Leptin Administration to Normal Rats Does Not Alter Catecholamine Responsiveness to Insulin-Induced Hypoglycemia

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We previously showed, through direct neural recording in conscious rats, that hypoglycemia increases adrenal sympathetic nerve activity (SNA) both acutely and 24 hours following the second of 2 daily antecedent hypoglycemic episodes. Nonetheless, antecedent hypoglycemia impaired catecholamine responsiveness to subsequent acute hypoglycemia. Here we hypothesized that antecedent, nonhypoglycemic adrenal sympathetic stimulation by leptin would impair acute adrenal catecholamine responsiveness to subsequent hypoglycemia. We also hypothesized that acute leptin administration (after 2 days of antecedent hypoglycemia) would enhance adrenal SNA and thereby enhance catecholamine responsiveness to concurrent hypoglycemia. Leptin or saline was administered to normal rats in repeated subcutaneous injections for 2 days prior to acute insulin-induced hypoglycemia. In contrast to our hypothesis, antecedent leptin did not change catecholamine responsiveness or glycemic change in response to subsequent acute insulin administration. In additional studies, intravenous leptin or saline was acutely administered beginning 1 hour before insulin-induced hypoglycemia. All rats had been exposed to antecedent hypoglycemia. In these experiments, acute leptin did not alter catecholamine responses to insulin or glycemic change during or after termination of insulin. We conclude that antecedent nonhypoglycemic sympathetic stimulation by leptin does not alter subsequent catecholamine or glycemic responses to insulin. Moreover, concurrent leptin does not enhance catecholamine responses to insulin in rats exposed to antecedent hypoglycemia.

IMPAIRED HYPOGLYCEMIC counterregulation and hypoglycemia unawareness are common in longstanding type 1 diabetes.[1,2] Moreover, even in recent onset diabetes or in the absences of diabetes, prior episodes of antecedent hypoglycemia impair recognition of and catecholamine responses to subsequent hypoglycemia, a syndrome termed hypoglycemia-associated autonomic failure (HAAF). If glucagon responses are also impaired, this will also result in impaired glycemic counterregulation.[3] The pathophysiology of HAAF is still unclear and effective means of treatment, other than scrupulous avoidance of hypoglycemia, are lacking.

Autonomic input plays a major role in regulating glucose metabolism and responsiveness to hypoglycemia. Neural signals in response to changes in glucose concentration appear to derive from the ventromedial and lateral hypothalamic areas.[4] Given these considerations it is plausible that centrally acting neuropeptides might modify hypothalamic responsiveness to hypoglycemia and could potentially be used to improve the impaired recovery from acute hypoglycemia following antecedent hypoglycemic events.

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We recently found, through direct adrenal nerve recording in normal conscious rats, that adrenal sympathetic nerve activity (SNA) is acutely increased by insulin-induced hypoglycemia.[5] Further, increased basal adrenal SNA was also noted 24 hours after the second of 2 daily antecedent episodes of insulin-induced hypoglycemia. But, in spite of this increase in adrenal SNA, plasma epinephrine in response to subsequent hypoglycemia was reduced.[5] Older animal studies show that insulin-induced hypoglycemia is, in fact, associated with rapid adrenal catecholamine depletion lasting at least 24 hours[6,7] and may take as long as 4 days to recover.[7] Moreover, after unilateral adrenal denervation, depletion of catecholamines was localized to the innervated gland.[6,7] Thus, prior adrenal sympathetic neural input secondary to hypoglycemia may eventually decrease the capacity of the adrenal for acute catecholamine release. If this is the case, then it is possible that antecedent nonhypoglycemic sympathetic stimulation may have similar effects to reduce subsequent catecholamine release and glycemic responses to insulin. In fact, hypotension and cold exposure enhanced the activity of tyrosine hydroxylase, the rate-limiting enzyme for catecholamine biosynthesis.[8] Tyrosine hydroxylase is upregulated under conditions of catecholamine depletion, suggesting that the prior sympathetic stress resulted in depleted adrenal catecholamine content.[9]

We previously showed that intravenous administration of the centrally acting adipose hormone leptin increases adrenal sympathetic nerve traffic within 3 hours.[10] Thus, we hypothesized that antecedent nonhypoglycemic sympathetic activation by leptin would alter subsequent catecholamine and glycemic responses to insulin. We also hypothesized that acute leptin administration would modify the catecholamine and glycemic responses to concurrent insulin.

Here we examined the effect of 2 days of repeated antecedent subcutaneous leptin administration on adrenal SNA, plasma catecholamine concentration, and glycemic change in response

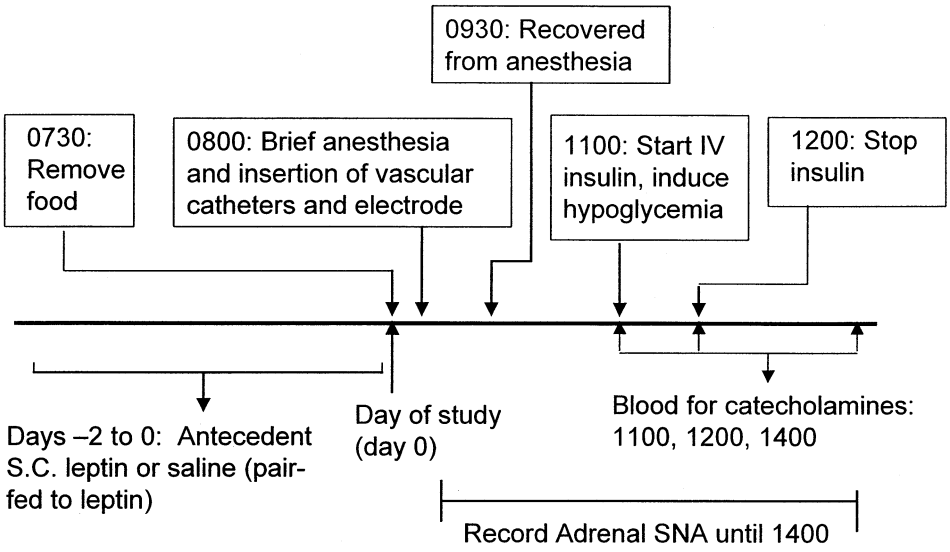

Fig 1. Schematic diagram depicting protocol followed by group I rats.

to subsequent insulin administration to conscious rats. We also examined the effect of acute leptin administration on adrenal SNA and catecholamine responses to hypoglycemia in conscious rats that had been exposed to prior hypoglycemia.

MATERIALS AND METHODS

Materials

Recombinant mouse leptin was kindly provided by Amgen, Inc, Thousands Oaks, CA. Other reagents and supplies were obtained as indicated or purchased from standard sources.

Animal Experiments

Rats were purchased from Harlan Sprague-Dawley, Indianapolis, IN. Animals were fed and maintained according to standard National Institutes of Health (NIH) guidelines and the protocol was approved by the University of Iowa Animal Care Committee. Room temperature was maintained at 25°C. Groups of animals were designated group I and group II. Male rats were used in all experiments.

Group I Animals (effect of antecedent leptin or vehicle on subsequent response to acute hypoglycemia)

Animal studies were performed as depicted in Fig 1. Rats were exposed to antecedent leptin (n = 11) or vehicle (phosphate-buffered saline [PBS]) (n = 12). Rats received subcutaneous injections of leptin (200 μg/injection) in 100 μL volumes at 9 AM and 7 PM on days −1 and −2 or an equivalent volume of vehicle (PBS). Animals exposed to antecedent leptin or saline were studied over the same time period on alternating days. Pair-feeding was accomplished by feeding saline-treated rats an equivalent weight of rat chow consumed by the leptin-treated rats determined on the preceding day.

Rats were transiently anesthetized at 8 AM on day 0 with intraperitoneal methohexital sodium (50 mg/kg). Additional methohexital sodium was administered intravenously every 10 minutes to sustain the level of anesthesia until 30 minutes after the surgical procedure was completed. Catheters were placed in the carotid artery and jugular vein. A nerve branch to the left adrenal nerve was exposed through a flank incision and the platinum-iridium electrode attached. When an adequate recording was obtained the electrode was fixed in place using silicon gel (Kwikcast, World Precision Instruments, Sarasota, FL) and the wound closed with 4.0 silk and skin glued shut with Vetbond tissue adhesive (3M, St Paul, MN). The vascular catheters and electrode wire were tunneled to exit the skin at the nape of the neck and protected by a spring tether connected to a swivel mount apparatus (Instech Laboratories, Plymouth Meeting, PA) at the top of the cage. The rats were loosely restrained allowing movement but not 180 degree rotation in order to protect the electrode lead. The procedure itself required approximately 20 minutes and recovery from anesthesia occurred over approximately 60 minutes. Adrenal SNA was continuously recorded until 2 PM.

At 11 AM, all rats received an intravenous bolus injection of 0.75 U human regular insulin (Lilly, Inc, Indianapolis, IN) followed by continuous intravenous infusion at 1.8 U/kg/h for 60 minutes. Glucose was measured using a Yellow Springs Instruments (YSI) glucose analyzer (Yellow Springs, OH) on a drop of whole blood obtained from the carotid artery at 5-minute intervals until noon. The protocol called for 25% glucose administration if the concentration decreased to 25 mg/100 mL or less; however, this degree of drop in glucose did not occur in any of the animals. The insulin infusion was terminated at noon and the blood glucose measured at 10-minute intervals for the next 120 minutes. A 1.0-mL blood sample was obtained for catecholamines from the carotid arterial line at 11 AM, noon, and 2 PM (0, 60, and 180 minutes relative to the start of the insulin infusion). Volume was replaced by infusing 1.0 mL of saline.

Group II Animals (effect of acute leptin or saline on responses to intravenous insulin in rats exposed to antecedent hypoglycemia)

Animal studies were performed as depicted in Fig 2. Rats were exposed to antecedent insulin-induced hypoglycemia on days −2 and −1 followed by subsequent acute hypoglycemia on day 0. At 1 PM, on days −2 and −1, all animals received subcutaneous injections of 1.5 U human regular insulin (Humulin, Eli Lily, Inc) and blood was sampled by tail vein puncture after 150 minutes for measurement of glucose by YSI analyzer. Vascular catheters and placement of the adrenal nerve electrode were carried out beginning at 8 AM on day 0 as described for the group I animals. Adrenal SNA was continuously recorded until 3 PM.

On day 0, rats received intravenous leptin, 250 μg/kg (n = 10), or an equivalent volume of saline (n = 9) as an initial bolus at 11 AM

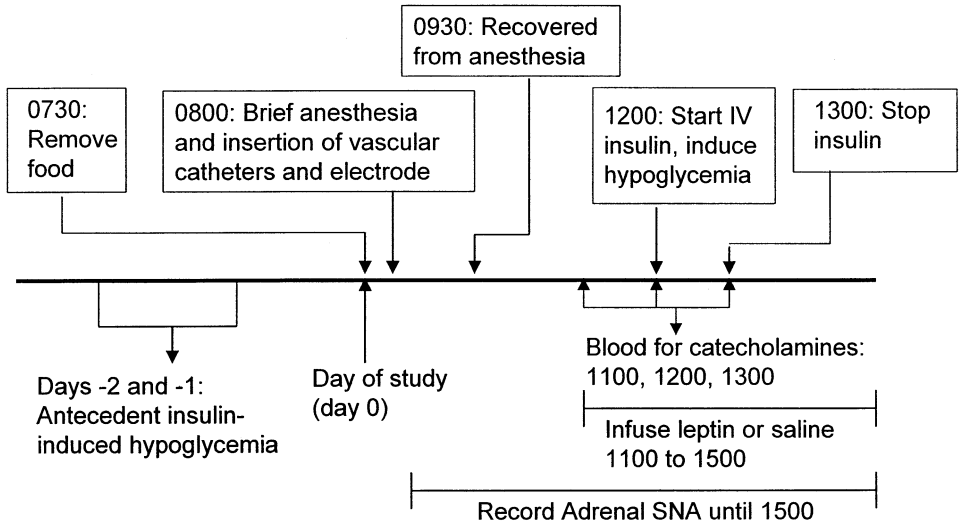

Fig 2. Schematic diagram depicting protocol followed by group II rats.

followed by 62.5 μg/kg/h of leptin or saline from 11 AM to 3 PM. Intravenous insulin was administered to all animals from noon to 1 PM as described for group I rats. Glucose was measured using the YSI analyzer at 15-minute intervals from 11 AM to noon, every 5 minutes from noon to 1 PM (time of insulin infusion), and every 10 minutes from 1 PM to 3 PM. Twenty-five percent glucose was administered during the insulin infusion to prevent the blood glucose from dropping below 35 mg/100 mL and was necessary in 2 of the leptin-treated and 2 of the saline-treated rats. A 1.0-mL arterial blood sample was obtained for determination of catecholamines at 11 AM, noon, and 1 PM (0, 60, and 120 minutes relative to the start of the leptin infusion).

In these studies, rats exposed to acute leptin or saline were studied over the same time period on alternating days.

Sympathetic Nerve Recordings

Sympathetic activity innervating the adrenal nerve was measured by multifiber recording as we have previously described.[10] SNA was recorded at 5-minute intervals as average activity over the preceding 1 minute. Using a dissecting microscope, a nerve branch to the left adrenal was carefully dissected and the bipolar electrode placed. After an optimum recording of SNA was obtained, the electrode was fixed in place using silicon gel. The electrode was connected to a high impedance probe (HIP-511, Grass Instruments, Washington, DC), amplified by 10^5, and filtered at low- and high-frequency cutoffs of 100 and 1,000 Hz with a nerve traffic analysis system (model 662-C, University of Iowa Bioengineering, Iowa City, IA). The filtered, amplified nerve signal was routed: (1) to an oscilloscope (model 54501A, Hewlett-Packard, Palo Alto, CA) for monitoring; (2) to a MacLab analogue-digital converter (CB Sciences, Milford, MA) for permanent recording of the neurogram on a Macintosh 9500 computer (Apple, Cupertino, CA); and (3) to a nerve traffic analyzer (model 706C, University of Iowa Bioengineering) that counts action potentials above a threshold voltage level set just above background (determined postmortem). To document that the nerve recordings represent sympathetic nerve impulses, ganglionic blockade was induced with chlorisondamine, 5 mg/kg intravenously at the end of each experiment. This reduced nerve activity to low-grade background "noise," which is subtracted from the recorded measurements. Also, there is a characteristic burst activity pattern seen as a result of sympathetic outflow, which, although subjective, provides a measure of confirmation. Further, in past studies[10] and in pilot experiments in rats under anesthesia, we transected adrenal nerves distal to the recording site (electrode). In 6 separate determinations, the neurograms were not altered documenting the efferent rather than afferent origin of the neural signals.

Blood Pressure and Heart Rate Determinations

Blood pressure and heart rate were continuously monitored along with adrenal SNA in all studies. This was accomplished using a pressure transducer (Gould Stathan P23ID, Oxnard, CA) attached to the carotid arterial line and the data acquired by computer through the MacLab analogue-digital converter. Blood pressure and heart rate were recorded every 5 minutes as average values over 1-minute intervals.

Catecholamine Determination

Plasma epinephrine and norepinephrine concentrations were determined by high-performance liquid chromatography with electrochemical detection.[11] The assay has an interassay and intra-assay coefficient of variation of 3.4% and 3.1%, respectively, and a lower limit of detection of 25 pg/mL.

Statistics

Data were analyzed by *t* test or analysis of variance (ANOVA) as indicated.

RESULTS

Antecedent Leptin Administration Does Not Alter Adrenal Sympathoexcitation or Catecholamine Responsiveness to Subsequent Insulin-Induced Hypoglycemia

These studies were performed in group I rats (Fig 1). Animals were treated with leptin or saline (pair-fed to leptin) by repeated subcutaneous injection for 2 days prior to insulin-induced hypoglycemia. Rats exposed to antecedent leptin lost significantly more weight over the 2-day period of subcutaneous leptin injections compared to pair-fed, saline-treated controls (Table 1).

As shown in Fig 3, 2 days of repeated subcutaneous leptin injections, compared to saline, had no significant effect on plasma epinephrine or norepinephrine concentrations when determined either prior to or in response to insulin-induced hy-

Table 1. Animal Characteristics by Group (mean ± SEM)

	Leptin	Saline	*P*
Group I	Antecedent	Antecedent	
Weight, day 0 (g)	415 ± 6	417 ± 5	NS
n	11	12	
Weight change, day −2 to day 0 (g)	−6.0 ± 1.3	0.0 ± 1.9	<.05
Group II	Concurrent	Concurrent	
Weight, day 0 (g)	434 ± 6	438 ± 13	NS
n	10	9	
Weight change, day −2 to day 0 (g)	10.9 ± 1.0	11.7 ± 1.6	NS
Glucose (mg/100 mL) after antecedent treatment (day −2)*	27 ± 3	22 ± 1	NS
Glucose (mg/100 mL) after antecedent treatment (day −1)*	29 ± 4	23 ± 2	NS

*Plasma glucose concentration determined 150 minutes after antecedent subcutaneous insulin; *P* values compare leptin *v* saline by 2-tailed, unpaired *t* test.

poglycemia. Prior leptin also did not alter adrenal SNA measured at baseline or in response to insulin-induced hypoglycemia (Fig 3). In addition, antecedent leptin did not alter the glucose response to intravenous insulin or glycemic recovery after insulin-induced hypoglycemia (Fig 3) and had no effect on mean arterial pressure or heart rate before, during, or after hypoglycemia (not shown).

Acute Leptin Administration Does Not Alter Catecholamine Responsiveness to Hypoglycemia After Antecedent Hypoglycemia

Normal rats (group II) were subject to 2 episodes of insulin-induced hypoglycemia on days −2 and −1 prior to acute hypoglycemia on day 0 (Fig 2). These rats gained weight over this 2-day period (Table 1). The mean blood glucose at 150 minutes after subcutaneous insulin on day −2 was 27 ± 3 mg/100 mL for rats subsequently exposed to acute leptin and 22 ± 1 for rats subsequently exposed to saline (*P* value not significant [NS]). The corresponding values on day −1 were 29 ± 4 and 23 ± 2 (*P* value NS).

To determine whether acute leptin altered catecholamine responsiveness to insulin-induced hypoglycemia or glycemia during and after insulin, leptin (or an equivalent volume of saline) was administered beginning 1 hour before insulin and continued for 4 hours (Fig 4). Treatment with leptin, compared to saline, significantly increased adrenal SNA as determined over the first hour of administration (time prior to initiation of insulin). The mean area under the curve for adrenal SNA over time 45 to 60 minutes was 3,954 ± 320 for leptin compared to 2,752 ± 497 for saline ($P < .05$ by unpaired, 2-tailed *t* test). Analysis of effects of time and treatment over the period 0 to 60 minutes by 2-factor ANOVA (repeated measures for time) revealed significant interaction with the treatment effect dependent on time ($P < .001$). However, acute leptin, compared to saline, had no significant effect on plasma epinephrine or norepinephrine responses to insulin-induced hypoglycemia (Fig 4). Acute leptin also had no effect upon the magnitude of

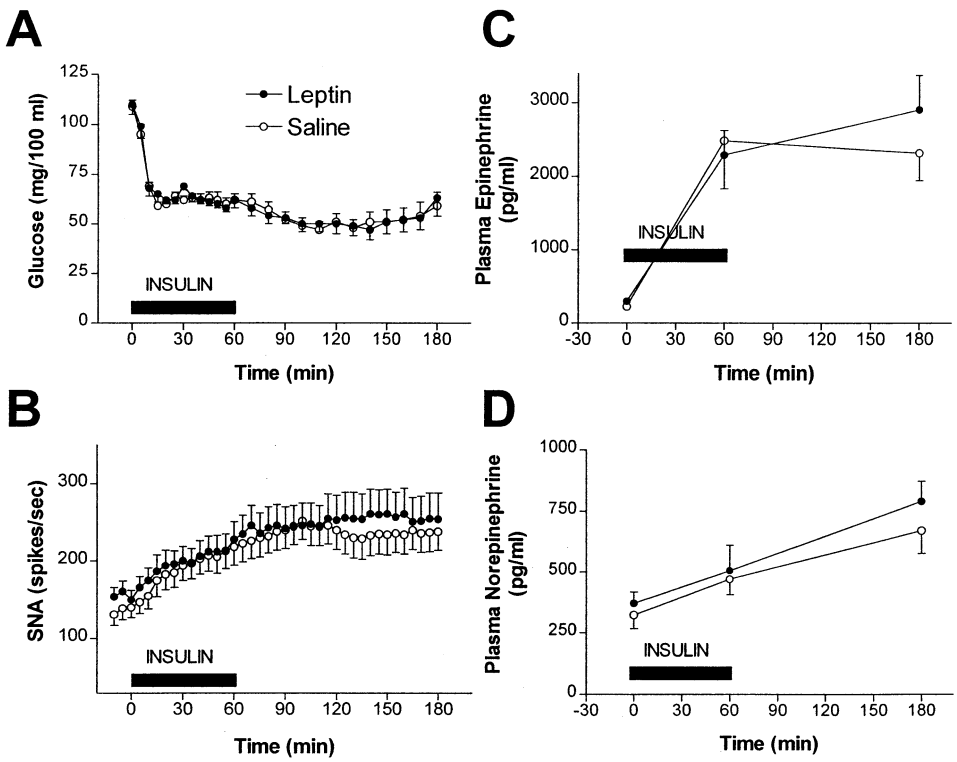

Fig 3. Effect of antecedent (days −2 and −1) leptin (n = 11) compared to vehicle (n = 12) on subsequent (day 0) responses to intravenous insulin. (A) Glucose, (B) adrenal SNA, (C) epinephrine, (D) norepinephrine.

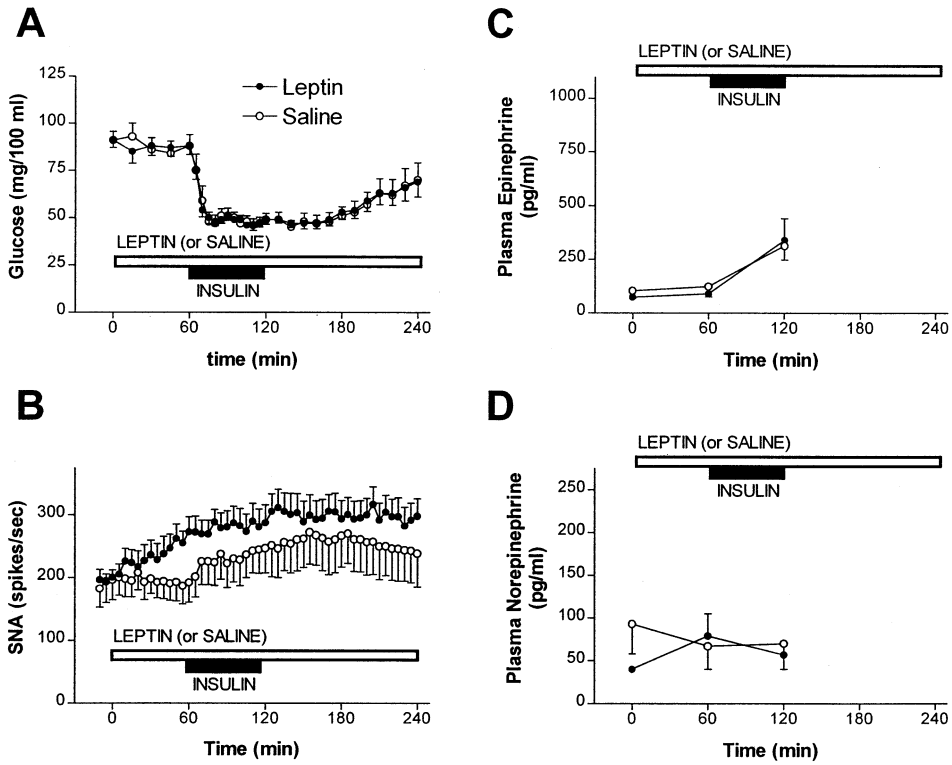

Fig 4. Effect of acute (day 0) leptin (n = 10) compared to vehicle (n = 9) on responses to intravenous insulin. (A) Glucose, (B) adrenal SNA, (C) epinephrine, (D) norepinephrine. All rats received subcutaneous injections of insulin on days −2 and −1 to induce antecedent episodes of insulin-induced hypoglycemia.

the glycemic drop after insulin or upon glycemic recovery (Fig 4). Finally, acute leptin compared to saline did not alter blood pressure or heart rate (data not shown).

DISCUSSION

Sympathetic stress in the form of antecedent hypoglycemia is well known to impair counterregulatory responses to subsequent hypoglycemia.[3] However, it is less clear whether antecedent nonhypoglycemic sympathetic stimuli have similar consequences. Our current data show that this is not necessarily the case. In the past, we showed that leptin activates sympathetic neural activity in peripheral nerves to multiple tissues including the adrenal.[10] Our current results show that 2 days of antecedent leptin treatment had no effect on catecholamine responses to hypoglycemia, glycemic change after insulin, or glycemic recovery upon termination of insulin. Although these effects were negative, the administered leptin did appear bioeffective. First, the group I rats treated with leptin lost weight even though they were of an age and size where weight gain would be expected. Second, the weight change in the leptin-treated group I rats was significantly different in comparison to the control pair-fed rats (Table 1), an effect reported in the past for leptin-treated rodents compared to pair-fed controls.[12]

There is evidence from human studies that antecedent sympathetic activation in the form of exercise may affect glycemic responses to subsequent hypoglycemia. Galassetti et al[13] showed that exercise decreases muscle SNA, catecholamine release, and endogenous glucose production in response to subsequent hypoglycemia. In another report, antecedent exercise in humans had a significant, but small effect, on subsequent hypoglycemia triggered epinephrine release.[14] Adrenal SNA could not be determined in these human studies. In contrast to the above, our data suggest that antecedent sympathetic stimulation by leptin, as opposed to insulin-induced hypoglycemia or exercise, does not alter glycemic or catecholamine responses to subsequent insulin-induced hypoglycemia. Hence, impaired catecholamine responsiveness to hypoglycemia after antecedent sympathetic activation is not a phenomenon that can be generalized to all sympathetic stimuli.

It has been suggested that the effect of antecedent exercise, like antecedent hypoglycemia, to impair subsequent catecholamine release to hypoglycemia might be dependent on cortisol secretion.[13] Our studies are consistent with this concept since leptin, unlike exercise or hypoglycemia, is not itself a stimulus to cortisol release. In fact, glucocorticoids at least in pharmacological doses enhance leptin release in humans[15] and directly increase leptin release from human fat cells in vitro,[16] whereas leptin appears to have direct action to reduce cortisol release from adrenocortical cells.[17]

We previously observed that, as in human studies,[18] catecholamine responses to hypoglycemia are impaired in normal rats after antecedent episodes of hypoglycemia.[5] Also, we previously found that intravenous leptin administration activates adrenal sympathetic neural activity by 3 hours.[10] Thus, we investigated the effect of acute leptin administration (compared to vehicle) on catecholamine and glycemic responses to intravenous insulin. We performed these studies in a group of rats that had been exposed, in like fashion to our past studies,[5]

to 2 prior episodes of antecedent hypoglycemia. Our data show that although acute leptin, compared to saline, increased adrenal SNA over the first hour of administration, leptin did not increase catecholamine responses to hypoglycemia or alter glycemic responses to insulin (Fig 4). In addition, leptin did not improve glycemic recovery from insulin-induced hypoglycemia (Fig 4).

In these experiments (Fig 4), antecedent (days −1 and −2) hypoglycemia was documented by tail vein puncture at 150 minutes after subcutaneous insulin (Table 1). Although a crude assessment, the animals clearly developed substantial hypoglycemia and the glucose values at 150 minutes post-insulin did not differ significantly between groups. Although the blood glucose was slightly lower (NS) in the subsequent saline-treated rats (*v* leptin), it is highly unlikely that this difference could account for the lack of effect of leptin to improve catecholamine recovery post-insulin (day 0). First, all rats experienced substantial hypoglycemia induced in the same way and any difference, by crude assessment, was small. Second, even if the saline-treated rats did have greater antecedent hypoglycemia than the leptin-treated rats, the expected effect of this, if any, would be to further impair catecholamine responsiveness to subsequent hypoglycemia. So the conclusion that the leptin-treated rats did no better in regard to catecholamine release (in spite of less severe antecedent glycemia) would still be valid.

With regard to glycemic recovery, we point out a limitation to the data. In past studies, we noted that, although epinephrine release was impaired in normal rats after antecedent insulin-induced hypoglycemia, glycemic recovery was not different.[5] This is most likely due to preservation of other glycemic counterregulatory responses (beyond epinephrine). In particular, glucagon release comes into play very early in the hierarchy of hypoglycemic counterregulatory mechanisms.[18] As opposed to normal rodents, human subjects with type 1 diabetes lose glucagon release early in the course of their diabetes and thus are susceptible to impaired glycemic recovery as well as catecholamine release after antecedent hypoglycemia.

Although we are reluctant to compare data between groups I and II, it appears evident that epinephrine responses to insulin-induced hypoglycemia were blunted in the group II rats compared to group I (compare Figs 3 and 4). This is as expected based on human studies[18] and our past studies in rodents.[5] Basal catecholamine and basal glucose concentrations on day 0 also appeared slightly lower in the group II versus group I rats. Although, this might reflect adrenal catecholamine depletion due to prior hypoglycemia, a phenomena reported in the past,[6,7] we cannot conclude that this is the case. First, adrenal catecholamine content may not reflect plasma concentrations. Second, we did not design these studies to compare groups I and II. Within groups (I and II), the rats were well matched for initial weight, studied on alternate days (one intervention *v* the other), studied at the same time of year and purchased at the same time. However, none of these conditions held between groups I and II and variations in these factors could impact circulating catecholamine and glucose concentrations. Finally, we did not see differences in basal catecholamine concentrations or glucose in past studies when rats with antecedent hypoglycemia were directly compared with those exposed to sham hypoglycemia.[5]

As shown in Fig 4, intravenous leptin, compared to saline, had no effect on plasma catecholamines measured 1 hour after initiation of infusion or at 2 hours (1 hour after insulin). This is somewhat in contrast to the findings of Satoh et al,[19] who observed an increase in epinephrine and norepinephrine within one-half to 3 hours of an intravenous injection of a large (0.25 to 1.0 mg per rat) bolus of leptin. Although, the difference may be that we studied rats exposed to prior hypoglycemia, there is also a matter of the leptin dosage. We administered an intravenous bolus of 0.25 mg/kg followed by 0.062 mg/kg/hr. In fact, at the more comparable lower dose administered by Satoh et al, there was only a minimal increase in plasma epinephrine. It is interesting that although leptin did not increase plasma catecholamines, SNA did increase (Fig 4). We can only speculate as to the reason for this. One possibility would be the prior hypoglycemia. Clearly, antecedent hypoglycemia blunts catecholamine release to subsequent insulin so it may have blocked or blunted (to a level we could not detect) leptin-induced catecholamine release. Possibly, adrenal catecholamine depletion secondary to antecedent hypoglycemia (see preceding paragraph) played a role. Further studies will be needed to examine this phenomena.

As evident in Figs 3 and 4, insulin-induced hypoglycemia appears to increase epinephrine preferentially to norepinephrine in the rat model studied. This differs from human studies, wherein hypoglycemia enhances concentrations of both catecholamines,[20] but is in accord with our past results.[5] Moreover hypoglycemia, at least in the rat, may preferentially stimulate adrenomedullary cells producing epinephrine compared to norepinephrine.[21] In addition, it is important to note that, although bioeffective plasma epinephrine derives from adrenomedullary chromaffin cells,[22] norepinephrine is also released by peripheral post-ganglionic sympathetic neurons. Hence, plasma norepinephrine reflects the balance between release and reuptake within multiple tissues and signifies more generalized sympathetic activation of sympathetic nerve terminals within multiple tissues.

In summary, antecedent, nonhypoglycemic sympathetic stimulation by leptin did not alter subsequent plasma catecholamine or glycemic responses to acute intravenous insulin. Thus, different antecedent sympathetic stimuli (leptin or hypoglycemia) do not necessarily have similar consequences towards subsequent counterregulation to acute hypoglycemia. Moreover, acute administration of leptin beginning 1 hour prior to and continuing concurrently with insulin did not alter catecholamine release in rats exposed to antecedent hypoglycemia.

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