

Title: Mechanisms of Hypoglycemia Associated Autonomic Failure

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SPECIFIC AIMS:

Intensive glucose control in type 1 diabetes mellitus (T1DM) is associated with clear health benefits (1). However, despite development of insulin analogs, pump/multi-dose treatment and continuous glucose monitoring, maintaining near-normal glycemia remains an elusive goal for most patients, in large part owing to the risk of hypoglycemia. T1DM patients are susceptible to hypoglycemia due to defective counterregulatory responses (CR) characterized by: 1) deficient glucagon release during impending/early hypoglycemia; 2) additional hypoglycemia-associated autonomic failure (HAAF) and exercise-associated autonomic failure (EAAF) that blunt the sympathoadrenal responses to hypoglycemia following repeated episodes of hypoglycemia or exercise as well as degrading other CR; and 3) hypoglycemia unawareness (HU), lowering the threshold for symptoms that trigger behavioral responses (e.g. eating). Thus, the risk of hypoglycemia in T1DM impedes ideal insulin treatment and leads to defaulting to suboptimal glycemic control (2). There are two approaches that could resolve this important clinical problem: 1) perfection of glucose sensing and insulin and glucagon delivery approaches (bioengineered or cell-based) that mimic normal islet function and precisely regulate glucose continuously, or 2) a drug to enhance or normalize the pattern of CR to hypoglycemia. Despite much research and important advances in the field, neither islet transplantation nor biosensor devices have emerged as viable long-term solutions for the majority of patients (3, 4). Over the past several years, our lab has explored the approach of enhancing CR by examining mechanisms responsible for HAAF/EAAF and searching for potential pharmacological methods to modulate the CR to hypoglycemia (5-11). Our work has led to a paradigm shift in the field of hypoglycemia, exemplified by the novel hypothesis and published experimental data supporting a role for opioid signaling that resulted in the initiation of exploratory clinical trials by other research groups.

In the prior project period of R01 DK079974, we elucidated the central role played by the opioid signaling system as a mechanism for the development of HAAF/EAAF. **We have demonstrated previously that opioid receptor blockade by acute infusion of naloxone during antecedent hypoglycemia can prevent experimentally induced HAAF in nondiabetic and T1DM subjects (JCEM 94:3372-80, 2009; JCEM 96:3424-31, 2011).** We have also shown that opioid receptor blockade also abolishes EAAF, and that both effects are regulated by the stress response (hypoglycemia and exercise, respectively). Furthermore, recently we have shown that activation of μ -opioid receptors with IV infusion of morphine reproduces some of the key biochemical and clinical features of HAAF in nondiabetic humans.

Taken together, these studies demonstrate that the opioid system plays a central role in hypoglycemia counterregulation and in HAAF. Originally, we proposed examining the efficacy of chronic opioid receptor blockade in decreasing hypoglycemia and preventing HAAF in patients with T1DM. However, a recent pilot study in subjects with T1DM showed no effect of short term oral naltrexone treatment on hypoglycemic symptoms or counterregulatory responses (J Diabetes Complications 29:1277-82, 2015).

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At chronic, low doses, naltrexone may have anti-inflammatory effects and result in an increase in opiate binding sites and thus supersensitivity to opioid agonists (Brain Res 289:223-34, 1983). Therefore, to avoid chronic use of naloxone, we instead propose to examine the effect of blocking opioid receptors specifically during hypoglycemia. Since this opioid antagonist is available in an intranasal, rapid active formulation (NARCAN® (naloxone hydrochloride) nasal spray, Adapt Pharma, 4mg/nasal spray), we propose to study whether acute administration of intranasal naloxone during antecedent hypoglycemia prevents HAAF.

In addition to our work, new avenues have emerged from other laboratories that offer approaches to augmenting the CR. Most importantly, based on studies showing that adrenergic activation has a modulatory effect on hypoglycemia CR (12), recent studies by the Cryer lab suggest that alpha-and beta-adrenergic receptor blockade with phentolamine and propranolol prevents HAAF in T1DM (13). Thus, the adrenergic system may also play a role in the development of HAAF, given the close functional interactions between opioid and adrenergic receptors controlling cellular signaling (14-16). Overall, in this application we propose that an underlying common mechanism linking opioidergic and adrenergic systems is responsible for the development of HAAF, and will pursue this concept with the following specific aims:

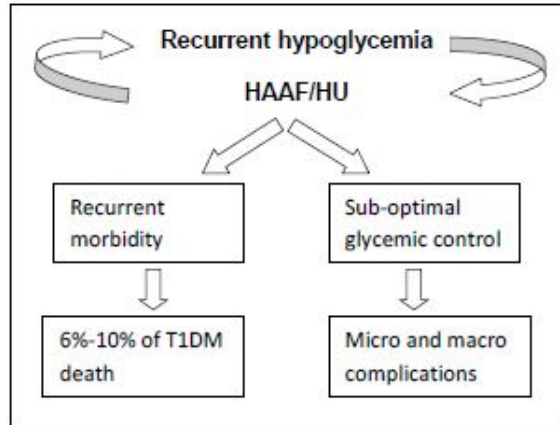
Aim 1. To examine whether activation of μ -opioid receptors, and/or adrenergic receptors, regulates HAAF in humans. We will examine the effects of morphine and/or epinephrine administered during euglycemia on subsequent CR and on the level of awareness to hypoglycemia. We hypothesize that pharmacological activation of μ -opioid receptors and adrenergic receptors (mimicking antecedent stress) will result in experimental HAAF. This experimental paradigm will enable us to dissect the independent and/or synergistic role of opioidergic and/or adrenergic system in the development of HAAF in nondiabetic subjects and in T1DM.

Aim 2. To establish the components of the adrenergic response responsible for modulating HAAF/EAAF and their association with the opioidergic system. We will analyze the effects of α -adrenergic blockade vs. β -adrenergic blockade during antecedent hypoglycemia/exercise on HAAF/EAAF in nondiabetic and in subjects with T1DM. We hypothesize that α -adrenergic blockade combined with β -adrenergic blockade will result in the most potent effect eliminating HAAF/EAAF.

Aim 3. To examine the efficacy of blocking opioid receptors during hypoglycemia and preventing HAAF in patients. We demonstrated that acute infusion of naloxone during antecedent hypoglycemia can prevent experimentally induced HAAF in nondiabetic and in T1DM subjects. We hypothesize that acute administration of intranasal, rapid active naloxone will also prevent HAAF. This proof-of-concept clinical study will be a step forward in designing a potential effective therapy to prevent severe hypoglycemia.

SIGNIFICANCE:

Iatrogenic hypoglycemia represents an important barrier to optimal insulin treatment in T1DM. Primary physiological responses to impending hypoglycemia include a decrease in insulin secretion, an increase in glucagon release, and activation of the adrenomedullary system. All three mechanisms are



responsible for reversing hypoglycemia by increasing endogenous glucose production (EGP), decreasing glucose uptake (GU) and promoting oral intake of carbohydrates (17). In patients with moderate-duration T1DM these mechanisms are defective; there is no physiological control on insulin secretion, there is no glucagon release in response to hypoglycemia, and the sympathetic adrenomedullary response is attenuated with compromised epinephrine release and decreased awareness of falling glucose levels. As a consequence, T1DM patients suffer from repeated diurnal and nocturnal episodes of hypoglycemia (18). Most of the hypoglycemic episodes are asymptomatic

(19), and when treated intensively the patients' risk for severe hypoglycemia is significantly increased (1). Furthermore, repeated episodes of hypoglycemia or exercise induce hypoglycemia-associated autonomic failure (HAAF) and exercise-associated autonomic failure (EAAF), respectively, characterized by further deterioration in glucose counterregulation and hypoglycemia unawareness (HU, (2)). Thus, a vicious cycle of recurrent hypoglycemia ensues, with added consequences: 1) a tendency to maintain suboptimal glycemic control, which may improve hypoglycemia counterregulation, however at the cost of increased risk for vascular complications (20), and 2) hypoglycemia per se causes recurrent morbidity and may even be fatal. Indeed, a recent review attributed 6 to 10% of deaths in patients with T1DM directly to hypoglycemic events (21).

The mechanism responsible for HAAF and EAAF is set up by repeated activation of the sympathoadrenal system. Similar activation occurs during a variety of stressful stimuli resulting in physiological and behavioral effects that may be useful during a short-term threat but potentially deleterious with prolonged exposure. Thus, altered or decreased defensive responses to recurrent, potentially damaging stressful stimuli are seen when recurrent or chronic exposure results in "stress habituation", possibly as a defensive adaptation (22). HAAF and EAAF may represent a state of "stress habituation" especially since attenuated epinephrine, defective glucose counterregulation and hypoglycemia unawareness can be reversed after 2–3 weeks of avoidance of hypoglycemia (23). Both hypoglycemia and stress stimuli involve activation of the opioid system. Studies in animals and humans have shown that blocking opioid receptors with naloxone during hypoglycemia induce an augmentation of the counterregulatory response (24, 25). These findings suggested that the opioid system play a modulatory role in hypoglycemia counterregulation and could be manipulated pharmacologically. Since the counterregulatory response to hypoglycemia represents a complex system with redundancy in hypoglycemia sensing, integration, and hormonal responses, other mechanisms may act synergistically to modulate counterregulation and HAAF (26). Indeed, recent studies have showed that adrenergic blockade can modulate hypoglycemia counterregulation (12, 27) and improve HAAF in humans (13). Taking into account the colocalization and relationship between the opioidergic and adrenergic systems (as discussed below), these two systems may represent a common arm in the counterregulatory response to hypoglycemia and HAAF.

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In this application we propose to analyze the interrelation of the opioidergic and adrenergic systems in the development of HAAF and EAAF, and to examine the effects of long term (orally administered) opioid receptor blockade on hypoglycemia counterregulation and on experimentally and non-experimentally-induced HAAF in T1DM patients. Correction or improvement of hypoglycemia counterregulation and restoring hypoglycemia awareness in patients with T1DM would represent an enormous step forward in the management of these patients, including the potential to prevent morbidities or death. Finally, we believe that the rationale for conducting these studies in humans is not only because of the clinical translation of our studies into potential therapy, but because the unique physiology of the counterregulatory system and the subtleties of HAAF and EAAF cannot be precisely matched in animal models.

INNOVATION:

Although opioid receptor blockade results in modulation of HAAF/EAAF (8-11), and recent data show that adrenergic receptor blockade prevents the effect of hypoglycemia to attenuate the sympathoadrenal response to subsequent hypoglycemia (13), the combined effect of opioid and adrenergic manipulation has not been investigated. This is of particular interest since opioidergic and adrenergic receptors show close functional interactions. Both morphine and norepinephrine induce major inhibitory effects in brain neurons and peripherally by activating G protein-coupled-receptors (GPCRs (15)). Additionally, heterodimerization of these receptors may activate common signal transduction pathways or confer them new functional proprieties that are different from the original receptor (16, 28, 29). Thus, in Aim 1 we will study the effects of nonselective opioidergic and adrenergic activation on HAAF, and in Aim 2, we propose to examine the selective effects of α -adrenergic receptor blockade and/or β -adrenergic receptor blockade - with and without μ -opioid receptor blockade, on HAAF/EAAF. These data will provide us with better understanding of the mechanism responsible for HAAF/EAAF and allow for further studies looking at pharmacological methods to eliminate HAAF/EAAF. Furthermore, although experimental HAAF can be prevented by administration of naloxone. Acute administration of opioid receptor blockade using intranasal naloxone during antecedent hypoglycemia and its effects on hypoglycemia counterregulation has not been investigated. Thus, in AIM 3 we propose to analyze the effects of acute administration of intranasal naloxone during antecedent hypoglycemia on hypoglycemia counterregulation and on induction of HAAF in patients.

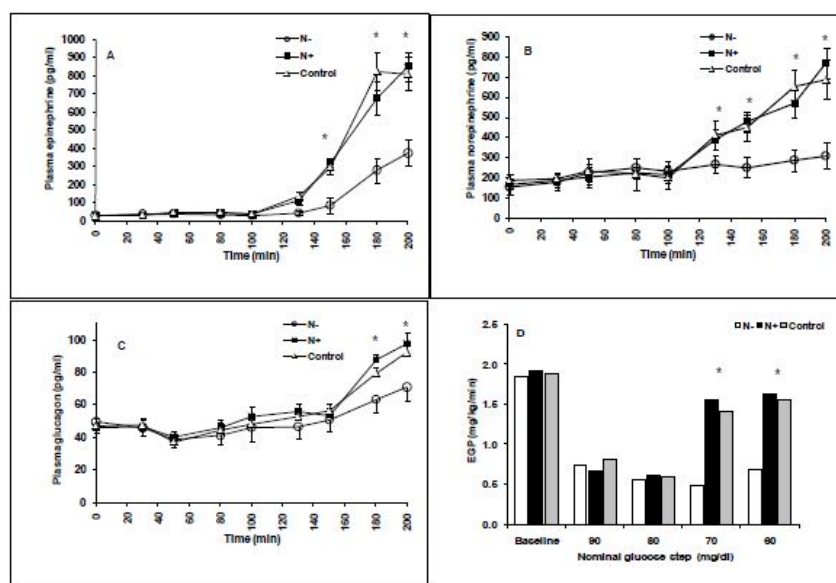


Figure 1. Plasma epinephrine(A), norepinephrine (B), and glucagon (C) concentrations, over time and endogenous glucose production (EGP, D) in the N+ (■), N- (○) and in the Control studies (Δ) on Day 2 of the studies. * $p < 0.01$ vs. N-.

APPROACH:

Progress report /preliminary data published in the first project period

1. Hypoglycemia associated autonomic failure is prevented by opioid receptor blockade (J Clin Endocrinol Metab. 94:3372-80, 2009): In this study, we examined whether opioid receptor blockade

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during antecedent hypoglycemia on Day 1 would prevent the development of HAAF on Day 2 in nondiabetic subjects (5 men, 3 women, age 28 ± 3.5 yr). Studies were performed on three occasions: Day 1 consisted of A) two 90-min hypoglycemic (60 mg/dL) clamps (N-); B) two 90-min hypoglycemic clamps plus naloxone (0.4 μ g/kg/min, N+); or C) two euglycemic clamps (C) as Control. Day 1 hypoglycemia caused marked deterioration of Day 2 hormonal responses to hypoglycemia consistent with HAAF – i.e., decreased plasma epinephrine, norepinephrine and glucagon compared to Control (374 ± 71 vs. 810 ± 94 , 307 ± 65 vs. 686 ± 98 and 71 ± 9 vs. 92 ± 7 pg/ml, respectively, $p < 0.01$), as well as in endogenous glucose production ([EGP], 24% vs. 163%, $p < 0.01$). In contrast, naloxone administration prevented the defective counterregulatory response; epinephrine, norepinephrine, and glucagon (852 ± 82 , 769 ± 77 , and 97 ± 7 pg/ml) and EGP recovery (167%) were identical to those following Day 1 euglycemia ($p = \text{NS}$ for all). These studies demonstrated for the first time that blocking opioid receptors with naloxone during antecedent hypoglycemia prevents HAAF in normal humans.

In order to exclude the possibility that improvement in counterregulation on Day 2 represents a carry-over effect from naloxone administration on Day 1, we performed another set of control studies with Day 1 identical to the control studies (C), however with naloxone infusion (C+). There was no difference in hypoglycemia counterregulation on Day 2 in the C+ studies compared to the control studies suggesting that a carry-over effect is unlikely. This proof of concept study demonstrates that the opioid system plays a central role in hypoglycemia counterregulation and in HAAF.

In light of these novel data, many questions remain unanswered. Recent translational research suggests that a complex cerebral network normally regulates the hypothalamic (and thus the systemic sympathoadrenal) response to hypoglycemia (30, 31) and that an inhibitory signal mediated through the thalamus might be involved in the pathogenesis of HAAF (30). Indeed, a recent study (13) demonstrated that in healthy humans, nonselective adrenergic blockade during hypoglycemia prevents the reduction of plasma catecholamine responses to subsequent hypoglycemia, suggesting that adrenergic activation mediates HAAF. The close relationship between opioid receptor activation and its modulatory effects on the autonomic system has been traditionally attributed to the presence of presynaptic κ , δ and μ -opioid receptors (32, 33). These mediate receptor-specific inhibitory effects on neurotransmitter release, with release of norepinephrine being predominantly under μ -opioid receptor modulation (33). Both adrenergic and μ opioid receptors (MORs) are members of the family A of G-protein-coupled receptors (GPCRs). Activation of these receptors results in inhibition of adenylylase, activation of K^+ currents, inhibition of Ca^{2+} channels and finally activation of MAP kinase phosphorylation. Both receptors may be co-localized, with G-proteins co-expressed and interact at multiple levels including receptor, G protein, or downstream signaling (16, 34). Furthermore, recent data suggests a close neuronal interaction between the μ - receptor and adrenergic receptors (α and β) that results in cross activation (15, 29). Alpha 2A-adrenergic receptors (α_2 -AR) modulate important physiologic responses, particularly in the CNS; they regulate sympathetic outflow and play a key role in systemic sympathetic activity and cardiovascular responses to CNS stimulation (35). Indeed, studies in α_2 -AR knockout mice showed that these receptors are primarily responsible for reduction of sympathetic tone and norepinephrine levels, reduction of blood pressure and heart rate, and sedation and analgesia (36). Furthermore, Vilardaga et

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al. recently showed that a conformational “cross talk” between $\alpha 2$ -AR and μ -opioid receptors controls cell signaling in humans (15).

The concept of heterodimerization of these two different receptors may be responsible for synergistic or antagonistic effects during dual receptor activation. Similar cross-talk between β -adrenergic receptor and MOR was also described with evidence that $\beta 2$ -adrenergic receptor and MOR can activate more than one G protein family (34). Taken together, these studies suggest that the interaction between the μ -receptor and the adrenergic receptor may represent a mechanism responsible for the modulatory effects of endorphins on the neuroadrenergic/sympathetic response to stress and possibly to hypoglycemia. Since both MOR blockade and adrenergic blockade result in improvement in HAAF, we hypothesize that both receptors represent a common pathway in the development of HAAF, and propose to analyze the effects of activation of the opioidergic and adrenergic systems (mimicking antecedent hypoglycemia) on subsequent development of HAAF.

2. Magnitude of exercise-induced β -endorphin response determines subsequent development of altered hypoglycemia counterregulation (J Clin Endocrinol Metab. 97:623-31, 2012): The regulation of glucose homeostasis during exercise has parallels to the counterregulatory response elicited by hypoglycemia (37, 38). Experimental evidence suggests that antecedent exercise (similar to antecedent hypoglycemia) in normal subjects and in T1DM patients attenuates autonomic counterregulatory responses during subsequent hypoglycemia, thereby contributing to the development of exercise associated autonomic failure (EAAF, (39-41)). This implies that shared mechanisms may be responsible for hypoglycemia-related and exercise-related autonomic failure. Since both hypoglycemia and exercise involve activation of the opioid system, we hypothesized that exercise-induced β -endorphin release will also result in deterioration of subsequent hypoglycemia counterregulation and that the attenuation of the counterregulatory response will negatively correlate with the degree of β -endorphin elevation. We studied 16 healthy subjects using 3 experimental paradigms on 2 consecutive days. Day 1 consisted of either 1) two 90-min hyperinsulinemic hypoglycemic clamps (60 mg/dl, [H]); 2) two 90-min hyperinsulinemic euglycemic clamps while subjects exercised at 60% $VO_{2max}[E]$; or 3) two 90-min hyperinsulinemic euglycemic clamps [C]. Day 2 followed with stepped hypoglycemic clamps (90, 80, 70 and 60 mg/dl plasma glucose steps, 50-min each). In order to evaluate the correlation between the rise in plasma β -endorphin levels during exercise and next day hypoglycemia counterregulation, we analyzed the association between plasma β -endorphin levels and the hormonal counterregulation using a statistical stepwise regression utilizing a mixed model. We discovered a significant inverse effect of plasma β -endorphin concentrations on Day 1 on epinephrine / norepinephrine responses to hypoglycemia on Day 2. Prior exercise with an exercise-induced rise in β -endorphin levels to above 25 ng/dl on Day 1 resulted in markedly reduced levels of epinephrine and norepinephrine compared to control (457 ± 56 pg/ml vs. 881 ± 104 pg/ml, and 328 ± 53 pg/ml vs. 489 ± 64 pg/ml, respectively, $p=0.01$ for both). The rate of EGP recovery in this group was also much lower than in control (42% vs. 89%, $p<0.01$). The maximal glucagon and cortisol responses were similar to the control. Thus, our results demonstrate that significant β -endorphin release during antecedent exercise is associated with deterioration of the counterregulatory response to subsequent hypoglycemia on the following day, similar to the effect of antecedent hypoglycemia.

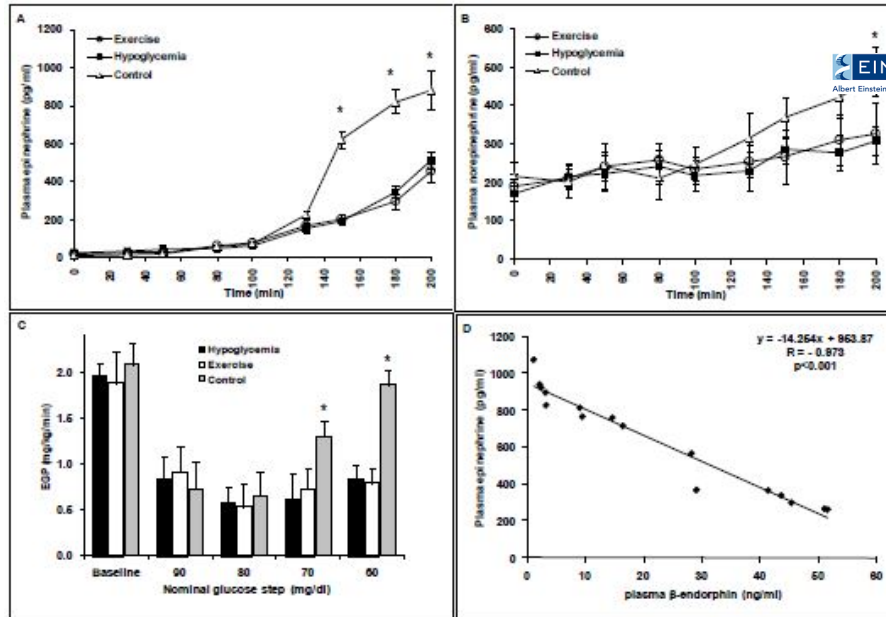


Figure 2. Day 2 plasma epinephrine ($*p < 0.001$ vs. Hypoglycemia, A), norepinephrine ($*p < 0.001$ vs. Hypoglycemia, B) concentrations, and EGP (C) in the antecedent exercise studies with significantly elevated plasma beta-endorphins concentrations (> 25 ng/ml). The correlation between plasma beta-endorphins concentrations (day 1) and day 2 plasma epinephrine in the exercise studies.

ventromedial hypothalamus and the arcuate nucleus (42, 43), suggesting that β -endorphin acts centrally to induce HAAF and EAAF. Repeated bouts of hypoglycemia result in suppression of genes in the hypothalamic neurons that inhibit glycolysis and stimulate fatty acid oxidation, including Pdk4, Angpt14, Cpt1a, and Gpd1 genes, which leads to the inability to shift from glycolysis toward alternate fuel use at the time of hypoglycemia (44) and may contribute to impaired recovery from hypoglycemia. However, this gene suppression is reversible with naloxone (44), implying that endogenous opioids are the mediators of these responses. Whether this gene regulation results directly from the action of endogenous opioids on the hypothalamic neurons or indirectly via opioid effect on sympathoadrenal system remains to be elucidated. Taken together, our data clearly demonstrate that the development of EAAF in non-diabetic subjects is dependent on, and inversely correlates with increments in plasma β -endorphin during antecedent exercise.

3. Opioid receptor blockade during antecedent exercise prevents exercise-associated autonomic failure in humans. Diabetes. 61:1609-15, 2012):

Since both hypoglycemia and exercise induce the release of β -endorphin and since higher levels of β -endorphin during antecedent exercise were associated with the development of exercise-associated autonomic failure (EAAF), we hypothesized that opioid receptor blockade during vigorous exercise (associated with significant increase in β -endorphin levels) can prevent EAAF. We studied healthy subjects (3 F, age 28 ± 5.3 yr) on 2 consecutive days, with each participating in 3 different studies in random order. Day 1 (at left) consisted of either A) two 90-min hyperinsulinemic euglycemic clamps (90 mg/dl) plus naloxone infusion ($0.4 \mu\text{g/kg/min}$ [Control]); or B) two 90-min hyperinsulinemic euglycemic clamps while the subjects exercised at 60% VO_2max , plus naloxone infusion ($0.4 \mu\text{g/kg/min}$, [N+]); or C) the same

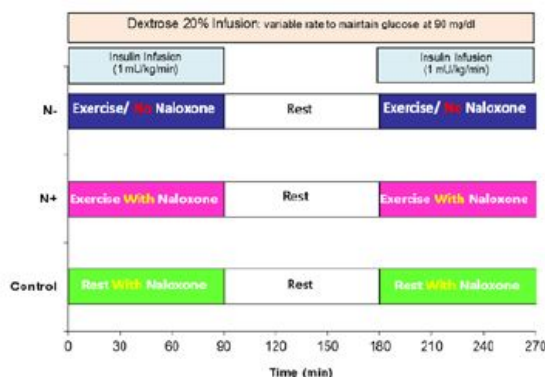


Figure 3. Experimental protocol on Day 1

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protocol as in N+ group, but with saline infusion replacing naloxone [N-]). Day 2 followed with stepped hypoglycemic clamps (90, 80, 70 and 60 mg/dl plasma glucose steps). Plasma β -endorphin levels at baseline (time 0) were 5.7 ± 1.3 , 7.2 ± 1.8 , and 5.2 ± 0.9 pg/ml in the N-, N+ and Control studies, respectively. At the end of the studies plasma β -endorphin concentrations increased significantly in the N- and N+ studies (41.7 ± 5.1 and 36.2 ± 4.6 pg/ml, respectively, $p < 0.001$ compared to time 0), however, remained unchanged in the Control studies. Plasma epinephrine concentrations were similar in all studies during the 90 and 80 mg/dl glucose steps (56.1 ± 11.1 pg/ml, 66.9 ± 8.5 pg/ml, and 52.1 ± 8.5 pg/ml, in the N-, N+, and Control studies, respectively, $p = \text{NS}$). Further reduction in plasma glucose to 60 mg/dl was associated with increments in plasma epinephrine in all studies, however, the N- studies demonstrated a significantly lower plasma epinephrine concentration compared to the N+ and Control studies (465 ± 47 pg/ml, vs. 702 ± 88 pg/ml and 768 ± 92 pg/ml in the N-, N+ and Control studies, respectively, $p < 0.01$). Plasma norepinephrine concentrations were equivalent during the 90 and 80 mg/dl glucose steps in all studies. However, during the 60 mg/dl glucose step, plasma norepinephrine increased slightly in the N- studies (283 ± 75 pg/ml) compared to the N+ and Control studies (562 ± 78 pg/ml and 486 ± 62 pg/ml, $p < 0.05$). Plasma glucagon and cortisol concentrations were equivalent in all studies at baseline and increased similarly with hypoglycemia in the N-, N+ and Control studies (data not shown). Mean baseline EGP was similar in all studies (2.2 ± 0.2 mg/kg/min, 2.1 ± 0.1 mg/kg/min and 2.1 ± 0.2 mg/kg/min, in the N-, N+, and Control studies, respectively, $p = \text{NS}$). With the initiation of insulin infusion EGP was equally suppressed by $\sim 65\%$ in all studies. During the 60 mg/dl glucose step EGP recovered by 53% in the N- studies, and by 92% and 85% in the N+ and Control studies, respectively ($p < 0.01$ vs. N-). Thus, we have clearly demonstrated here that opioid blockade with naloxone during antecedent exercise significantly improves the counterregulatory responses to hypoglycemia during subsequent hypoglycemia in nondiabetic subjects. These data establish the important role the opioidergic system plays in the development of EAAF and prove that pharmacological intervention during exercise can prevent EAAF in humans.

4. Opioid receptor blockade improves hypoglycemia-associated autonomic failure in type 1 diabetes mellitus (J Clin Endocrinol Metab. 96:3424-31, 2011):

Since we had already demonstrated that blockade of opioid receptors with naloxone can reverse HAAF and EAAF in nondiabetic subjects, we examined whether opioid receptor blockade can affect HAAF in T1DM patients who suffer from impaired hypoglycemia counterregulation. We studied 8 subjects with T1DM (mean age 34 yr, HbA1c $7.3 \pm 1.1\%$) on 2 consecutive days and on 3 separate occasions. Day 1 consisted of either: 1) two 90-min hypoglycemic clamps (60 mg/dl, N-); 2) two 90-min hypoglycemic clamps (60 mg/dl) with concomitant

naloxone infusion (N+); or 3) two 90-min euglycemic clamps (90 mg/dl) with concomitant naloxone infusion (Control). Day 2 consisted of stepped hypoglycemic clamps (90, 80, 70 and 60 mg/dl plasma glucose steps). Antecedent hypoglycemia in N- group resulted in a markedly decreased

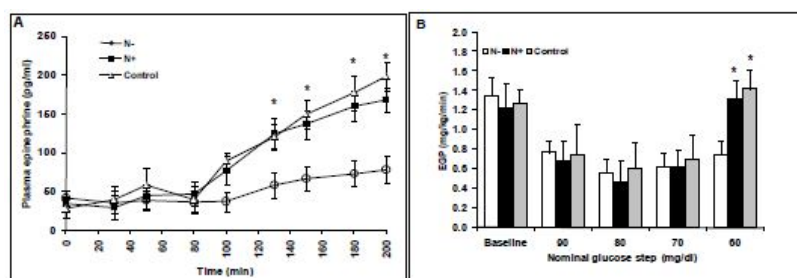


Figure 4. A. Plasma epinephrine concentrations (Day 2) over time in the N- (white circles), N+ (black squares), and control studies (white triangles, * $p < 0.05$ vs. N+ and control). B. Endogenous glucose production (EGP, Day 2) averaged for the final 30 minutes of each glucose step (* $p < 0.01$ vs. N-).

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epinephrine response and a lower rate of endogenous glucose production (EGP) during subsequent hypoglycemia compared to Control (75.2 ± 17.0 pg/ml vs. 187.1 ± 20.6 pg/ml, $p < 0.05$ and 0.8 ± 0.1 mg/kg/min vs. 1.4 ± 0.2 mg/kg/min, $p < 0.05$, respectively). In contrast, in the N+ studies, plasma epinephrine was 163.6 ± 17.8 pg/ml and EGP was 1.3 ± 0.2 mg/kg/min during subsequent hypoglycemia, both levels similar to those seen in Control studies ($p = \text{NS}$ vs. Control). Plasma glucagon did not increase with hypoglycemia. Although others have also noted improvement in hypoglycemia counterregulation with naloxone infusion (24, 25), we have demonstrated here, for the first time, that naloxone infusion during antecedent hypoglycemia induces amelioration in subsequent hypoglycemia counterregulation in T1DM patients. This effect of naloxone was hypoglycemia-dependent and could not be considered a carry-over effect since there was no change in HAAF in the Control studies.

In this study, we did not observe either a further impairment in, or a rescue by naloxone of absent glucagon secretion in response to hypoglycemia. However, we had previously shown that naloxone infusion augmented the glucagon response to subsequent hypoglycemia in nondiabetic subjects (see above, (8)), confirming other studies that suggest that the loss of glucagon response to hypoglycemia in T1DM may not be solely dependent on opioid release during hypoglycemia; different mechanisms are likely involved in the regulation of catecholamine and glucagon release in response to hypoglycemia (45, 46). Endogenous opioids are secreted by the proopiomelanocortin (POMC) neurons of the pituitary gland (47, 48) in response to a variety of stressors, including hypoglycemia and exercise (49-51). Central nervous system (CNS) signals that mediate the response to hypoglycemia may be of major importance in glucose counterregulation. In the CNS, opioids contribute to the development of HAAF likely via activation of opioid receptors, localized to areas in the thalamus and hypothalamus responsible for glucose sensing, including the ventromedial hypothalamus, arcuate nucleus and dorsal medial thalamus (42, 43, 52, 53). Administration of β -endorphin directly into rat brain was shown to inhibit hypothalamic responses to hypoglycemia (54). In parallel, accumulating evidence suggest that endogenous opioids produced peripherally by the adrenal medulla may lead to glucose lowering in streptozocin-induced diabetic rats by increasing glucose uptake and decreasing hepatic gluconeogenesis (55, 56). Finally, various studies have demonstrated that β -endorphin can modulate glucose homeostasis by its action on insulin release (57-59). In vitro, β -endorphin (primarily μ -opioid receptor targeting) inhibits insulin release from isolated islets (60), and in vivo, β -endorphin also attenuates insulin release when administered by iv infusion (61).

The glucose lowering effects of β -endorphin in a type 1-like diabetic rat model are due to an increase in GLUT 4 gene expression leading to higher glucose utilization and decreased PEPCK gene expression leading to a decline of hepatic gluconeogenesis (62, 63). Furthermore, it has recently been shown that β -endorphin release from the adrenal gland is activated by α_1 -adrenoreceptor stimulation (56); phenylephrine stimulation caused an increase in β -endorphin concentrations whereas α -antagonists administration resulted in a decrease in β -endorphin levels (56, 62). Endogenous opioids, in turn, induce suppression of catecholamine release from the adrenal gland, suggesting secretory negative feedback between adrenal catecholamine release and opioid secretion (64-66). Importantly, this opioid effect on the adrenal medulla is reversed in vitro with naloxone administration (67). Taken together, these data suggest that the modulation of the counterregulatory response to hypoglycemia occurs centrally and

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peripherally, and that the opioid system plays a pivotal role in both locations. It is noteworthy, that activation of both β -adrenergic receptors and α -adrenergic receptors at the level of the hypothalamus modulates glucose regulation. Szepietowska et.al. (12) recently showed in the rat that activation of VMH β -adrenergic receptor (BAR) results in enhanced counterregulation to hypoglycemia characterized by significant increment in epinephrine release and increased glucagon response. Blocking BAR in the VMH induced a blunted epinephrine response (12). Other studies suggest that central α 2-receptors play a role in glucose regulation. Smythe et. al. reported that yohimbine, an α 2-receptor antagonist, markedly inhibited stress induced hyperglycemia, and clonidine (central α 2-receptor agonist) administration induces hyperglycemia (68-70). Repeated activation may lead to adrenergic receptor down regulation/desensitization which results in tachyphylaxis (71). These findings are further supported by recent data in humans that show that blunting of the catecholamine response to hypoglycemia could be prevented by pre-treatment with adrenergic blockade during antecedent hypoglycemia (13). In view of the close relationship between the opioidergic and adrenergic systems and the cross talk between α and β -adrenergic receptor with the opioid receptor (see above), we hypothesize that a common mechanism is responsible for activation of hypoglycemia counterregulation and to the development of HAAF in T1DM.

Specific Aim 1. To examine whether activation of μ -opioid receptors, and/or adrenergic receptors, regulate HAAF in humans.

Summary: We will administer morphine with and without co-infusion of epinephrine on Day 1, followed by a stepped hypoglycemia clamp (euglycemia \rightarrow 90 mg/dl \rightarrow 80 mg/dl \rightarrow 70 mg/dl \rightarrow 60 mg/dl) on Day 2. This experimental paradigm will enable us to test the effects of nonselective activation of opioidergic or opioidergic + adrenergic receptor on the counterregulatory responses to subsequent hypoglycemia in nondiabetic and in intensively treated T1DM subjects.

Experimental Protocol: Two groups of subjects will be studied: 12 healthy controls and 12 subjects with intensively-treated T1DM (see Human Subjects section) matched for age, gender and body mass index (BMI). Each subject will participate in four, two-day study sets, separated by an interval of five weeks. Day 1 will include administration of either morphine alone, epinephrine alone, morphine + epinephrine, or normal saline (Control, C). Day 2 will be identical in all studies and will include a stepped hypoglycemia clamp. The sequencing of studies will be randomized in both groups of subjects.

Day 1: Nondiabetic subjects will be admitted to the Clinical Research Center (CRC) at 06:00 A.M. Subjects with T1DM will be admitted to the CRC the evening prior to the study, and a variable-dose infusion of insulin (Humulin Regular, Eli Lilly, Indianapolis, IN) will be initiated at 10:00 P.M. via a peripheral vein and will continue overnight. The infusion rate of insulin will be adjusted according to hourly plasma glucose, to assure a gradual normalization of plasma glucose levels (72). At 07:00 A.M. on the study day, all subjects will have two indwelling cannulae inserted, one in an antecubital vein for infusions and the second placed in a retrograde fashion in a distal hand vein of the contralateral forearm for blood sampling. To obtain arterialized venous blood samples, this hand will be maintained at 55° C in a thermoregulated Plexiglas box. Subjects with T1DM will continue their basal insulin infusion and a variable infusion of 20% dextrose will maintain their plasma glucose at euglycemia. At 9:00 A.M., a

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morphine infusion will be initiated at a rate of 0.5 $\mu\text{g/kg/min}$, for two hours. At the end of the 2h infusion all subjects will receive a small snack (15 grams of carbohydrate) and will rest for the next two hours. At that point we will restart the morphine infusion at the same rate, for an additional 2h period. After completion of the second 2h infusion, subjects will receive a meal, a bedtime snack, and remain for an overnight fast. In the T1DM subjects a variable-dose infusion of insulin will continue overnight, as described above. All subjects will receive 5 ml/kg body water of $2\text{H}_2\text{O}$ (99.9% 2H ; Isotec, Miamisburg, OH), divided into three equal portions and given at 08:00 P.M., 11:00 P.M. and again at 03:00 A.M. Additional water ingested during the fast will be enriched to 0.5% with $2\text{H}_2\text{O}$ to prevent dilution of the isotopic steady state. The three other sets of studies will be similar with the exception that at 9:00 A.M., we will initiate a continuous infusion of epinephrine at a rate of 0.03 $\mu\text{g/kg/min}$, morphine + epinephrine at rates of 0.5 $\mu\text{g/kg/min}$, and 0.03 $\mu\text{g/kg/min}$, respectively, or 0.9% NaCl, (Control) during each of the 2h clamps.

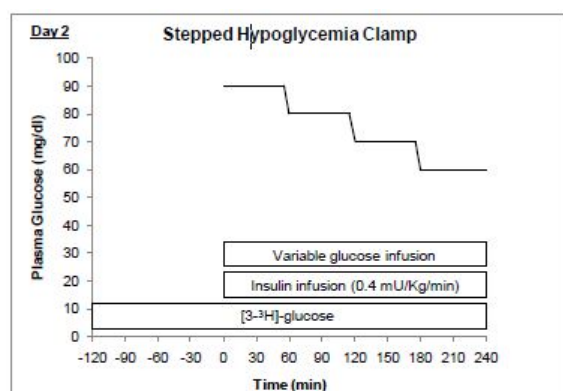


Figure 5. Experimental protocol of Day 2 stepped hypoglycemia clamp. This study will be identical in all protocols.

Day 2: At 07:00 A.M. all subjects will have two indwelling cannulae inserted. T1DM subjects will continue their insulin infusion, maintained at baseline. At $t = -120$ min, a primed-continuous infusion of HPLC-purified $[3\text{-}^3\text{H}]\text{-glucose}$ will be initiated with a bolus of 21.6 μCi followed by continuous infusion of 0.15 $\mu\text{Ci/min}$ for the entire period of study. The specific activity of infused dextrose will be kept equivalent to plasma glucose specific activity by addition of $[3\text{-}^3\text{H}]\text{-glucose}$ to the infusate, using the method of Finegood et al. (73). At $t = 0$ min, a primed-continuous infusion of insulin will be initiated at a rate of 0.4 mU/kg/min throughout the study, and a variable infusion of 20%

dextrose will be begun to maintain the plasma glucose concentration at 90 mg/dl for 60 minutes (step 1 of the clamp). At $t = +60$ min, and every 60 minutes thereafter, the plasma glucose concentration will be decreased by 10 mg/dl decrements for 60 minutes each by reducing the dextrose infusion rate accordingly. Plasma glucose will be clamped at the desired range by varying the dextrose infusion according to plasma glucose measured at 5-minute intervals with targets of 90, 80, 70 and 60 mg/dl. As we increase the insulin levels, we will administer oral potassium chloride tablets in order to prevent hypokalemia during the insulin infusion with a maximal daily dose of up to 40 mEq on Day 2. At each plasma glucose step subjects will be evaluated for symptoms of hypoglycemia (74), and plasma samples for $[3\text{-}^3\text{H}]\text{-glucose}$, insulin, C-peptide, glucagon, epinephrine, norepinephrine, cortisol, growth hormone, free fatty acids, lactate and glycerol and $\beta\text{-endorphins}$ will be obtained at 10-min intervals. 2H enrichment of the hydrogen bound to C2 and C5 of blood glucose will be collected at 30-min intervals (see Detailed Experimental Methods). At the end of the clamp ($t = 240$ min), all the infusions will be discontinued, and the subject will be given a meal and discharged from the CRC.

Rationale and Interpretation of the Results: Activation of $\mu\text{-opioid}$ receptors with morphine on Day 1 will mimic the effects of stress (hypoglycemia and exercise), however, excluding all other factors that

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may contribute to the modulation of HAAF. We will compare the counterregulatory response on Day 2 after administration of morphine vs. after administration of saline (C). This will enable us to quantify the specific role of opioid receptor activation on the development of HAAF. Similarly, activation of (both α - and β -) adrenergic receptors with epinephrine will enable us to quantitatively define its role in the development of HAAF (when compared to C). Finally, the counterregulatory responses to subsequent hypoglycemia after administration of a combination of morphine and epinephrine will further elucidate the contribution of each system in HAAF, and enable us to ascertain whether there are additive or synergistic effects of combined receptor activation. We chose to administer morphine, epinephrine or saline (on Day 1) in two-2h intervals in order to mimic our previous studies in which we experimentally induced HAAF by either two-2h bouts of hypoglycemia or exercise. The epinephrine dose selected will result in a plasma epinephrine that is typically seen during an appropriate counterregulatory response to hypoglycemia (75-77) and has been previously employed by us. The insulin infusion rate on Day 2 (0.4 mU/kg/min) is tailored to induce only moderate ($\sim 50\%$) suppression of endogenous glucose production (EGP, as we have determined previously, (8-10)), and will enable us to measure the effects of counterregulation on EGP recovery ([3-3H]-glucose). Furthermore, using the 2H2O methodology to quantitate gluconeogenesis (GNG), we will be able to determine the contribution of GNG to EGP recovery during this paradigm of hypoglycemia counterregulation (as we previously reported, (8)). Subtracting GNG from EGP will provide an indirect measurement of the rate of net hepatic glycogenolysis (8). Determination of hepatic glycogenolysis during recovery from hypoglycemia and its contribution to EGP is of particular interest since intensively treated T1DM patients may have variation in their hepatic glycogen content, and depleted hepatic glycogen due to repeated episodes of hypoglycemia counterregulation may affect EGP (we have previously shown that hepatic glycogenolysis represents the initial source for glucose-6-phosphate for EGP during hypoglycemia counterregulation, (78)).

This study will not determine the exact location at which morphine/epinephrine act in order to modulate hypoglycemia counterregulation. However, since morphine can cross the brain blood barrier (BBB) and epinephrine does not, obtaining a significant effect on hypoglycemia counterregulation induced by epinephrine would suggest a peripheral action. Additionally, although morphine interacts predominantly with the μ -opioid receptor, it also may act as a κ -opioid and δ -opioid receptor agonist. Similarly, epinephrine is a nonselective agonist of both α - and β -adrenergic receptors. Thus, these studies will not be able to discern the subtype receptor activated by these drugs. Answers to some of these questions will be addressed in Aim 2.

Specific Aim 2. To establish the components of the adrenergic response responsible for modulating HAAF/EAAF and their association with the opioidergic system.

Summary: We will induce α -adrenergic blockade, β -adrenergic blockade, or a combination of α -plus β -blockade during antecedent hypoglycemia or exercise and analyze their effects on subsequent hypoglycemia counterregulation in nondiabetic and in T1DM subjects..

Experimental Protocol: A. Antecedent hypoglycemia: Two groups of subjects will be studied: 10 nondiabetic and 10 subjects with intensively treated T1DM matched for age, gender and BMI with the

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control group. **Day 1:** Nondiabetic subjects will be admitted to the CRC at 06:00 A.M. Subjects with T1DM will be admitted to the CRC the evening prior to the study, and a variable-dose infusion of short acting insulin will be initiated at 10:00 P.M. via a peripheral vein and will continue overnight. The infusion rate of insulin will be adjusted according to hourly plasma glucose, to assure a gradual normalization of plasma glucose levels. At 07:00 A.M. on the study day, all subjects will have two indwelling cannulae inserted, one in an antecubital vein for infusions and the second placed in a retrograde fashion in a distal hand vein of the contralateral forearm for blood sampling. To obtain arterialized venous blood samples, this hand will be maintained at 55° C in a thermoregulated Plexiglas box. At $t = -30$ min, a insulin infusion (Humulin Regular, Eli Lilly, Indianapolis, IN) will be initiated at a rate of 1.5 mU/kg/min, and a variable infusion of 20% dextrose will maintain the plasma glucose concentration either at euglycemia (in the control studies) or at 60 mg/dl for a duration of two hours. At the end of the 2h clamp insulin infusion will be stopped (in T1DM insulin infusion will be reduced to baseline/pre-clamp infusion rate), the subjects will receive a small snack (15 grams of carbohydrate) and their plasma glucose will be maintained at euglycemia by the use of an exogenous glucose infusion (as needed) for further 2h. At that point insulin will be restarted at a rate of 1.5 mU/kg/min, and a variable infusion of 20% dextrose will be administered for a second 2h hyperinsulinemic-euglycemic (control) or hyperinsulinemic-hypoglycemic clamp. After completion of the second 2h clamp subjects will consume a large meal, bedtime snack, and remain for an overnight fast. In the T1DM subjects a variable-dose infusion of short acting insulin will continue overnight, as described above. All subjects will receive 5 ml/kg body water of 2H₂O (99.9% 2H; Isotec, Miamisburg, OH), divided into three equal portions and given at 08:00 P.M., 11:00 P.M. and again at 03:00 A.M. Additional water ingested during the fast will be enriched to 0.5% with 2H₂O to prevent dilution of the isotopic steady state. These studies will be used as control (i.e. antecedent euglycemia and antecedent hypoglycemia. Each subject will be required to participate in the following additional studies: 1. Identical protocol as above with the exception that studies will be with or without phentolamine mesylate (70mg/kg, followed by 7.0 mg/kg/min) infusion, propranolol hydrochloride (14 mg/kg, followed by 1.4 mg/kg/min), or phentolamine plus propranolol, beginning at $t = -30$ min and continued throughout the period of the clamps. **Day 2:** a stepped hypoglycemia clamp will be induced, identical to the protocol in Aim 1.

B. Antecedent exercise: At least two weeks before the initial study, all subjects will be admitted to the CRC to perform an incremental exercise test on a stationary cycle ergometer to determine their VO₂max. Expired gases will be collected and analyzed using computerized open-circuit indirect calorimetry (SensorMedics VMax-29, Yorba Linda, CA). VO₂max will be determined when at least two of the following three criteria will be met: 1) the subject is too tired to continue, 2) the respiratory exchange ratio is >1.0, or 3) there is a plateau in oxygen consumption with increasing workloads. **Day 1:** Nondiabetic subjects will be admitted to the CRC at 06:00 A.M. on the day of study. Subjects with T1DM will be admitted to the CRC the evening prior to the study. In T1DM, a variable-dose infusion of short acting insulin will be initiated at 10:00 P.M. via a peripheral vein and will continue overnight. The infusion rate of insulin will be adjusted to achieve plasma glucose concentrations of 100-120 mg/dl prior to the study the next morning. At 07:00 A.M. subjects will be assigned to either perform 90 min of exercise at 50% VO₂max at 60–70 rpm on a reclining cycle ergometer, or to sit in a chair (control). During exercise and while resting in the chair (control), T1DM subjects will continue to receive an insulin

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infusion, adjusted (according to 5 min interval measurements of plasma glucose during exercise and 20 min intervals during rest), to achieve euglycemia. This will be followed by a 120-min resting period and a second 90-min exercise period at the same exercise intensity as performed in the morning. During the first 30 min of the break period between morning and afternoon exercise or control periods, a small snack (15 grams of carbohydrate) will be administered. Each subject will be required to participate in the following additional studies: 1. identical studies as above with the exception that studies will be with or without phentolamine mesylate (70mg/kg, followed by 7.0 mg/kg/min) infusion, propranolol hydrochloride (14 mg/kg, followed by 1.4 mg/kg/min, or phentolamine plus propranolol, initiated at the beginning of exercise and continued throughout the period of both exercise/control clamps All subjects will receive 5 ml/kg body water of 2H₂O (99.9% 2H; Isotec, Miamisburg, OH), divided into three equal portions and given at 08:00 P.M., 11:00 P.M. and again at 03:00 A.M. Additional water ingested during the fast will be enriched to 0.5% with 2H₂O to prevent dilution of the isotopic steady state. In the T1DM subjects, a variable-dose infusion of short acting insulin will be continue overnight to achieve plasma glucose concentrations of 100-120 mg/dl prior to the study the next morning. **Day 2:** A stepped hypoglycemia clamp will be induced, similar as in Aim 1.

Rationale and Interpretation of the Results: These studies will enable us to differentiate the effects of α -adrenergic vs. β -adrenergic blockade (vs. combined blockade) during antecedent hypoglycemia/exercise on development of subsequent HAAF/EAAF in nondiabetic and T1DM subjects. Additionally, we will be able to quantify the contribution of additional μ -opioid receptor blockade to these effects, by comparing the counterregulatory responses of each study protocol. We anticipate that adrenergic and opioidergic blockade will result in normalization of hypoglycemia counterregulation after antecedent hypoglycemia or exercise. We anticipate that phentolamine and propranolol plus naloxone will achieve the most significant effect in eliminating HAAF/EAAF. We include euglycemic control studies in order to exclude the possibility that insulin may play a role in HAAF modulation. Similarly, we include non-exercise (rest) euglycemic studies for the same reason. The control studies will be also used as baseline experimentally-induced HAAF/EAAF for quantifying changes in all the other study arms in Aim 2. These studies, however, will not differentiate between the effects of α - and β -adrenergic subtype blockade due to lack of specificity of the phentolamine and propranolol. Based on our findings, however, further analyses of subtype of adrenergic receptor classes involved in the mechanism of HAAF/EAAF may be pursued. Exercise studies will be conducted on a reclining cycle ergometer with continuous blood pressure and heart rate monitoring.

Aim 3. To examine the efficacy of blocking opioid receptors during hypoglycemia and preventing HAAF in patients. *Summary: We propose to analyze the effects of acute intranasal naloxone during antecedent hypoglycemia on hypoglycemia counterregulation and on induction of HAAF in patients..*

Experimental Protocol: We will study 18 healthy subjects (see details in the Protection of Human Subjects Section). Each subject will participate in two, two-day study sets, separated by an interval of five weeks. Day 1 will include the induction of two, two-hour episodes of hypoglycemia, with a two hour rest in between during which the subject will receive a snack (no more than 10 grams of carbohydrate). Either intranasal naloxone or an intranasal placebo will be administered twice during each hypoglycemia episode on Day 1, at the start of insulin administration and after one hour. Day 2 will include a single

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two-hour hypoglycemic clamp. The order in which studies take place will be randomized. **Day 1:** At 08:00 A.M. all subjects will have two indwelling cannulae inserted. At 8:30 A.M., a primed-continuous infusion of insulin at a rate of 1.0 mU/kg/min will be given to rapidly lower the plasma glucose to 54 mg/dl and continued for a duration of two hours. The rate of insulin will not vary unless the subject does not attain a nadir of 54 mg/dl – in which case the insulin rate will be increased to a maximum infusion of 2.0 mU/kg/min by increments of 0.2 mU/kg/min. This is consistent with insulin infusion rates used in similar hypoglycemia studies in the literature. A variable infusion of 20% dextrose will be used as needed to prevent plasma glucose concentration from falling below 54 mg/dl. As we increase the insulin levels, we will administer oral potassium chloride tablets in order to prevent hypokalemia during the insulin infusion with a maximal daily dose of up to 80 mEq (split into two doses) on Day 1 and up to 40 mEq on Day 2. Blood glucose will be monitored every 5 minutes. Subjects will be given 4mg NARCAN® Nasal Spray in one nostril at the start of insulin infusion and again after 60 min.

At the end of the 2h insulin infusion, the insulin will be discontinued and dextrose will be infused to bring glucose concentration back to 90 mg/dl. All subjects will receive a small snack (10 grams of carbohydrate) and will rest for the next two hours, as in the original protocol. At that point we will repeat the primed continuous infusion of insulin and variable infusion of 20% dextrose as above for an additional 2 hour period. Subjects will again be given 4mg NARCAN® Nasal Spray in one nostril at the start of insulin infusion and again after 60 min. After completion of the second 2h infusion, insulin will be discontinued, subjects will receive a meal, and be monitored until glucose concentration returns to 90 mg/dl. They will then be allowed to return home but will have another overnight fast in preparation for Day 2.

The other set of studies will be similar with the exception that subjects will be given a placebo saline nasal spray in one nostril at the start of insulin infusion and again after 60 min.

Day 2: A single two-hour hypoglycemic clamp will be induced. At 08:00 A.M. all subjects will have two indwelling cannulae inserted. At $t = -120$ min, a primed-continuous infusion of 6-6 glucose (D2G) will be initiated with a bolus of 200mg/m² bolus followed by continuous infusion of 3.9mg/min for the entire period of study. The specific activity of infused dextrose will be kept equivalent to plasma glucose specific activity by addition of [3-3H]-glucose to the infusate, using the method of Finegood et al. (73). At $t = 0$ min, a primed-continuous infusion of insulin will be initiated at a rate of 1.0 mU/kg/min throughout the study, but may be raised to 2.0 mU/kg/min by increments of 0.2 mU/kg/min if needed to attain a plasma glucose of 54 mg/dl. A variable infusion of 20% dextrose will begin to prevent the plasma glucose concentration from falling below 54 mg/dl. As we increase the insulin levels, we will administer oral potassium chloride tablets in order to prevent hypokalemia during the insulin infusion with a maximal daily dose of up to 80 mEq (split into two doses) on Day 1 and up to 40 mEq on Day 2. During hypoglycemic episodes, subjects will be evaluated for symptoms of hypoglycemia (74), and plasma samples for 6-6-glucose, insulin, C-peptide, glucagon, epinephrine, norepinephrine, cortisol, growth hormone, free fatty acids, lactate and glycerol and β -endorphins will be obtained at 10-min intervals. 2H enrichment of the hydrogen bound to C2 and C5 of blood glucose will be collected at 30-min intervals (see Detailed Experimental Methods). At the end of the clamp ($t = 120$ min), all the infusions will be discontinued, and the subject will be given a meal and discharged from the CRC.

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Rationale and Interpretation of the Results: The Day 1 study involves two, two-hour episodes of hyperinsulinemia hypoglycemia at 54 mg/dl – designed to induce HAAF. This protocol mimics our previous studies in which HAAF was experimentally induced (or aggravated) in T1DM subjects (11). During Day 1 we will measure baseline counterregulatory responses to hypoglycemia including serum cortisol, growth hormone, glucagon, epinephrine, norepinephrine, insulin, c-peptide. Day 2 study is planned to assess subsequent hypoglycemia counterregulation (i.e. HAAF). Thus, during this visit we will again measure hormonal counterregulatory response including serum cortisol, growth hormone, glucagon, epinephrine, norepinephrine, insulin, c-peptide. Additionally using a standardized questionnaire we will monitor level of awareness of hypoglycemia by asking about eleven specific symptoms at each glucose step. Subjects will receive either intranasal naloxone (8mg) during each episode of hypoglycemia or a placebo saline nasal spray, in random order. We will compare the effects of counterregulatory response to hypoglycemia and awareness to hypoglycemia in subjects after receiving opioid receptor blockade during antecedent hypoglycemia vs placebo.

Sub-Aim of Aim 3:

SIGNIFICANCE:

Significance of HAAF in T1D: Hypoglycemia is a frequent, dangerous, and costly consequence of intensive insulin therapy. The benefits of attaining near-normal glycemic control have been well established by numerous studies (1). Despite technologic advances in insulin therapy provided by cutting edge insulin pumps and novel insulin formulations, many patients are still unable to achieve this goal due to the risk of iatrogenic hypoglycemia (2). The fear of hypoglycemia may lead to negative compensatory behaviors and poor compliance, undermining attempts at glycemic control (1). Additionally, hypoglycemia contributes to significant morbidity and even mortality, as it is estimated that 6-10% of deaths in patients with T1D may be attributed to hypoglycemia (3). Hypoglycemic events also take an enormous economic toll on the health care system. Each year, hypoglycemia accounts for an estimated 100,000 emergency room visits and 30,000 hospital admissions, with each event costing thousands of health care dollars in addition to lost productivity for the patients themselves (4). The risks of hypoglycemia are exacerbated by the presence of HAAF. As part of this syndrome, patients exposed to recent antecedent hypoglycemia may develop a blunting of the normal hormonal counterregulatory responses to hypoglycemia as well as loss of symptoms, ie. hypoglycemia unawareness (5). These two defects lead to a vicious cycle of recurrent hypoglycemia, which puts patients with HAAF at a 25-fold increased risk for severe hypoglycemia (5).

Significance of the Role of the Opioid System in HAAF: The ventromedial hypothalamus (VMH) contains a high concentration of pro-opiomelanocortin (POMC) neurons, which are glucose responsive neurons that produce beta-endorphins. Beta-endorphins, whose levels rise in response to hypoglycemia, bind μ -opioid receptors and postsynaptically modulate the excitability of local γ -aminobutyric acid (GABA) and dopamine neurons (6). Increased GABAergic tone in the VMH mediates the development of HAAF in rats, which is modulated by K_{ATP} channels (7). Of note, naloxone inhibits all three isoforms of the μ -opioid receptor (6). It has been determined by our research group and others that activating and inhibiting μ -opioid receptors can modulate HAAF in patients with T1D, leading to the promise of novel alternative

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therapies to ameliorate this condition (8). We and others have demonstrated that intravenous naloxone, an opioid receptor antagonist, ameliorates HAAF (8). Intriguingly, while overnight administration of oral naltrexone increased epinephrine responses to hypoglycemia in T1D (9), twice daily oral naltrexone for one month had no effect on hypoglycemic symptoms or counterregulatory responses (10). Such chronic, low doses of naltrexone may up-regulate opiate binding sites and lead to heightened sensitivity to endogenous beta-endorphins (11). **These findings underlie the rationale for the proposed use of intranasal naloxone only at the onset of a hypoglycemic episode rather than on a habitual daily basis.**

Significance of the Role of K_{ATP} channels in HAAF: It has been demonstrated in numerous rodent studies that hypothalamic K_{ATP} channels play an important role in detecting hypoglycemia (12). Specifically, POMC neurons in the VMH express a unique complement of inwardly rectifying potassium channels (Kir6.2) that allow them to respond to changes in ambient glucose. Diazoxide activates Kir6.2 channels in glucose-responsive neurons of the VMH, leading to neuronal hyperpolarization (6). Recently it was shown that T1D patients who received oral diazoxide over a 12 hour period prior to a hyperinsulinemic hypoglycemic clamp had heightened hormonal responses to hypoglycemia. Furthermore, the subjects who responded best to diazoxide carried an activating E23K polymorphism in the K_{ATP} channel, collectively suggesting that activation of K_{ATP} channels improves counter-regulatory responses in T1D patients with established HAAF (13). Intriguingly, in addition to Kir6.2 channels, other subtypes of POMC neurons express Kir3.1-3.4 channels that are complexed to the μ -opioid receptor in these cells. Indeed, some POMC neurons respond to both μ -opioid receptor activation and K_{ATP} channel activation by diazoxide, while others respond either to μ -opioid activation alone or to diazoxide alone (14). We have previously studied the impact of activating K_{ATP} channels with diazoxide on the regulation of endogenous glucose production by a brain–liver pathway in humans.

INNOVATION: Complementary neuronal response patterns raise the exciting possibility that μ -opioid receptor antagonism together with activation of K_{ATP} channels could have synergistic effects in patients with HAAF, and forms the justification for the study design in this proposal. The newly available intranasal formulation of naloxone offers the ability for patients to self-administer naloxone immediately at the onset of a hypoglycemic episode, with theoretical advantages of rapid systemic availability as well as a more targeted delivery toward the VMH. The combined use of these agents, a novel therapeutic approach that has never previously been investigated, will be studied in a rigorous manner using state-of-the-art hypoglycemic clamp methodologies which will provide crucial data on the effectiveness of this intervention.

RESEARCH APPROACH:

We will employ a randomized, placebo controlled, double blinded study design to examine the impact of intranasal naloxone and oral diazoxide (alone and in combination) on symptomatic and counter-regulatory responses to an episode of hypoglycemia in subjects with HAAF.

SPECIFIC AIM 1: To examine the efficacy of blocking opioid receptors with **intranasal naloxone** to prevent subsequent HAAF in normal subjects, we will analyze the effects of acute intranasal naloxone during antecedent hypoglycemia on hypoglycemia counterregulation, i.e. on induction of HAAF.

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Our **preliminary data** in n=12 non-diabetic subjects indicate that two 120 minute intervals of intravenous morphine (0.1µg/kg/min; a dose designed to replicate the beta endorphin rise during hypoglycemia) impacted their ability to respond to a 200 minute stepped hypoglycemic episode (nadir 60mg/dl) on the following day (15). Compared with saline, morphine induced a reduction in hypoglycemia-associated plasma epinephrine responses (30% reduction, at 60 mg/dL glucose step, 419.4 ± 20.4 pg/mL in the control studies and 292.5 ± 15.7 pg/mL in the morphine studies, $p=0.02$, **Figure 1**), in plasma glucagon responses (30% reduction, at 80 mg/dL glucose step, 31.7 ± 5.9 in the control studies vs. 22.2 ± 2.9 pg/ml in the morphine studies, $p=0.03$, **Figure 2**), a small reduction in Endogenous glucose production that reached significance at the 80 mg/dl glucose step ($p=0.04$, **Figure 3**) and hypoglycemic symptoms ($p=0.03$, **Figure 4**).

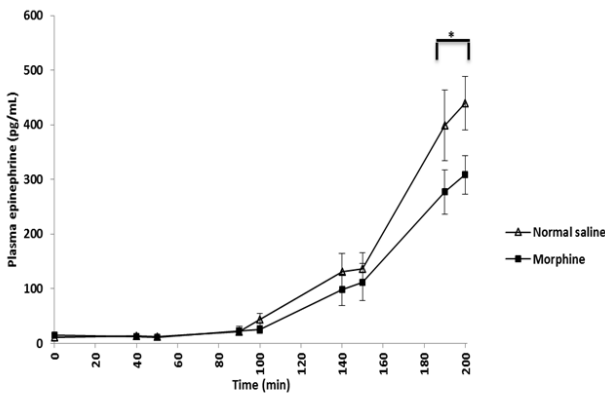


Figure 1 – Plasma epinephrine Concentrations

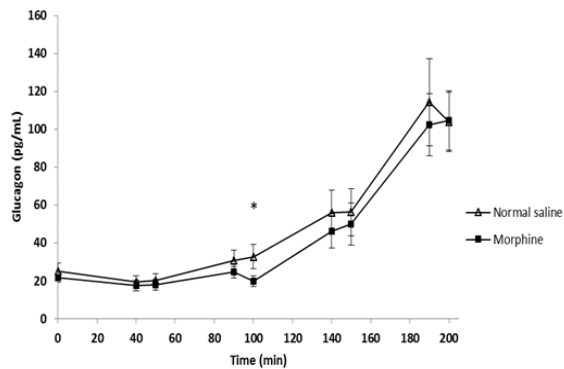


Figure 2- Plasma glucagon concentrations

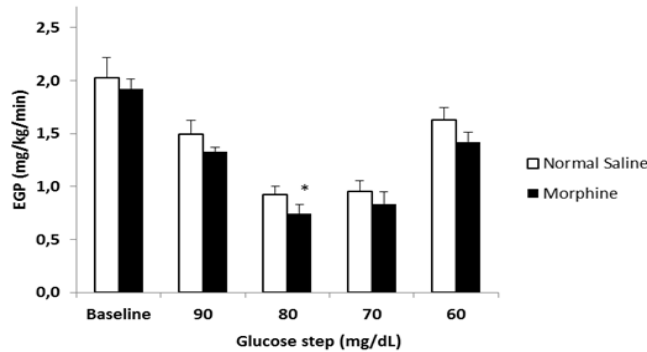


Figure 3- Endogenous Glucose Production

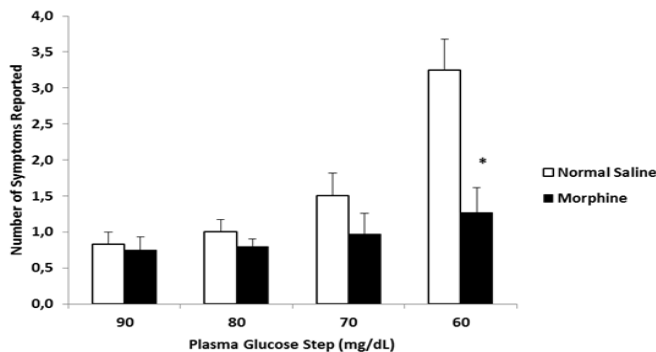


Figure 4- Hypoglycemia Symptoms Score

SPECIFIC AIM 2: To determine **whether diazoxide has synergistic effects with naloxone** to augment counter-regulatory responses during an episode of hypoglycemia and to prevent HAAF in normal subjects. We propose that the K_{ATP} channel activator diazoxide would have synergistic effects with naloxone in promoting counterregulatory responses to hypoglycemia, and we will therefore study the impact of diazoxide alone and in combination with naloxone in a placebo-controlled fashion on responses to hypoglycemia.

EXPERIMENTAL METHODOLOGIES:

SUBJECT RECRUITMENT: The subjects will be recruited by local and online advertising as well as from the Clinical Research Center (CRC) database. The purpose, nature, risks and benefits of the study will be explained to all subjects in the CRC prior to their enrollment in the study, and their voluntary, informed, written consent will be obtained. As described below, all subjects will have an initial screening visit to allow for the clinical evaluation of the subjects, including history, physical examination, hematologic, lipid, and chemistry screening (including fasting glucose levels), baseline EKG, and consent procedures.

STUDY POPULATION: Nondiabetic subjects 21-60 years of age and in general good health, taking no medications, with no family history of diabetes, and not participating in another study. All subjects with a history of diabetes mellitus, hyperlipidemia, hypertension, heart disease, cerebrovascular disease, seizures, bleeding disorders, or smoking will be excluded.

EXPERIMENTAL PROTOCOLS:

Screening: This visit will include a full history and physical examination and additional laboratory tests to determine eligibility, including serum electrolytes, BUN and creatinine,

PT/PTT, liver function tests and lipid profile, as well as a screening urinalysis, and baseline EKG.

Randomization: The pharmacist providing naloxone, diazoxide, and matched placebos to the CRC will ensure that each subject receives the experimental agents in random order, and the study agents will be identical in appearance (and in the case of diazoxide, in taste) with relevant placebos. The subject and the investigators will be blinded as to which agent the subject is receiving.

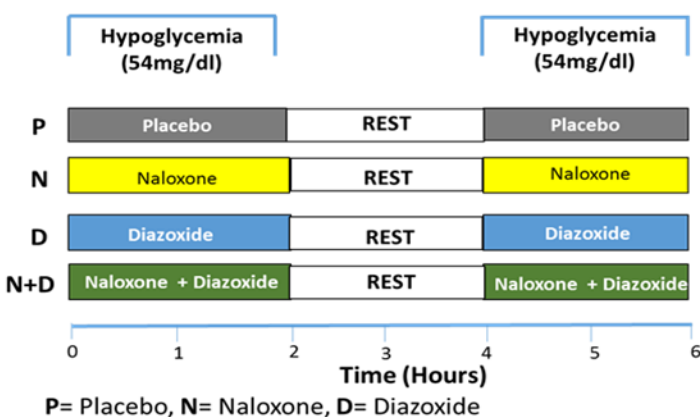
Specific Aim 1. We will determine whether administering intranasal naloxone vs. identically appearing saline spray (placebo) will prevent the onset of HAAF in normal subjects during the third of three episodes of experimental hypoglycemia.

Specific Aim 2. We will determine whether administering diazoxide vs. oral placebo will have synergistic effects with intranasal naloxone in preventing the onset of HAAF in normal subjects during the third of three episodes of experimental hypoglycemia.

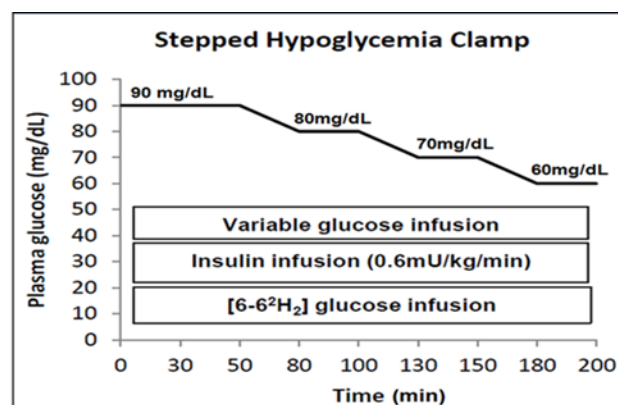
We will study 15 healthy non-diabetic subjects in each Aim. Each subject will participate in four, 2-day study sets, separated by an interval of five weeks. **For both Aims, descriptions of Days 1 and 2 are as follows:**

Day 1 will involve the induction of two, 2-hour episodes of hypoglycemia, with an intervening 2-hour rest period. Either intranasal naloxone or an intranasal placebo spray will be administered twice during each hypoglycemia episode on Day 1, at the start of insulin administration and after one hour.

At 08:00 A.M. all fasting subjects will have two indwelling cannulae inserted. At 8:30 A.M., a primed-continuous infusion of insulin at a rate of 1.0 mU/kg/min will be given to rapidly lower the plasma glucose to 54 mg/dl and continued for a duration of two hours (**Figure 5: Experimental Protocol Day 1**).



Experimental Protocol on Day 1.



Experimental Protocol on Day 2.

Figure 5. Experimental Protocol Day 1 and 2

The rate of insulin will not vary unless the subject does not attain a nadir of 54 mg/dl, in which case the insulin rate will be increased to a maximum infusion of 2.0 mU/kg/min by increments of 0.2 mU/kg/min. This is consistent with insulin infusion rates used in similar hypoglycemia studies in the literature (10). A variable infusion of 20% dextrose will be used as needed to prevent plasma glucose concentration from falling below 54 mg/dl. As we increase the insulin levels, we will infuse 20 mEq of potassium in order to replete potassium levels. Blood glucose

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will be monitored every 5 minutes. At the end of each 2h period of insulin infusion, the insulin will be discontinued and dextrose will be infused to bring the glucose concentration back to 90 mg/dl. All subjects will receive a small snack (10 grams of carbohydrate) and will rest for the next two hours. At that time we will repeat the primed continuous infusion of insulin and variable infusion of 20% dextrose as above for an additional 2 hour period. After completion of the second 2h infusion, insulin will be discontinued, subjects will receive a meal, and will be monitored until glucose concentration returns to 90 mg/dl. They will then be allowed to return home but will have another overnight fast in preparation for Day 2. Additionally, the following describe the medications that will be administered on Day 1 in the two Aims:

- **Aim 1.** We will determine whether administering intranasal naloxone will help to prevent HAAF. On Day 1, intranasal naloxone (4mg NARCAN® Nasal Spray) or placebo spray will be administered in one nostril twice during each hypoglycemia episode: at the start of insulin administration and after one hour. During the second period of hypoglycemia, subjects will again be given 4mg NARCAN® Nasal Spray or placebo spray in one nostril at the start of insulin infusion and again after 60 min.
- **Aim 2.** We will determine whether administering oral diazoxide (7 mg/kg orally), alone or together with intranasal naloxone, will help to prevent HAAF. On Day 1, subjects will be given oral diazoxide alone (7 mg/kg orally) or an oral placebo three hours before the first hypoglycemic episode ($t=-3h$). The timing of oral diazoxide ingestion is based on the available literature indicating that its hypotensive and antihypoglycemic effect lasted ~3–12 h, with a peak action at ~5 h (17). We will then determine whether administering intranasal naloxone together with diazoxide with hypoglycemia on Day 1 will have synergistic effects on counter-regulatory and/or symptomatic responses to hypoglycemia on Day 2. On Day 1, intranasal naloxone (4mg NARCAN® Nasal Spray) or placebo spray will be administered twice during each hypoglycemia episode on Day 1, at the start of insulin administration ($t=0h$ and $t=4h$) and after one hour ($t=1h$ and $t=5h$). *Oral diazoxide (7 mg/kg orally) or matched oral placebo will be administered three hours before the first hypoglycemia episode on Day 1 ($t=-3h$). Hence, the study types in Aim 2 will be: diazoxide with placebo spray, diazoxide with naloxone spray, and oral placebo with placebo spray.*

Day 2 will consist of a **stepped hyperinsulinemic hypoglycemic clamp**, as follows: euglycemia → 90 mg/dl → 80 mg/dl → 70 mg/dl → 60 mg/dl), with quantification of hormonal responses and glucose kinetics.

Stepped Hypoglycemic Clamp Studies: See the schematic depiction shown in **Figure 5 (Experimental Protocol Day 2)**. At 0800 h, subjects will have two indwelling catheters inserted. At $t = -120$ min, a primed continuous infusion of 6-6 glucose (D2G) tracer will be initiated (200mg/m² bolus followed by 3.9mg/min for the entire study period) to measure glucose fluxes. At $t = 0$ min, a primed continuous infusion of insulin will be initiated at a rate of 1.0 μ U/kg/min for the first 10 minutes and thereafter will be continued at 0.6 μ U/kg/min throughout the study. At $t = 10$ min, a variable infusion of 20% dextrose will also begin to maintain the plasma glucose concentration at 90mg/dL for 50 min. The specific activity of infused dextrose will be kept equivalent to plasma glucose specific activity by addition of 6-6 glucose to the infusate. At $t = 50$ min, and every 50 min thereafter, the plasma glucose concentration will be decreased by decrements of 10mg/dL for 50 min by reducing the dextrose infusion rate accordingly. Plasma glucose will be clamped at the desired range according to plasma glucose measured at 5-min intervals with targets of 90, 80, 70, and 60mg/dL. Blood samples will be obtained for determinations of plasma insulin, C-peptide, glucagon, epinephrine, norepinephrine, and cortisol, as well as for glucose turnover. Symptoms of hypoglycemia will be measured at each glucose step using the Edinburgh Hypoglycemia Score (16). At $t = 200$ min all infusions will be discontinued, a meal will be provided and plasma

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glucose will be monitored for at least 1 hour to ensure restoration of euglycemia prior to subjects being discharged.

RATIONALE AND INTERPRETATION OF DATA:

The Day 1 study involves two, 2-hour episodes of hyperinsulinemic hypoglycemia with a target plasma glucose level of 54 mg/dl. This protocol mimics previous studies by our group and others in which HAAF was experimentally induced (or aggravated) in T1D and non-diabetic subjects (5, 8). In our current studies, plasma glucose levels fall in a steady, rapid manner to 54 mg/dl over about 35 to 40 minutes and are then maintained tightly at that target, such that hypoglycemia of ~54 mg/dl is maintained for nearly 90 minutes.

During Day 1 we will measure baseline counterregulatory responses to hypoglycemia including serum cortisol, growth hormone, glucagon, epinephrine, norepinephrine, insulin, C-peptide.

The Day 2 study is planned to assess subsequent hypoglycemia counterregulation (i.e. HAAF) and symptomatic responses to hypoglycemia. Thus, during this visit we will again measure hormonal counterregulatory response including serum cortisol, growth hormone, glucagon, epinephrine, norepinephrine, insulin, and C-peptide. Additionally we will monitor level of awareness of hypoglycemia using a standardized questionnaire regarding eleven specific symptoms at each glucose step (16). We will compare the effects of counterregulatory response to hypoglycemia and awareness to hypoglycemia in the same subjects after receiving opioid receptor blockade versus placebo during the antecedent two intervals of hypoglycemia.

It is important to note that **these efficacy studies** will examine the impact of these agents alone and in combination on the development of HAAF in response to experimentally-induced hypoglycemia in non-diabetic subjects. The ultimate proposed approach for actual T1D subjects in the 'real world' setting is that naloxone would be administered intranasally right at the onset of hypoglycemia, while oral diazoxide would be given chronically. While, as noted above, there is evidence for compensatory upregulation of opioid receptors in response to their chronic inhibition, there is no evidence for such a phenomenon with longterm diazoxide. Furthermore, giving diazoxide chronically to patients with T1D would not be associated with the prominent effects of diazoxide on insulin secretion that would be observed in non-diabetic subjects.

Analytical Methods: Plasma glucose will be measured at the bedside during clamp studies by a Beckman glucose analyzer (Beckman Coulter, Fullerton, CA), using the glucose oxidase method. Measurements of plasma insulin, C-peptide, glucagon and cortisol concentrations will be measured by radioimmunoassay in the Diabetes Research Center Hormone Assay Core, as previously reported (18). For the D-[6,6-²H₂] glucose determinations, Gas Chromatography-Mass Spectroscopy (GC-MS) analysis will be performed at **the Albert Einstein College of Medicine Diabetes Research Center Core Facility** as previously described (19). Plasma samples for GC-MS will be derived after protein precipitation to the aldehyde penta-acetate with hydroxylamine hydrochloride acetic anhydride. Plasma epinephrine and norepinephrine levels will be determined using high performance liquid chromatography (Quest Diagnostics, Chantilly, VA).

Steele's equation will be used for calculation of glucose turnover (20). Values for endogenous glucose production (EGP) and glucose rate of disappearance (R_d), will be obtained at 10-min intervals, were averaged over the final 30-min of each glucose step for each individual subject. The glycemic threshold for activation of a particular hormone will be calculated as the glycemic level at which there was an increase of more than 2 SD values above basal concentration.

Statistical Analyses and Power Calculation: All of the statistical analyses will use a two-tailed alpha of 0.05 for statistical significance and will be performed with STATA and SPSS

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software. In the event that the data for any of these analyses violate the normality assumptions of the ANOVA, non-parametric analyses will be used. Statistical analyses will be performed with the assistance of the CTSA biostatistician Hillel W. Cohen, Dr. Ph., with whom we have collaborated in the past.

Aim 1. The primary outcomes are epinephrine release, EGP and hypoglycemic symptoms after antecedent hypoglycemia with intranasal naloxone or placebo administration. In our prior study, using IV naloxone, during the 60-mg/dl glucose step, plasma epinephrine increased significantly in the Naloxone group compared with the placebo group (459% vs. 196% of baseline values, $P < 0.05$)(8). We would anticipate a 3-fold increase in epinephrine release with intranasal naloxone. A sample size of 15 would therefore achieve 80% power to detect a difference between conditions in this aim.

Aim 2. The primary outcomes are epinephrine release, EGP and hypoglycemic symptoms at a glucose level of 54 mg/dl after antecedent hypoglycemia with oral diazoxide or intranasal naloxone and oral diazoxide administration. Prior rodent studies showed a ~3-fold increase in counterregulatory epinephrine release after ICV diazoxide compared with vehicle. Since humans may utilize alternate fuel sources in the brain in the face of hypoglycemia, we may not see as dramatic a response to diazoxide. Thus, a conservative estimate would anticipate a 1.5-2 fold increase in epinephrine release with diazoxide. A sample size of 12 would achieve 80% power to detect such a difference between conditions in this aim.

POTENTIAL FOR TRANSLATION INTO THERAPY: Based upon the Specific Aims proposed above, we anticipate that the combined administration of intranasal naloxone and oral diazoxide will have synergistic effects on hypoglycemia-associated hormonal and symptomatic responses. This combined regimen should, therefore, have considerable therapeutic potential for ameliorating HAAF in patients with T1D.

DETAILED EXPERIMENTAL METHODS

EGP: We have decided to use [3-3H]-glucose as a glucose tracer (instead of deuterated glucose which is non-radioactive) in order to permit the co-analysis of gluconeogenesis (see 2H₂O method). In case there will be no significant difference in the contribution of gluconeogenesis to EGP between the T1DM and nondiabetic subjects, we will use deuterated glucose (D-glucose-6,6-2d₂) for EGP determinations. Plasma [3-3H]-glucose radioactivity will be measured in duplicate on the supernatants of barium hydroxide-zinc sulfate precipitates (Somogyi procedure) of plasma samples after evaporation to dryness to eliminate tritiated water. Plasma tritiated water specific activity will be determined by liquid scintillation counting of the protein-free supernatant (Somogyi filtrate) before and after evaporation to dryness. These determinations will be performed in the PI's laboratory. Rates of glucose appearance (Ra) and glucose disappearance (Rd, or glucose uptake) will be calculated using Steele's steady state equation (81). Rates of endogenous glucose production (EGP) will be determined by subtracting rates of glucose infusion from the tracer-determined Ra.

²H₂O method: Body water will be calculated as 50% of body weight in women and 60% in men (82). Enrichment of the hydrogen at C2 and C5 of blood glucose will be determined by procedures employed by us previously (78). ZnSO₄ (0.3N) and Ba (OH)₂ (0.3N) will be added to the blood to precipitate proteins and the mixture will be centrifuged. The supernatant will be deionized by passage through a column of AG 1-8 X in the formate form and AG 50W –8 X in the hydrogen form (Bio-Rad Laboratories,

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Hercules, CA). Glucose in the effluent will be isolated by HPLC using a Bio-Rad HPX –87P column with water as solvent at 80 °C and a flow rate of 0.5 ml/min. To convert plasma glucose into monoacetone glucose (MAG), the dried residue will be suspended in 10ml acetone containing 0.4 ml concentrated sulfuric acid (for 20 ml v). The mixture will be stirred for 4 h at room temperature to yield diacetone glucose. After filtering off any remaining precipitate and adding 10 mL of water, the pH will be adjusted

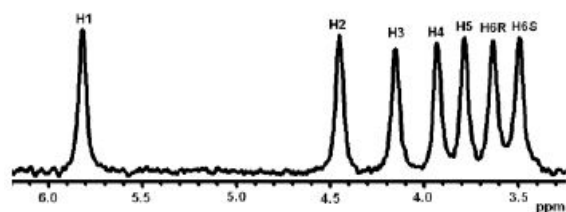


Figure 8. ^2H -NMR spectrum of monoacetone glucose (MAG) derived from human plasma glucose

to 2.0 by dropwise addition of NH_4OH solution. The mixture will be stirred for 24 h at room temperature to convert diacetone glucose into MAG. The pH will be then further increased to 8.0 using NH_4OH and the sample will be dried. MAG will be extracted with 3mL of hot ethyl acetate. These solutions will be combined and the ethyl acetate will be removed by vacuum evaporation. The resulting MAG will be further purified by passage through a 2-mL cartridge using

100% acetonitrile as eluant, followed by dl water. The effluent will be freeze-dried and stored dry prior to NMR analysis (83). The dried MAG sample will be dissolved in 120 ml deuterium-depleted water and 420 ml HPLC acetonitrile into 5mm NMR tube. A ^2H NMR spectrum will be collected at 14.1 T (92 MHz for ^2H) on a Bruker DRX600 system equipped with a 5-mm deuterium probe with the ability to do ^1H decoupling and ^{19}F lock and with a z-gradient. Deuterium will be tuned to the ^2H carrier frequency. Shimming will be performed on the ^1H signal using the automated gradient shimming routine. ^1H Waltz-16 decoupling will be used during acquisition to remove proton-deuterium J coupling. Data will be collected over a spectral width of 1.8 KHz at 47°C using the 90° pulse, 1.1-sec acquisition time and an average of 5000-10,000 acquisition over 2-4 h. Peak areas are analyzed using the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR Inc., Fremont CA). Given that the acquisition time is 1.1 sec and the longest T_1 of any deuterium in MAG is less than 0.25 sec, no postacquisition correction of peak areas is necessary (84).

$^2\text{H}_2\text{O}$ -determined rates of total glycogenolysis and gluconeogenesis: Fractional contributions of GNG and total glycogenolysis will be estimated from the ratios of the ^2H enrichments of the hydrogens bound to C5 and C2 of blood glucose after oral intake of $^2\text{H}_2\text{O}$ (as we previously reported, (78)). Rates of GNG will be calculated by multiplying those fractional contributions by the rates of glucose production. The rate of total hepatic glycogenolysis will be calculated by subtracting the rates of GNG from the rate of glucose production.

Threshold and magnitude of hormone release calculations: The glycemic thresholds for activation of counterregulatory hormone secretion (epinephrine, glucagon, norepinephrine, growth hormone and cortisol) will be calculated as the glycemic concentration at which there was a significant increase in the plasma hormone concentration as described previously (6). We will define a significant increase in plasma hormone concentration as an increase of at least two standard deviations above the baseline level (before the initiation of hypoglycemia). The magnitude of hormone release will be determined as the highest plasma concentration achieved during the hypoglycemia clamp, and we will also calculate

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the area under the hormone / time curve (using the trapezoidal method) to express the magnitude of hormone release over time.

Infusions: Epinephrine (Adrenalin; JHP Pharmaceuticals, Parsippany, NJ), Morphine sulfate (Hawkins Pharmaceutical Group, Minneapolis, MN), Naloxone (Narcan; Du Pont Pharmaceuticals, Wilmington, DE), Propranolol HCl (Inderal®, Wyeth Ayerst Laboratories, Philadelphia, PA), and Phentolamine Mesylate (Regitine®) - all will be diluted in normal saline under sterile conditions.

Plasma Hormone Assays: Plasma hormone assays (cortisol, growth hormone, glucagon, insulin, C-peptide) will be performed in the Hormone Assay Core Laboratory of the DRTC, as previously reported (85). Plasma epinephrine and norepinephrine will be assayed using a single radioimmunoassay technique in the PI's laboratory as previously reported (9). Plasma β -endorphin will be measured using ELISA Kit (MD Bioproducts, St Paul, MN) in the Analytic Core Laboratory of the ICTR/CTSA.

Intranasal Naloxone: Intranasal naloxone (NARCAN® (naloxone hydrochloride) Nasal Spray; Adapt Pharma, Radnor, PA) may be associated with nasal dryness, nasal edema, nasal congestion and nasal inflammation. This route of administration of naloxone would be expected to reduce the risks relative to intravenous administration of this agent, as described in the original grant.

Potassium Chloride Tablet: Rarely, potassium chloride tablets may cause stomach upset (such as nausea, vomiting, diarrhea, abdominal pain, discomfort) and extremely rarely they can lead to stomach or bowel ulceration, bleeding, perforation, or obstruction.

STATISTICAL ANALYSES AND POWER CALCULATION

Aim 1. The primary parameter of interest is the EGP response after antecedent morphine, epinephrine or a combination of morphine and epinephrine infusion. We will perform three analyses of this response. The difference in Day 2 EGP (compared to Control) with and without - morphine, epinephrine and morphine plus epinephrine infusion on Day1, will be analyzed with one way repeated measures analysis of variance. Statistically significant results will be analyzed using post-hoc paired tests. For the sample size estimation we chose this last comparison because in the preliminary studies this analysis had the smallest effect size, with a difference of Δ 's between groups of 0.4 ± 0.2 mg/kg/min (mean \pm SD, EGP). A sample size of 10 subjects per group will yield 89% power for this comparison.

Aim 2. The primary parameter of interest is the EGP response after antecedent hypoglycemia or exercise with and without infusion of phentolamine, propranolol, and phentolamine+propranolol. As with aim 1 above, we will perform three analyses of this response with one way repeated measures analysis of variance. The difference between these Δ 's with and without co-infusion of naloxone will be analyzed as the interaction effect of a two-way analysis of variance with repeated measures. A sample size of 12 subjects per group will yield 83% power for this comparison.

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Aim 3. The primary parameter of interest is the EGP response after antecedent hypoglycemia with intranasal naloxone or placebo administration. For this comparison we will use an unpaired student's t-test. A sample size of 18 subjects will yield 92% power for this comparison.

Sample size calculations for the above analyses were performed by the ICTR/CTSA biostatistician Hillel W. Cohen, Dr. Ph, using NCSS-PASS software. All of the statistical analyses will use a two-tailed alpha of 0.05 for statistical significance and will be performed with STATA and SPSS software. In the event that the data for any of these analyses violate the normality assumptions of the ANOVA, non-parametric analyses will be used.

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