Carvedilol prevents counterregulatory failure and impaired hypoglycaemia awareness in non-diabetic recurrently hypoglycaemic rats

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Abstract

Aims/hypothesis—This study evaluates whether the non-selective β-blocker, carvedilol, can be used to prevent counterregulatory failure and the development of impaired awareness of hypoglycaemia (IAH) in recurrently hypoglycaemic rats.

Methods—Sprague Dawley rats were implanted with vascular catheters and intracranial guide cannulas targeting the ventromedial hypothalamus (VMH). These animals underwent either three bouts of insulin-induced hypoglycaemia or received three saline injections (control group) over 3 days. A subgroup of recurrently hypoglycaemic animals was treated with carvedilol. The next day, the animals underwent a hypoglycaemic clamp with microdialysis without carvedilol treatment to evaluate changes in central lactate and hormone levels. To assess whether carvedilol prevented IAH, we treated rats that had received repeated 2-deoxyglucose (2DG) injections to impair their awareness of hypoglycaemia with carvedilol and measured food intake in response to insulin-induced hypoglycaemia as a surrogate marker for hypoglycaemia awareness.

Results—Compared with the control group, recurrently hypoglycaemic rats had a ~1.7-fold increase in VMH lactate and this was associated with a 75% reduction in the sympathoadrenal response to hypoglycaemia. Treatment with carvedilol restored VMH lactate levels and improved the adrenaline (epinephrine) responses. In 2DG-treated rats compared with control animals

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receiving saline, food intake was reduced in response to hypoglycaemia and increased with carvedilol treatment.

Conclusion/interpretation—We conclude that carvedilol may be a useful therapy to prevent counterregulatory failure and improve IAH.

Keywords

Introduction

Hypoglycaemia remains a major barrier to properly managing glucose levels in people with type 1 diabetes [1, 2]. However, loss of the ability to secrete glucagon in the early stages of diabetes, compounded by the fact that antecedent hypoglycaemia results in loss of the sympathoadrenal response to hypoglycaemia, not only reduces effective recovery from hypoglycaemia but also reduces symptomatic awareness of hypoglycaemia—a condition called hypoglycaemia-associated autonomic failure (HAAF) [3]. The mechanisms leading to HAAF are not known, but several mechanisms have been proposed, including, but not limited to, repeated activation of the adrenergic system and impairments in central glucose-sensing mechanisms [4-6]. The data from the current study suggest these two mechanisms may be linked.

We have reported that repeated activation of adrenergic receptors in the ventromedial hypothalamus (VMH) in the absence of peripheral hypoglycaemia raised local lactate concentrations and suppressed the counterregulatory responses (Sejling et al., unpublished data). Noradrenaline (norepinephrine) enhances lactate production from astrocytes through the activation of β_2-adrenergic receptors (β2ARs) and can also increase lactate uptake into neurons by enhancing monocarboxylic acid transporter (MCT) expression [7-9]. Normally, in response to acute bouts of hypoglycaemia, VMH noradrenaline levels rise and act through β2ARs to enhance the sympathoadrenal response [10-12]. Less is known about the effects of recurrent hypoglycaemia on the VMH noradrenaline system or how repeated activation of β2ARs impacts the counterregulatory responses. It has been reported that VMH noradrenaline responses to hypoglycaemia remain intact in recurrently hypoglycaemic rats [13] and that the activity of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis, is not impaired by recurrent hypoglycaemia [14]. This suggests that recurrent hypoglycaemia does not impair the VMH noradrenaline response to subsequent bouts of hypoglycaemia, but, rather, defects leading to counterregulatory failure may lie downstream of noradrenaline release. In support of this finding, adrenergic blockade during antecedent hypoglycaemia prevents counterregulatory failure in healthy humans [15]. Therefore, while the acute activation of VMH adrenergic receptors may be beneficial in enhancing the counterregulatory response, repeated activation of this neurotransmitter system may contribute to counterregulatory failure. Hence, β-blockers may be a useful therapeutic strategy to help prevent the onset of HAAF. The objectives of the current study were to evaluate whether the non-specific β-blocker, carvedilol, can be used to prevent the development of counterregulatory failure and impaired awareness of hypoglycaemia (IAH)

in rodents. We chose these models because the rat model of recurrent hypoglycaemia is an established model of HAAF that replicates many of the same features of HAAF found in humans, while the rodent model of IAH possesses the same counterregulatory defects as the recurrent hypoglycaemia model, but it also exhibits changes in feeding responses that allow us to objectively evaluate hypoglycaemia awareness.

Methods

Animals

Adult male Sprague Dawley rats (CD:SD, strain 001; Charles River, Wilmington, MA, USA) of 7–8 weeks of age and weighing ~300 g were individually housed in conventional rat cages in the University of Utah's Comparative Medicine Center in temperature- (22±2°C) and humidity-controlled rooms. Cages were lined with wood chip bedding and the animals were provided with enrichment in the form of a red acrylic tube and a gnawing block. The animals had free access to rodent chow (Envigo Teklad; Madison, WI, USA) and water and were acclimatised to a 12 h light/dark cycle (lights on between 07:00 hours and 19:00 hours) for 1 week before experimental manipulation. The principles of laboratory animal care were followed, and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Carvedilol treatment

Carvedilol treatment (Fig. 1a,b) was initiated 1 day prior to the start of the recurrent hypoglycaemia or recurrent 2-deoxyglucose (2DG; Sigma-Aldrich, St Louis, MO, USA) treatment regimens described below. The animals were administered a single daily i.p. injection of carvedilol (3 mg/kg) dissolved in saline (154 mmol/l NaCl) at 08:00 hours in the laboratory. On each of the days that either insulin or 2DG was given, carvedilol was administered 1 h prior to the insulin or 2DG injection. Carvedilol treatment continued throughout the 3 days of recurrent hypoglycaemia or 2DG treatment, but the drug was not given on the day of the clamp or food-intake studies. Typical doses of carvedilol used in rodent studies range from 1 mg/kg to 30 mg/kg, with benefits evident even at the lower doses of 1 and 2 mg/kg [16-19]. As the goal of our study was to identify doses of carvedilol that would temper the β-adrenergic effects and not completely block β-adrenergic signalling, we evaluated two low doses, 1 and 3 mg/kg; we noted that the optimal dose for preventing counterregulatory failure in this rodent model of recurrent hypoglycaemia was 3 mg/kg (electronic supplementary material [ESM] Fig. 1).

Study 1: evaluating the counterregulatory hormone responses following carvedilol treatment

Vascular surgery—After the acclimatisation period, the animals underwent aseptic surgery under 2-2.5% isoflurane anaesthesia to have vascular catheters implanted into the left carotid artery and right jugular vein as described previously [20]. Animals were monitored every 15 min throughout the surgery. Compared with injectable anaesthetics, isoflurane allowed for more rapid recovery from surgical procedures.

Stereotaxic surgery—Subsequently, the animals were placed into a stereotaxic frame and bilateral stainless steel guide cannulas for microdialysis (Azuma, San Diego, CA, USA) were inserted to the level of the VMH (from bregma: 2.6 mm posterior, 3.8 mm lateral and 8.7 mm ventral at an angle of 16°) and secured with screws and dental acrylic as described previously [21]. The animals were then returned to their home cages for 4 days to recover before being randomly assigned to one of the following treatment groups: (1) saline control (n=6); (2) recurrent hypoglycaemia (n=7); and (3) recurrent hypoglycaemia + carvedilol (n=7). Treatment groups underwent the clamping procedure in random order.

Recurrent hypoglycaemia—Hypoglycaemia was induced using a single i.p. injection of insulin (5-10 IU/kg, Humulin R, Eli Lilly, Indianapolis, IN, USA). Insulin doses were tapered daily from 10 U/kg on day 1 to 8 U/kg on day 2 to 5 U/kg on day 3 to account for the development of counterregulatory failure and to avoid severe hypoglycaemia and seizures [22]. Plasma glucose concentrations were monitored through a tail nick every 30 min over 3 h using an AlphaTRAK 2 glucometer (Abbott Laboratories, Chicago, IL, USA). Target glucose levels were maintained between ~1.7 and 2.8 mmol/l for at least 2 h during this time. During recurrent hypoglycaemia, animals had access to water but not food. Following each episode of hypoglycaemia, plasma glucose levels were recovered to euglycaemic levels by providing free access to food and administering 0.5-1 ml 20% dextrose i.p., as needed. Once recovered, the animals were returned to their home cages. This procedure was repeated for 3 consecutive days (Fig. 1a). This treatment regimen was shown to effectively reduce the counterregulatory hormone responses to hypoglycaemia [22].

Hypoglycaemic clamp—Following an overnight fast, bilateral 1 mm microdialysis/microinjection probes (Azuma) were inserted through the guide cannulas of the animals and their vascular catheters were connected to the infusion pumps. After a 2.5 h recovery period, baseline blood samples were collected and the collection of baseline microdialysate samples was initiated for 45 min. Once baseline microdialysate samples were collected, a constant insulin (50 mU kg^{-1} min^{-1}) and variable 20% glucose infusion was used to lower and maintain plasma glucose levels at 2.5±0.3 mmol/l for 90 min. Blood samples were collected every 5 min to assess plasma glucose levels (Analox GM9, Analox Instruments, Stourbridge, UK) and at 30, 60 and 90 min during the clamping period to assess plasma lactate and hormone responses. Once the plasma was collected and frozen, the erythrocytes were re-suspended in an equivalent volume of artificial plasma and re-infused back into the animal to prevent volume depletion and anaemia.

Probe placement—At the end of the experiment, the animals were euthanised with intravenous sodium pentobarbital. The brains were rapidly removed, frozen and stored at −80°C until they were analysed. Accuracy of probe position was histologically verified and only those animals with properly positioned probes were included in the analysis. The primary endpoint of the clamp procedure was to determine whether carvedilol prevented the development of counterregulatory failure in recurrently hypoglycaemic rats.

Study 2: determining the effects of recurrent hypoglycaemia and carvedilol treatment on VMH *Mct* expression

To evaluate the effects of recurrent hypoglycaemia and carvedilol treatment on expression of the monocarboxylic acid transporter in the VMH, groups of control rats (n=8), recurrently hypoglycaemic rats (n=9) and recurrently hypoglycaemic rats that received carvedilol (n=8) were killed on the day following their last treatment regimen. The VMH was micropunched and quantitative (q)RT-PCR was used to quantify mRNA levels of *Mct1* (also known as *Slc16a1*; Taqman Assay ID: Rn00562332_m1) and *Mct2* (also known as *Slc16a7*; Assay ID: Rn00568872_m1). Total RNA was extracted (Qiagen RNA extraction kit; Qiagen, Germantown, MD, USA) and reverse transcription was conducted using Taqman primers and RT-PCR assays. Briefly, reverse transcription was performed by adding 10 μl Taqman master mix to 125 ng RNA and running the following thermocycler settings: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and 4°C hold. PCR reactions were carried out by adding 18 μl reaction mix to 2 μl cDNA and running the following reactions: 50°C for 2 min, 95°C for 2 min, 95°C for 1 s, 60°C for 20 s for 40 cycles. Normalisation was carried out using β-actin (Assay ID: Rn01410374_m1) as the endogenous control. The $2^{-\Delta\Delta C_2}$ method was used to calculate relative fold change in gene expression between control and experimental groups [23].

Study 3: evaluating hypoglycaemia awareness

Impaired hypoglycaemia awareness model—In humans, IAH is determined using questionnaires querying the extent to which individuals feel various hypoglycaemic symptoms [24, 25]. This procedure is not feasible in animals and a model of IAH needed to be developed. In response to hypoglycaemia, animals typically seek and consume food [26]. Therefore, food consumption may be a useful and quantitatively objective surrogate marker of IAH in rodents. We anticipate that in such a model, those animals that are hypoglycaemia aware would consume more food when blood glucose levels decline, whereas those that are impaired aware would eat less. It has been reported that recurrent insulin-induced hypoglycaemia does not elicit a reduced feeding response to hypoglycaemia, whereas recurrent glucose deprivation using 2DG does [27, 28]. Of note, both recurrent insulin-induced hypoglycaemia and recurrent glucose deprivation lead to impairments of the counterregulatory response to hypoglycaemia [29]. The reason for this dichotomy in the feeding response is not fully understood, but it may be due to a more profound and/or prolonged reduction in utilisable glucose (at the cellular level) in the 2DG model. Therefore, we induced 'IAH' in our animals by administering a s.c. injection of 2DG (200 mg/kg) once daily for 3 consecutive days. Control animals received s.c. saline injections. During recurrent 2DG or saline administration, the animals had access to water but not food. Animals were randomly allocated to these treatment conditions.

Effects of carvedilol treatment on hypoglycaemia awareness—To evaluate whether carvedilol improved awareness of hypoglycaemia, we treated rats made 'IAH' with carvedilol and measured food intake in response to insulin-induced hypoglycaemia on day 4 as a surrogate marker of hypoglycaemia awareness (Fig. 1b). Again, carvedilol treatment was started prior to the start of the 2DG-treatment regimen and carvedilol was given 1 h prior to each injection of 2DG. On day 4, non-fasted rats were given either a single s.c.

injection of saline or 25 U/kg NPH insulin (Lilly). This dose of insulin was necessary to induce and maintain the hypoglycaemic stimulus for the entire 4 h period that food consumption was evaluated. Lower insulin doses were insufficient to keep glucose levels at ~2.8 mmol/l once the animals started to eat. Moreover, this dose of insulin was comparable with the total amount of insulin used to maintain hypoglycaemia using insulin-clamp procedures while food consumption was evaluated (data not shown). Tail vein glucose and food consumption was evaluated every 2 h and total food intake over the course of 4 h was measured. The rats were randomly assigned to one of the following treatment groups (recurrent treatment + day 4 treatment): (1) saline + saline (n=6); (2) saline + insulin (n=8); (3) 2DG + insulin (n=8); and (4) 2DG and carvedilol + insulin (n=6). The primary endpoint of the awareness study was to determine whether carvedilol prevented the development of IAH.

Study 4: effects of carvedilol on body weight and feeding behaviour

To control for the potential non-specific effects of carvedilol on feeding behaviour, we administered carvedilol (3 mg/kg; i.p.) once daily for 4 consecutive days to a group of animals (n=8) and monitored its effects on body weight and daily food consumption. This group was compared with a group administered a daily saline injection (n=8).

Hormone and microdialysate analysis—Plasma catecholamine concentrations were analysed by ELISA (Abnova, Taipei, Taiwan), while plasma glucagon concentrations were determined using commercial radioimmunoassays (MilliporeSigma, Burlington, MA, USA). Plasma lactate concentrations were determined using an Analox GL6 analyser (Analox Instruments). Microdialysate lactate concentrations were analysed using a fluorometric assay (Biovision, Milpitas, CA, USA).

Statistical analysis

Treatment effects were analysed using Student's t-test, one- (for lactate, glucagon, adrenaline and food-consumption data) or two-way (for plasma glucose and glucose infusion rate [GIR] data) ANOVA for independent or repeated measures as appropriate, followed by Tukey's pairwise comparisons using Prism GraphPad 7.0 statistical software. A value of p 0.05 was set as the criterion for statistical significance. The sample sizes required for all studies were determined using statistical power calculations based on our experience with these experiments, with a $p<0.10$ for the β error and $p<0.05$ for the α error.

Results

Exogenous GIRs with recurrent hypoglycaemia

Plasma glucose levels were matched between treatment groups during the hypoglycaemic clamp (Fig. 2a). Despite similar plasma glucose concentrations, exogenous glucose requirements were significantly higher between 70 and 90 min in the recurrently hypoglycaemic group compared with the control group (Fig. 2b; $p<0.05$, control vs recurrent hypoglycaemia). Carvedilol treatment reduced GIRs to normal ($p<0.05$, recurrently hypoglycaemic vs recurrently hypoglycaemic + carvedilol).

Hormone response with recurrent hypoglycaemia

No significant differences in baseline hormone levels were noted between treatment groups (Table 1). As expected, recurrent hypoglycaemia raised baseline extracellular lactate concentrations in the VMH by 66% ($p<0.05$ vs control group; Fig. 2c). Treatment with carvedilol normalised VMH lactate concentrations. In contrast, plasma lactate concentrations were slightly, but significantly, lower in both recurrently hypoglycaemic and carvedilol-treated animals ($p<0.05$ vs control group; Fig. 2d). Peak glucagon responses, which generally occurred between 30 and 60 min after the start of insulin, were reduced by 42% in the recurrently hypoglycaemic group ($p<0.05$ vs control group; Fig. 2e). Although carvedilol improved the glucagon responses in recurrently hypoglycaemic animals, the improvement failed to reach statistical significance ($p=0.09$ vs recurrently hypoglycaemic). Peak adrenaline (epinephrine) concentrations, which generally occurred between 60 and 90 min after the start of insulin, were reduced by 75% compared with the control group ($p<0.05$ vs control group; Fig. 2f) and carvedilol fully restored the sympathoadrenal response in recurrently hypoglycaemic animals ($p<0.001$ vs recurrently hypoglycaemic).

Mct expression in the VMH with recurrent hypoglycaemia

Recurrent hypoglycaemia did not significantly alter the level of *Mct1* mRNA (Fig. 3a) in the VMH, but it increased the level of *Mct2* mRNA ($p<0.05$ vs control group; Fig. 3b). Treatment with carvedilol reduced *Mct2* mRNA levels to normal.

Carvedilol treatment prevents the development of IAH

Plasma glucose levels were matched between all treatment groups during hypoglycaemia induction (Fig. 4a). Rats treated with saline for 3 days consumed significantly more food in response to insulin-induced hypoglycaemia than was consumed by saline-treated rats administered saline on day 4 (Fig. 4b). This demonstrated hypoglycaemia induces a significant feeding response. In contrast, 2DG-treated animals ate significantly less than the saline-treated animals during day 4 hypoglycaemia ($p<0.05$ vs saline + insulin), suggesting IAH. Treatment with carvedilol prevented the development of IAH in the 2DG-treated animals as they ate as much as the saline + insulin group, suggesting hypoglycaemia awareness was restored to normal.

Effects on feeding and body weight

Carvedilol treatment did not increase body weight (Fig. 5a) or overall food consumption (Fig. 5b) over the course of the 4 day regimen.

Discussion

The treatment and prevention of hypoglycaemia is critical if we are to help individuals with diabetes reach and maintain their glycaemic targets. Previous research suggests that repeated activation of the adrenergic system during recurrent episodes of hypoglycaemia may potentially contribute to the development of HAAF [15]. The current study shows that carvedilol may be a useful therapy to prevent the development of HAAF.

Activation of VMH β2ARs is important for augmenting the counterregulatory responses to an acute bout of hypoglycaemia [11, 12]. This is consistent with observations by Beverly and colleagues who showed noradrenaline levels in the VMH rose during hypoglycaemia [10, 30]. However, it was noteworthy that recurrent hypoglycaemia did not alter this neurotransmitter response, despite the development of a defective sympathoadrenal response [13]. Consistent with these observations, we showed that repeated activation of VMH β2ARs in the absence of hypoglycaemia raised VMH lactate levels and induced counterregulatory failure (Sejling et al, unpublished data), suggesting the suppressive effects of noradrenaline are most likely mediated through β2ARs. In a subgroup of animals treated with noradrenaline in that prior study, we administered α-cyano-4-hydroxy-cinnamic acid (4CIN) into the VMH to block the uptake of lactate into neurons immediately prior to the hypoglycaemic clamp. In this group, we saw the suppressive effects of noradrenaline treatment on glucose counterregulation were completely abolished. Hence, these data suggest that repeated activation of VMH β2ARs plays an important role in the development of counterregulatory failure, in part by enhancing central lactate production. Therefore, the use of β-blockers may be a promising therapeutic strategy to help preserve the hormone responses to hypoglycaemia.

The current study shows that when carvedilol is administered in low doses, it can effectively prevent the development of counterregulatory failure in recurrently hypoglycaemic rats. Carvedilol-treated recurrently hypoglycaemic rats required less exogenous glucose during the hypoglycaemic clamp compared with recurrently hypoglycaemic animals treated with vehicle and, more importantly, we saw improvements in the sympathoadrenal response to hypoglycaemia in the carvedilol-treated recurrently hypoglycaemic animals that corresponded to the reduced GIRs, suggesting more effective recovery from hypoglycaemia. Of interest is the fact that carvedilol is a lipophilic drug that gains rapid access to the brain. It was reported that the level of ^{11}C-labelled carvedilol reaches a peak in the brain within 10 min of systemic administration in mice and remains in the circulation with a half-life of 7-10 h [31]. In our studies, we administered carvedilol 1 h prior to inducing hypoglycaemia and this was sufficient to prevent the onset of counterregulatory failure in recurrently hypoglycaemic animals. The ability of carvedilol to penetrate and act within the brain was evident when examining lactate levels in the VMH—although recurrent hypoglycaemia raised lactate levels in the VMH, animals treated with carvedilol exhibited normal brain lactate levels. These changes occurred despite plasma lactate concentrations being slightly lower in recurrently hypoglycaemic animals, indicating that elevated brain lactate levels are generated locally and not taken up from the periphery, as lactate generally travels along its concentration gradient.

Carvedilol treatment did not affect plasma lactate levels in recurrently hypoglycaemic animals. This important observation underscores the potential for carvedilol to prevent some of the central adaptations that occur during recurrent hypoglycaemia. Elevated brain lactate levels have been implicated in the development of HAAF in both humans and rodents [32-37], but the mechanism(s) are still not known. De Feyter et al reported elevated brain lactate concentrations in people with type 1 diabetes that were not associated with increased lactate oxidation [32, 38]. In contrast, Wiegers et al reported enhanced brain lactate utilisation in individuals with type 1 diabetes with IAH, as evidenced by a decline in brain

lactate levels during a hypoglycaemic clamp that was not observed in healthy individuals [35, 37]. Using exercise to raise peripheral lactate levels physiologically prior to a hypoglycaemic clamp, the same group also showed that lactate levels decreased to 30% below baseline levels during hypoglycaemia in people with type 1 diabetes with IAH compared with healthy individuals and people with type 1 diabetes with normal hypoglycaemia awareness, for whom brain lactate levels decreased to baseline levels [36]; this suggests increased lactate oxidation may contribute to IAH. This observation is consistent with our data showing enhanced VMH lactate uptake plays a major role in suppressing the counterregulatory response by enhancing GABAergic neurotransmission [34]. The increase in local lactate production is likely driven by repeated adrenergic activation as carvedilol prevented the rise and the development of IAH. Although the mechanism(s) through which noradrenaline enhances central lactate levels is not entirely clear, we speculate that if VMH noradrenergic neurotransmission remains intact following recurrent hypoglycaemia, then activation of VMH adrenergic receptors could potentially enhance glucose uptake [39] and stimulate the breakdown of glycogen into lactate [40-45] and the long-term re-synthesis of glycogen [46]. Importantly, as brain lactate levels remain chronically elevated and brain glycogen supplies are limited, an increase in glucose uptake may help replenish and sustain brain glycogen stores in recurrently hypoglycaemic animals. Whether these effects are mediated directly through β2ARs on astrocytes or indirectly through actions on other neurons [47] is less clear. These mechanisms need to be investigated in more detail.

Elevated brain lactate levels in recurrently hypoglycaemic animals were accompanied by increased *Mct2* mRNA expression, suggesting recurrent hypoglycaemia leads to adaptations that enhance the capacity to transport lactate into neurons. The increase in MCT expression is consistent with those observed in rodent models of ischaemia, in which brain lactate levels are also elevated [48]. It is notable that *Mct2* expression in recurrently hypoglycaemic animals was reduced with carvedilol treatment, coincident with the reduction in extracellular lactate levels.

Consistent with the animal data, studies in healthy humans showed that adrenergic blockade with propanolol during antecedent episodes of hypoglycaemia prevented suppression of the catecholamine responses [15]. Together, the rodent and human data support the idea that the development of counterregulatory failure and the potential loss of hypoglycaemia awareness stem from repeated activation of βARs. We therefore evaluated whether mild to moderate adrenergic blockade was a suitable therapeutic strategy to prevent the development of IAH in rodents. To address this question, we used repeated injections of 2DG to impair hypoglycaemia awareness in our animals. Hypoglycaemia triggers the onset of a number of different symptoms including tremulousness, increased heart rate and hunger. Hunger or food-seeking behaviour may be an objective and reliable indicator of hypoglycaemia awareness in rodents as it is both measurable and can be reliably triggered by hypoglycaemia. While hypoglycaemia awareness involves cognitive processes to recognise when one is low, that recognition should arguably translate into behavioural actions to correct hypoglycaemia (i.e. food seeking or eating). We therefore used food intake as a surrogate marker of hypoglycaemia awareness with the premise being that if the animal is aware that it is hypoglycaemic, it would consume more food compared with one that is less

aware. Notably, Sanders et al demonstrated that recurrent insulin-induced hypoglycaemia was not able to suppress the feeding response to subsequent bouts of hypoglycaemia in rats [27], whereas recurrent glucose deprivation using 2DG did [28]. We therefore used recurrent 2DG treatment to induce IAH in our rats and quantified food consumption in response to insulin-induced hypoglycaemia. While the 2DG stimulus is not entirely the same as insulin-induced hypoglycaemia, glucose deprivation at the cellular level is likely to be similar between the two conditions, despite the hyperglycaemia generally found in the 2DG model. We showed that hypoglycaemia significantly increased food intake in saline-treated animals, whereas 2DG treatment significantly reduced food intake in response to hypoglycaemia, suggesting 2DG animals were less aware of hypoglycaemia. Treatment with carvedilol prevented the reduction in food intake in 2DG animals, suggesting the animals were more hypoglycaemia aware. Our data are consistent with studies in humans conducted by Hirsch and colleagues which showed that total symptom scores improved in the presence of propanolol, although the improvement did not reach statistical significance [49]. Importantly, neuroglycopenic symptoms were not reduced by propranolol.

It is important to note that treatment of diabetic individuals with beta-blockers is controversial because of the perceived potential to attenuate hypoglycaemic symptoms. However, there is a lack of convincing clinical evidence to support this notion. In the recent GEMINI (Glycemic Effect in Diabetes Mellitus: Carvedilol–Metoprolol Comparison in Hypertensives) study, which evaluated the use of two different beta-blockers in individuals with type 2 diabetes and hypertension, carvedilol was shown to effectively reduce overall hypoglycaemia burden scores [50]. Likewise, the majority of clinical studies show that beta-blocker treatment of non-diabetic individuals and those with type 1 diabetes improves counterregulatory hormone responses without increasing the frequency of hypoglycaemia or impairing hypoglycaemia awareness [51-56], although loss of tremulousness and reduced tachycardia were reported in some studies. In a prospective study of 150 people with diabetes, 50 of whom were on beta-blockers, no difference in the incidence of hypoglycaemic unconsciousness was noted between those taking beta-blockers and those not, suggesting the use of beta-blockers in type 1 diabetes does not increase the incidence of severe hypoglycaemia [52] and may be a safe therapeutic option that can be used in conjunction with insulin.

In conclusion, our data suggest that partial adrenergic blockade with carvedilol during antecedent hypoglycaemia may be useful for preventing IAH and the development of counterregulatory failure. To our knowledge, this is the first study to investigate the use of carvedilol as a treatment to prevent IAH. Future studies will be directed towards evaluating this therapy for the treatment of HAAF in the setting of diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

2DG	2-Deoxyglucose
β2AR	β_2-Adrenergic receptor
GIR	Glucose infusion rate
HAAF	Hypoglycaemia-associated autonomic failure
IAH	Impaired awareness of hypoglycaemia
MCT	Monocarboxylic acid transporter
qRT-PCR	Quantitative RT-PCR
VMH	Ventromedial hypothalamus

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Research in context

What is already known about this subject?

- Recurrent hypoglycaemia does not affect the release of noradrenaline in the ventromedial hypothalamus, despite impairments in the counterregulatory hormone responses

- Increased lactate in the ventromedial hypothalamus may contribute to counterregulatory failure in recurrently hypoglycaemic animals

- Repeated adrenergic activation induced by hypoglycaemia progressively impairs the sympathoadrenal response to hypoglycaemia in healthy humans
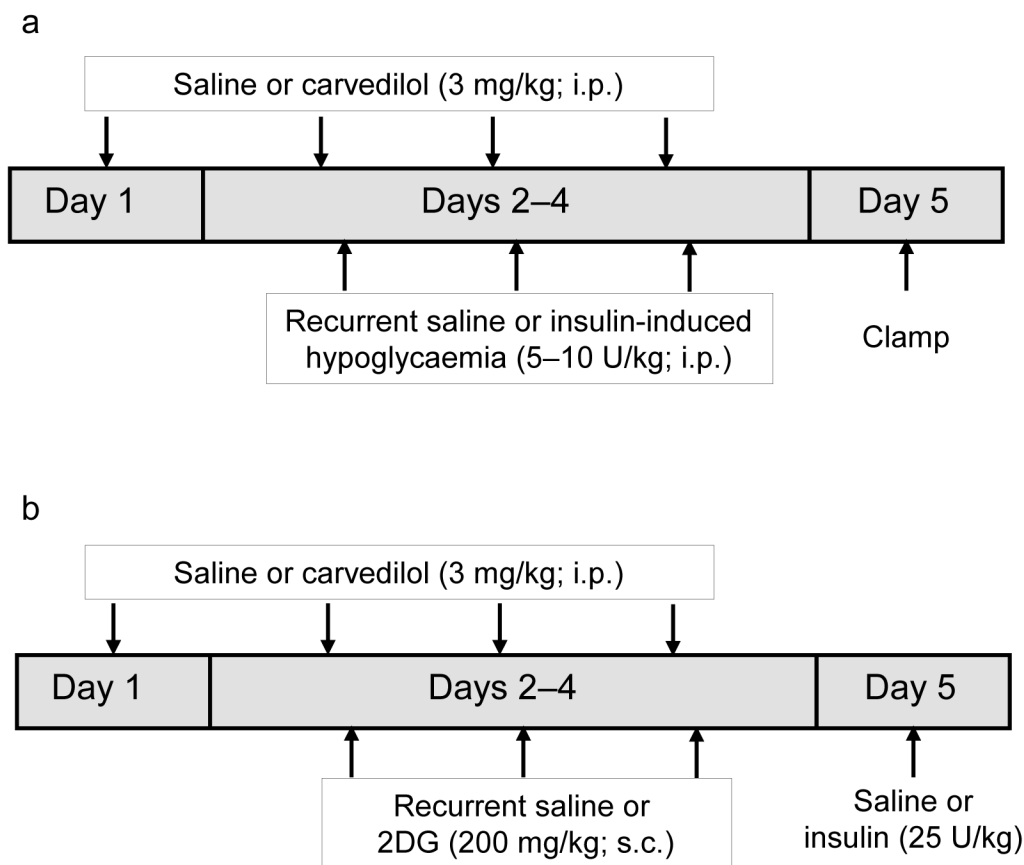
What is the key question?

- Can low doses of the β-blocker carvedilol be used to improve hypoglycaemia awareness or prevent the development of counterregulatory failure?

What are the new findings?

- Systemic treatment of Sprague Dawley rats with low doses of carvedilol reduces lactate levels in the ventromedial hypothalamus

- Low-dose carvedilol treatment prevents the development of counterregulatory failure in recurrently hypoglycaemic rats

- Low-dose carvedilol treatment improves hypoglycaemia awareness in rats

How might this impact on clinical practice in the foreseeable future?

- Low doses of carvedilol may be a promising clinical therapy to improve hypoglycaemia awareness in diabetic individuals with impaired awareness of hypoglycaemia

a

Saline or carvedilol (3 mg/kg; i.p.)

Day 1	Days 2–4	Day 5

Recurrent saline or insulin-induced
hypoglycaemia (5–10 U/kg; i.p.)

Clamp

b

Saline or carvedilol (3 mg/kg; i.p.)

Day 1	Days 2–4	Day 5

Recurrent saline or
2DG (200 mg/kg; s.c.)

Saline or
insulin (25 U/kg)

Fig. 1.
Study design. (**a**) Schematic diagram showing carvedilol and recurrent saline or insulin treatment during days 1–4. All animals underwent a hypoglycaemic clamp on day 5. (**b**) Schematic diagram showing the 2DG treatment regimen. Carvedilol and 2DG were administered from day 1 to 4. On day 5, insulin was given to induce hypoglycaemia and total food consumption (g) in response to the hypoglycaemic challenge was measured over 4 h
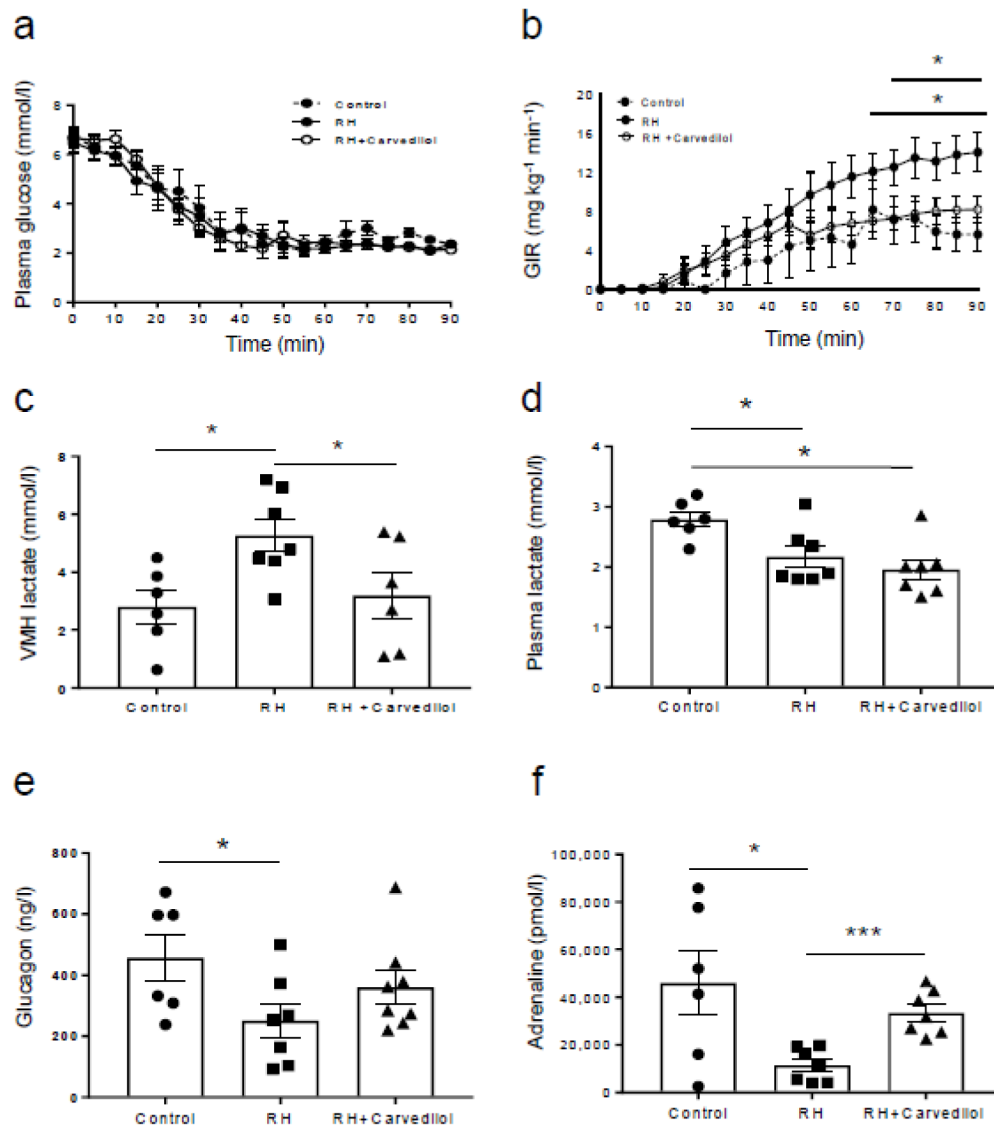
Fig. 2.

Evaluation of VMH lactate and the counterregulatory hormone responses. Plasma glucose (**a**) and GIR (**b**) during the hypoglycaemic clamp; control group (n=6); recurrently hypoglycaemic (RH) rats (n=7); carvedilol-treated RH rats (n=7). GIRs at the end of the clamp were significantly higher in the RH group compared with the control group (*p<0.05, control vs RH, for time points shown below the bar). Carvedilol treatment reduced the GIR of RH animals to normal ($^{†}p$<0.05, RH vs RH + carvedilol, for time points shown below the bar). (**c**) Basal extracellular (VMH) lactate levels were 66% elevated in the RH rats (n=7; *p<0.05 vs control) compared with the controls (n=6) and these levels were reduced to normal with carvedilol treatment (n=6; *p<0.05 vs RH). (**d**) Baseline plasma lactate concentrations were lower in RH animals (n=7) compared with controls (n=6; *p<0.05, control vs RH and RH + carvedilol). No differences were observed between carvedilol-treated RH animals (n=8) and the RH group. (**e**) In comparison with controls (n=6), peak plasma glucagon levels were reduced by 42% in the RH (n=7) group (*p<0.05 vs control).

(**f**) Peak adrenaline concentrations during the hypoglycaemic clamp were significantly suppressed by 75% in the RH (n=7) group compared with controls (n=6) (*p<0.05 vs control). Treatment with carvedilol (n=7) restored these hormone responses to normal (***p<0.001 vs RH animals). Plasma glucose and GIR data were analysed using two-way repeated measures ANOVA and microdialysate and hormone data were analysed using one-way ANOVA with post hoc Tukey honest significant difference (HSD) test. Data are presented as mean ± SEM. RH, recurrently hypoglycaemic
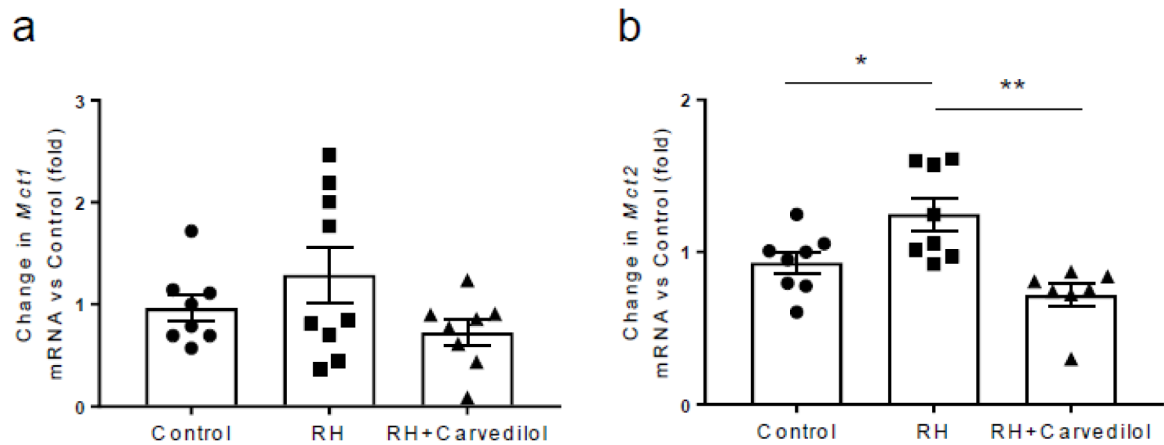
Fig. 3.
Mct1 and *Mct2* mRNA levels in the VMH. (**a**) In comparison with the control group (*n*=8), *Mct1* mRNA level in the VMH was not significantly altered by recurrent hypoglycaemia (*n*=9). Treatment of recurrently hypoglycaemic (RH) rats with carvedilol (*n*=8) reduced the *Mct1* mRNA levels slightly, although the difference just failed to reach statistical significance (*p*=0.09 vs RH). (**b**) *Mct2* mRNA expression in the VMH was significantly upregulated in RH animals (*n*=8) compared with controls (*n*=8) (**p*<0.05 vs control); treatment with carvedilol (*n*=7) restored these levels to normal (***p*<0.01 vs RH). Data were analysed using one-way ANOVA with post hoc Tukey honest significant difference (HSD) test and presented as mean ± SEM. RH, recurrently hypoglycaemic
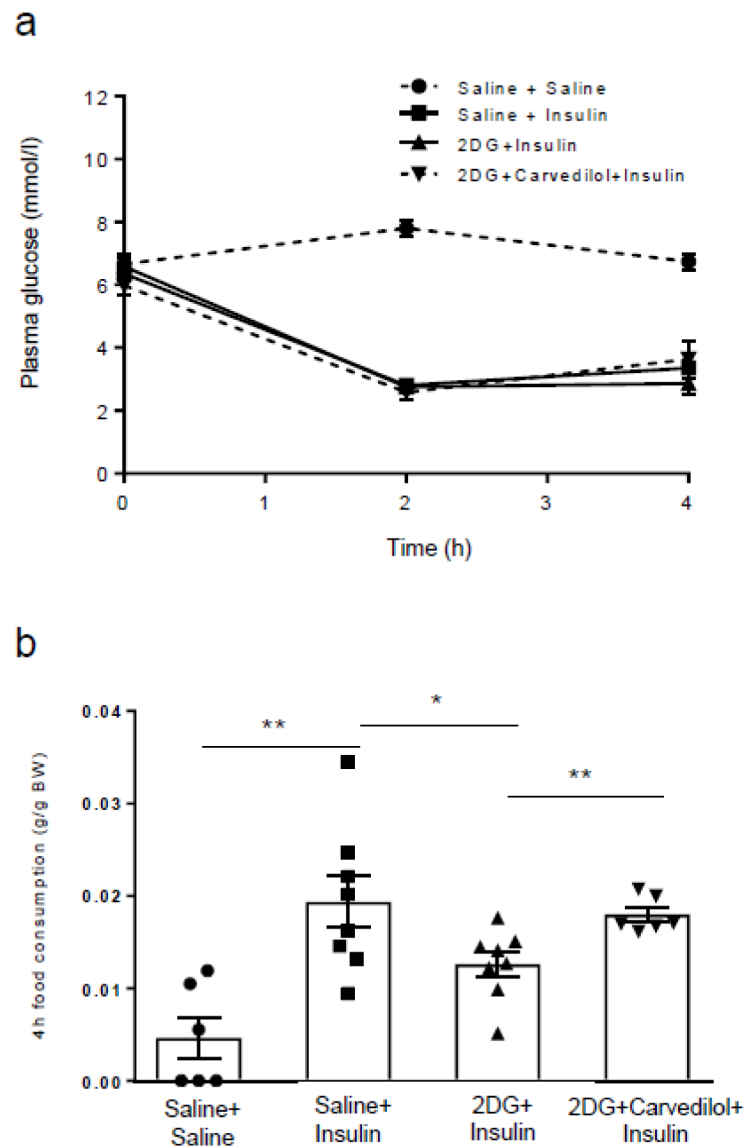
Fig. 4.
Evaluation of hypoglycaemia awareness. (**a**) Plasma glucose levels during the induction of hypoglycaemia on day 5. (**b**) Hypoglycaemia-naive rats made hypoglycaemic (saline + insulin; n=8) on day 4 consumed more than four times more food than hypoglycaemia-naive rats given a saline injection (saline + saline; n=6) (**$p<0.01$, saline + insulin vs saline + saline). Rats treated with 2DG (2DG + insulin; n=8) ate 35% less food when hypoglycaemic compared with the saline + insulin group (*$p<0.05$, 2DG + insulin vs saline + insulin). In contrast, when the 2DG animals were treated with carvedilol (n=6), the animals consumed as much food as the insulin group (**$p<0.01$, 2DG + insulin vs 2DG + carvedilol + insulin). Data were analysed using one-way ANOVA with post hoc Tukey honest significant difference (HSD) test and presented as mean ± SEM. BW, body weight
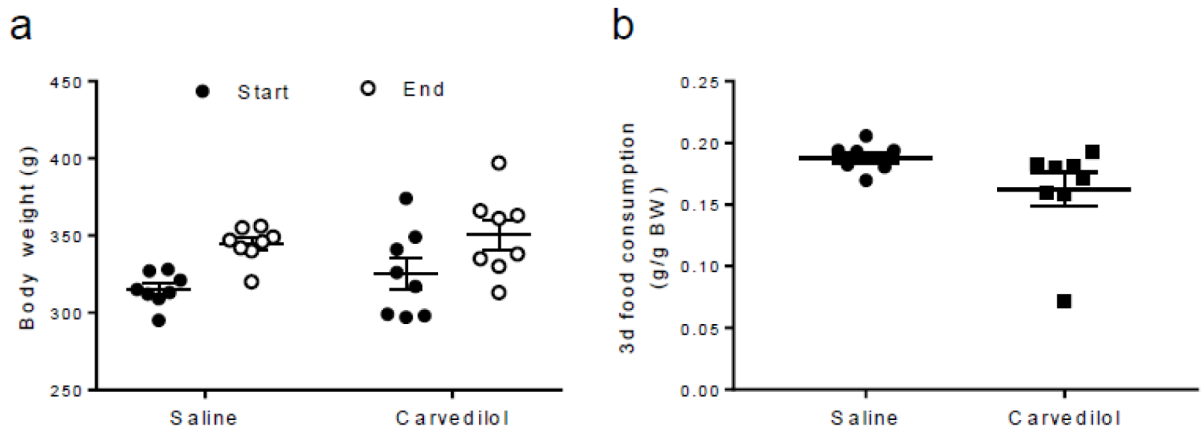
Fig. 5.
Carvedilol treatment did not affect body weight or food consumption. Treatment with low doses of carvedilol over the course of 4 days did not increase (**a**) body weight gain from the start of treatment ($n=8$) until the end of the treatment ($n=8$; p=NS, saline vs carvedilol) and nor did it affect (**b**) total food consumption between saline ($n=8$) and carvedilol-treated animals ($n=8$) over the course of the treatment (p=NS, saline vs carvedilol). Unpaired Student's t tests were used for these comparisons. Data are presented as mean ± SEM. BW, body weight

Table 1

Baseline hormone concentrations in groups of rats

Variable	Control (n=6)	Recurrently hypoglycaemic (n=7)	Recurrently hypoglycaemic + carvedilol (n=7)
Body weight (g)	315.8±9.4	294.1±19.1	323.4±5.0
Plasma glucose (mmol/l)	6.7±0.4	6.4±0.4	6.7±0.4
Glucagon (ng/l)	67.4±15.0	49.8±11.0	73.2±14.4
Adrenaline (pmol/l)	746.1±151.5	922.1±406.6	923.8±257.2

Data reported as mean ± SEM. No significant differences in baseline hormone levels were noted between treatment groups