denmark. Somatostatin was purchased from Ferring Pharmaceuticals, Switzerland.

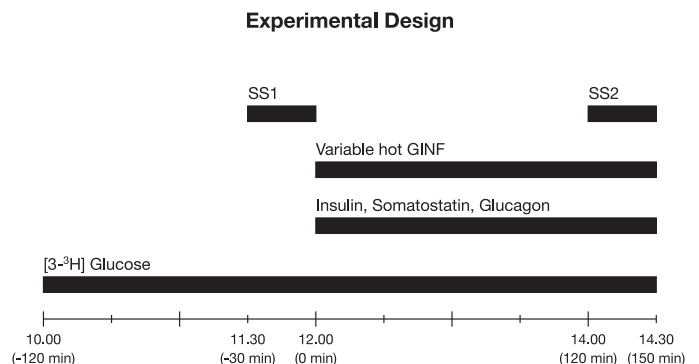


Figure 1. Experimental design. Glucose kinetics was determined in the baseline condition (SS1) from –30 to 0 min and at steady state during the hypoglycemic, hyperinsulinemic clamp (SS2). Infusions of glucose (GINF), insulin, glucagon and somatostatin were started at 0 min. The plasma concentration of glucose during the hypoglycemic hyperinsulinemic clamp did not differ between hepatic denervated and sham pigs.

Blood sample analyses

Arterial plasma glucose concentrations were measured every 5–10 min throughout the glucose clamp. Blood samples for measurement of plasma glucose, insulin, glucagon, epinephrine and norepinephrine were obtained throughout the study. Blood samples were placed immediately on ice, centrifuged at 4 °C, and plasma samples were stored at –20 °C until assay. Plasma concentration of glucose was measured using the glucose oxidase method by a Beckman glucose analyzer (Beckman Instruments, Inc., Fullerton, California). Specific activity of [^3H]-glucose was measured by high-performance liquid chromatography. Plasma and liver tissue concentrations of epinephrine and norepinephrine were determined by high performance liquid chromatography followed by electrochemical detection. With an intra assay coefficient of variation between 0.5 and 10 % [25]. Plasma samples for determination of epinephrine and norepinephrine were frozen immediately at –80 °C and analyzed within 2 weeks. Serum insulin was measured by an enzyme-linked immunosorbent assay employing a two-site immunoassay [26] with an intra-assay coefficient of variation of 2.0% ($n = 75$) at a plasma level of 200 pm. Plasma glucagon was measured by RIA using wick chromatography as described [27] with the modification that polyethylene glycol (PGE) was used for separation before determination and that plasma was extracted with ethanol. The intra-assay coefficient of variation was 4–6.8% at plasma concentration of glucagon about 150 pmol/L with a recovery higher than 90% and no detectable cross-reactivity. Serum free fatty acids (FFAs) were measured enzymatically using a Wako NEFA (nonesterified fatty acid) test kit (Wako Chemicals, Neuss, Germany).

Calculations

All data are expressed as the mean \pm SEM. Rate of glucose infusion (GIR), EGP and Rd are expressed per kg total body weight. Tracer-determined rates of EGP and Rd were calculated using the equations for non-steady state as described by Steel et al. [28]. Rate of glucose appearance (Ra) equals the sum of exogenous glucose infusion (GIR) and endogenous glucose production (EGP), i.e. $Ra = GIR + EGP$. Consequently, EGP was calculated by subtracting GIR from the tracer determined rate of glucose rate of appearance ($EGP = Ra - GIR$). Mean values during the 30 min before the start of the clamp (i.e. –30 to 0 min) were considered as baseline values. Mean values during the hyperinsulinemic hypoglycemic clamp (120–150 min) were considered as steady state values. The volume of distribution of glucose was assumed to be 200 mL/kg, with a pool correction factor of 0.65. Glucose specific activity was calculated as the ratio between the tracer concentration and the prevailing plasma glucose concentration.

Histology

The liver tissue samples were immediately fixed in 10% buffered formalin and embedded in paraffin after 24 hours. Subsequently, slides were prepared for immunohistochemistry. In a pilot study a number of antibodies to human neural antigens were tested on liver biopsies from control pigs. The following antibodies were found to give the same staining reactions in human and porcine liver: (1) Protein gene product 9.5 (PGP) (polyclonal, Biogenesis, England), (2) neurofilament protein (NFP) (clone 2F11, DAKO, Denmark) and (3) glial fibrillary acidic protein (GFAP) (polyclonal, DAKO, Denmark). PGP and NFP have been demonstrated in neurons and neuroendocrine cells (11, 32) whereas GFAP has been demonstrated in Schwann cells (21).

Statistical analyses

Sample size calculation was based on a power of 80%. Statistical type 1 error was set to 5% and type 2 to 20%. Anticipating a

MERIDIF of EGP of 1 mg/kg/min and a SD of 0.7 mg/kg/min a non-paired analysis would require 12 pigs in each group, which was the number enrolled. Data are expressed as mean \pm standard error of the mean (SEM). ANOVA for repeated measurements and, in the case of significant time effects, Student's T-test (paired or unpaired as relevant) were used as statistical tests for significance of differences. Wilcoxon test was used for data not fulfilling criteria for normal distribution. A p-value less than 0.05 was considered to indicate statistically significant difference.

Results

Glucose, insulin, FFA, glucagon, epinephrine and norepinephrine concentration

Baseline plasma concentration of glucose (4.60 ± 0.18 vs 5.02 ± 0.29 mmol/L; $P = 0.22$), insulin (11 ± 2 vs 12 ± 3 pmol/L; $P = 0.72$) and FFA (0.38 ± 0.05 vs 0.33 ± 0.06 mmol/L; $P = 0.54$) did not differ between DN and sham pigs (Tables 1 and 2). During the euglycemic clamp insulin increased similarly in DN and sham pigs (712 ± 106 vs 712 ± 44 pmol/L; $P = 0.99$) and plasma concentrations of glucose were allowed to fall to the same moderately hypoglycemic levels in both groups (3.00 ± 0.06 vs 3.07 ± 0.08 mmol/L; $P = 0.47$). During the clamp, the glucose infusion rate (GIR) did not differ between the groups (Fig. 2) (8.51 ± 0.77 vs 6.70 ± 0.65 mg/kg/min; $P = 0.09$) demonstrating no difference in net insulin action. FFA concentrations fell similarly in both groups (Table 2).

Baseline plasma concentrations of glucagon (151 ± 29 vs 179 ± 27 pmol/L; $P = 0.50$), epinephrine (215 ± 51 vs 99 ± 27 pg/mL; $P = 0.08$) and norepinephrine (167 ± 24 vs 151 ± 23 pg/mL; $P = 0.63$) were not significantly different between DN and sham pigs. In sham pigs, the clamp resulted in significant increments in plasma concentrations of both epinephrine (99 ± 27 vs 521 ± 290 pg/mL; $P = 0.046$) and norepinephrine (151 ± 23 vs 285 ± 57 pg/mL; $P = 0.028$) (Table 1). In DN the clamp resulted in significant increments in plasma concentrations of norepinephrine (167 ± 24 vs 265 ± 48 pg/mL; $P = 0.028$), whereas plasma concentrations of epinephrine (215 ± 51 vs 195 ± 63 pg/mL; $P = 0.46$) remained unaltered. The hypoglycemic clamp did not change plasma concentrations of glucagon in

Table 1

Plasma concentrations of epinephrine, norepinephrine and glucagon following an overnight fast (baseline) and during insulin-induced hypoglycemia (insulin) in DN and sham pigs

		Epinephrine (pg/mL)	Norepinephrine (pg/mL)	Glucagon (pg/mL)
DN	Baseline	215 \pm 51	167 \pm 24	151 \pm 29
	Insulin	195 \pm 63	265 \pm 48*	102 \pm 32
Sham	Baseline	99 \pm 27	151 \pm 23	179 \pm 27
	Insulin	521 \pm 290*	285 \pm 57*	228 \pm 68

* Statistical difference ($P < 0.05$) within the group (baseline vs insulin).

**Statistical difference ($P < 0.05$) between groups.

Table 2

Plasma concentrations of insulin and FFA following an overnight fast (baseline) and during insulin-induced hypoglycemia (insulin) in DN and sham pigs. The glucose infusion rate (GIR) at steady state (120–150 min; SS2) is a measure of net insulin action determined during the hypoglycemic hyperinsulinemic clamp

		Insulin (pmol/L)	FFA (pmol/L)	GIR (mg/kg/min)
DN	Baseline	11 \pm 2	0.38 \pm 0.05	–
	Insulin	712 \pm 106*	0.07 \pm 0.02*	7.64 \pm 0.85
Sham	Baseline	12 \pm 3	0.33 \pm 0.06	–
	Insulin	712 \pm 44*	0.10 \pm 0.02*	6.22 \pm 0.67

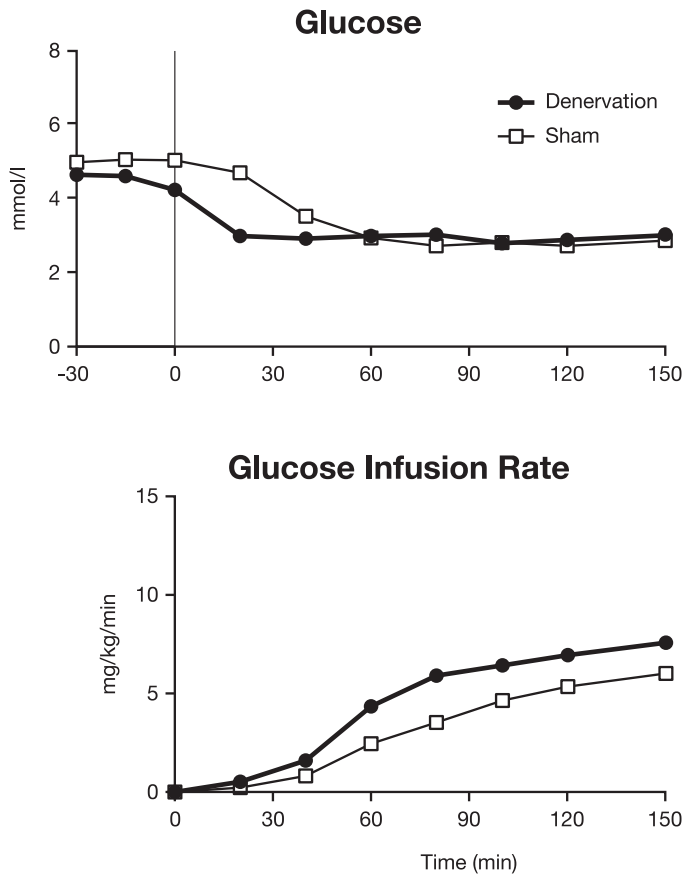


Figure 2. Time course of plasma concentration of glucose (top) and glucose infusion rate (GIR) (bottom). Insulin infusion was started at 0 min. Plasma concentration of glucose (top) and glucose infusion rate (bottom) at baseline and during the clamp did not differ between hepatic denervated and sham pigs.

neither DN (151 ± 29 vs 102 ± 32 pg/mL; $P = 0.11$) nor in sham operated animals (179 ± 27 vs 228 ± 68 pg/mL; $P = 0.56$).

Endogenous glucose production (EGP) and glucose utilization (Rd)

At baseline, there were no difference between DN and sham of EGP (2.83 ± 0.30 vs 3.32 ± 0.30 mg/kg/min; $P = 0.26$) or Rd (3.16 ± 0.26 vs 3.11 ± 0.32 mg/kg/min; $P = 0.89$) (Fig. 3). During the clamp, Rd increased similarly in DN and sham pigs (7.76 ± 0.61 vs 7.13 ± 0.53 mg/kg/min; $P = 0.44$). In contrast, EGP remained higher in sham than in DN pigs (1.13 ± 0.48 vs 0.01 ± 0.20 mg/kg/min; $P = 0.04$).

Histology

In the liver tissue samples taken before the denervation there were numerous PGP (protein gene product 9.5) positive fibers along the sinusoids and around the central veins. A few NFP (neurofilament protein) and GFAP (glial fibrillary acidic protein) positive fibers were seen in the periportal areas of the lobules. In the portal tracts large nerves as well as small bundles of nerve fibers mainly around arteries, veins and bile ducts were detected. These nerves showed strong reactions for PGP, NFP and GFAP. More small fibers were detected in the PGP stained samples than in the GFAP stained samples. In the liver tissue samples taken 2 weeks after the denervation there were only small GFAP positive nerve bundles in the portal tracts, whereas NFP and PGP staining were negative.

Liver tissue norepinephrine

Prior to the hepatic denervation liver tissue norepinephrine was 165 ± 28 ng/mL liver tissue and after the denervation it was 2.0 ± 0.0 ng/mL liver tissue ($P < 0.001$).

Discussion

The main finding of this study in anesthetized pancreatic hormone blocked pigs is that the ability of the liver to increase its EGP in

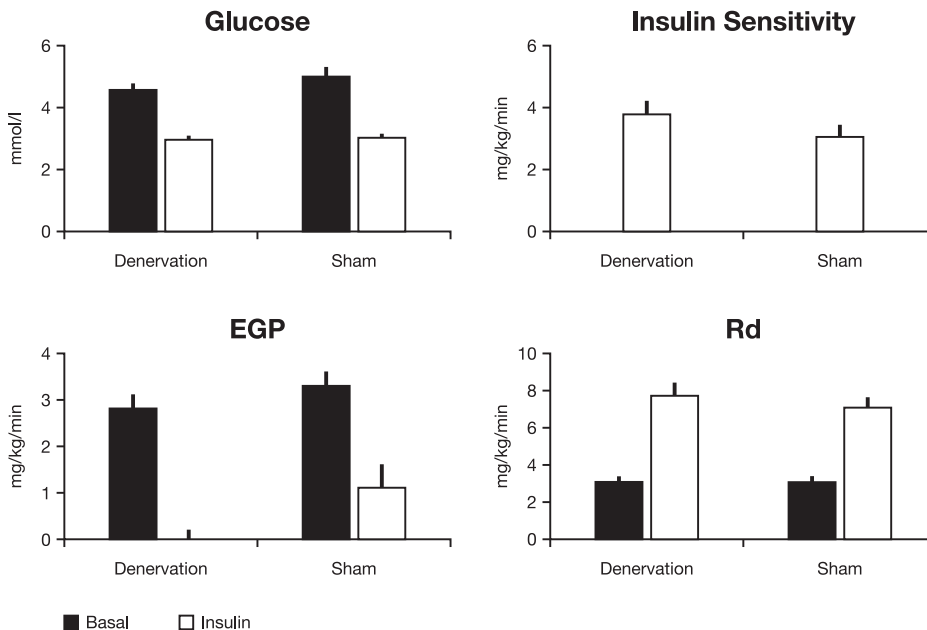


Figure 3. Glucose concentration, insulin sensitivity, endogenous glucose production (EGP) and glucose uptake (Rd) following an overnight fast (■) and during insulin-induced hypoglycemia (□) in pigs undergoing either hepatic denervation or a surgical sham procedure.

response to insulin-induced hypoglycemia was nearly abolished by the total hepatic denervation. While it is well established that glucose sensing receptors in the brain stimulate neurons innervating the liver during hypoglycemia, the existence of hepatic glucose receptors and afferent neuronal stimulation of the brain is less clear [8,10,16,21,29]. This shows that the effect of hypoglycemia on EGP is mediated not only through humoral, but also through neuronal pathways.

As expected, hyperinsulinemia increased whole body glucose uptake and suppressed plasma concentration of FFA with none of these effects being altered by hepatic denervation. In both groups EGP was suppressed by insulin [30]. However, whereas the EGP in the hepatic denervated pigs was nearly completely suppressed by hyperinsulinemia, EGP in the sham animals was only suppressed from baseline by 67%. This finding demonstrates that in the anesthetized pigs, the normal liver maintains its ability to increase EGP in response to insulin-induced hypoglycemia, whereas the denervated liver nearly completely lost this ability. This shows that intact hepatic nerve supply is necessary to maintain an appropriate hepatic response to hypoglycemia.

In addition to the present experiments, a variety of animal studies have examined the role of the liver in maintaining glucose homeostasis. In experiments by Donovan et al. [15] peripheral hypoglycemia was induced by insulin infusion and the metabolic response observed in the presence or absence of portal vein hypoglycemia. These studies imply the presence of glucose sensing receptors in the portal vein, and the results suggest in accordance with our observation that neural impulses from the liver are required for a complete counterregulatory response to hypoglycemia. This observation has subsequently been confirmed by Donovan et al. [16] and by Hevener et al. [10] in rats. Moreover, a recent study by Hevener et al. suggests that the portal vein is generating afferent signals to glucose sensing sites in the central nervous system and that these signals are critical for hypoglycemic detection and for the normal sympathoadrenal counterregulatory response [8]. In contrast to these observations are results from canine experiments by Wasserman [23] and Jackson et al. [21] in which hepatic denervation failed to alter the hepatic response to hypoglycemia, and by Mikines et al., who reported a similar findings [31].

In the present study we used somatostatin to inhibit endogenous insulin and glucagon secretion. This may in our opinion provide an explanation for the discrepancy between our and the results reported by other investigators. Stable concentrations of glucagon were obtained by an exogenous glucagon infusion. This design was chosen, because glucagon secretion is increased during hypoglycemia. Glucagon stimulates EGP [32–34]. Therefore, if endogenous secretion had not been inhibited the invariable rise in glucagon concentration during hypoglycemia would have made it impossible to determine whether the observed difference in EGP is due to a rise in glucagon secretion or due to an altered hepatic response to hypoglycemia. Furthermore, in studies where somatostatin was not infused, plasma glucagon concentrations are likely to differ from those obtained in the present study. This difference is likely to have affected the hepatic response and thus explain the difference in EGP observed in response to hypoglycemia.

In the present study glucagon was infused into a peripheral vein and not the portal vein, which results in comparable portal and peripheral vein concentrations. Because endogenous glucagon secretion was inhibited with somatostatin, glucagon concentrations were maintained at constant and stable levels. Consequently, glucagon action was unaltered during the glucose clamp arguing against that the absence of a glucagon gradient may have had an effect on the present findings.

Somatostatin also inhibits endogenous growth hormone (GH) secretion, but we did not replace GH. This design was chosen because in a previous study in humans GH replacement did not affect EGP

following near-complete blockade of GH secretion by a somatostatin analog [2].

Under normal conditions the metabolic counterregulatory response to hypoglycemia is thought to result in an inhibition of insulin secretion followed by a rise in plasma concentrations of glucagon, epinephrine, norepinephrine and eventually cortisol and growth hormone concentrations. The present study was designed to maintain insulin and glucagon concentrations at steady levels during hypoglycemia. The difference in EGP between the sham and DN animals is therefore likely a result of the difference in epinephrine and norepinephrine concentrations rather than a result of the counterregulatory effects of glucagon and insulin. Epinephrine and norepinephrine both have effects on EGP predominantly through activation of α_1 - and β_2 adrenergic receptors [35,36]. In sham operated animals hypoglycemia resulted in a significant increase in epinephrine and norepinephrine concentrations, while this response was abolished in DN animals. Noteworthy, a five-fold increase in epinephrine concentration was observed, whereas the rise in norepinephrine, although significant, was less pronounced. This strongly implies that epinephrine plays an important role for the hepatic counterregulatory response to hypoglycemia. This observation is in line with a recent study by Ionut et al., who examined the counterregulatory response following portal vein denervation in dogs during a hypoglycemic hyperinsulinemic clamp [19]. As in our study, portal vein denervation resulted in a reduced epinephrine response confirming our observation that epinephrine plays a key role in the hypoglycemic counterregulatory response to hypoglycemia. A likely explanation may be that the denervation procedure has interrupted the afferent pathway of the hypoglycemic counterregulatory circuit from the liver, normally relaying the message of hypoglycemia to the brain and the subsequent signaling from the brain to the suprarenal glands.

In conclusion, the present experiments show that hepatic innervation is necessary for the liver to maintain its capacity to increase glucose production in response to hypoglycemia, which constitutes a pivotal defense mechanism against the harmful effects of hypoglycemia. The hepatic response to hypoglycemia by increasing EGP was severely blunted by hepatic denervation. This underlines the importance of neuronal impulses for the complete counterregulatory response of the liver to hypoglycemia. Possible clinical implications of our findings are that patients with hepatic denervation following liver transplantation or patients with autonomic neuropathy due to long term complications to type 1 diabetes mellitus may be vulnerable to hypoglycemia.

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Conflict of interest

The authors declare they have no conflicts of interest.

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