Study Title: Novel Approach for the Prevention of Hypoglycemia Associated Autonomic Failure (HAAF)

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Novel approach for the prevention of hypoglycemia associated autonomic failure (HAAF)

OVERVIEW:

Intensive glucose control in type 1 diabetes (T1D) is associated with clear health benefits. However, despite the 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. T1D patients are susceptible to hypoglycemia due to defective counterregulatory responses (particularly blunting of peak epinephrine levels), and in fact recurrent episodes of hypoglycemia lead to hypoglycemia-associated autonomic failure (HAAF) in ~2/3 of individuals. Thus, in addition to the mortality and morbidity of hypoglycemia itself, the risk of hypoglycemia in T1D impedes ideal insulin treatment and leads to suboptimal glycemic control. Over the past several years, our group and others have explored mechanisms responsible for HAAF in a quest for pharmacological approaches to modulate the counterregulatory response to hypoglycemia.

Both hypoglycemia and stress stimuli lead to activation of the opioid system. Our recently published data, presented below, indicate that activation of opioid receptors with morphine down-regulates the adrenergic response to a subsequent episode of hypoglycemia. Furthermore, studies in animals and humans have shown that blocking opioid receptors with intravenously (IV) administered naloxone during hypoglycemia induces an augmentation of the counterregulatory response. **These findings suggest that the opioid system plays a modulatory role in hypoglycemia counterregulation and could be manipulated pharmacologically.**

Since the counterregulatory response to hypoglycemia represents a complex system of hypoglycemia sensing, integration, and hormonal responses, other mechanisms may act synergistically to modulate counterregulation and HAAF. Indeed, it has been demonstrated in rodent studies that hypothalamic K_{ATP} channels play an important role in detecting hypoglycemia. Specifically, neurons in the ventral medial hypothalamus (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, and administration of diazoxide reduces HAAF in rodent models and in patients with T1D. Intriguingly, some subtypes of VMH neurons express K_{ATP} channels that are complexed to the μ-opioid receptor in these cells. Indeed, some VMH 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.

The ***overarching goal*** of this proposal is to develop a novel and clinically feasible strategy to ameliorate HAAF in T1D. We believe that recent advances in the study of HAAF offer new approaches that could act synergistically to prevent or reduce HAAF in the outpatient setting. Specifically, we will employ a newly available intranasal formulation of the μ-opioid receptor antagonist naloxone to rapidly inhibit beta-endorphins during hypoglycemia, thereby heightening counter-regulatory responses to hypoglycemia. Intranasal administration couples rapid systemic absorption with the potential for increased delivery to the VMH, where the pathological processes leading to HAAF occur. Such rapid delivery would permit patients to self-administer naloxone at the onset of a hypoglycemic episode, thereby impacting responses to subsequent episodes. Furthermore, given the apparently complementary role of K_{ATP} channels and opioid receptors in hypothalamic glucose-sensing neurons, we propose that the K_{ATP} channel activator diazoxide will have synergistic effects with naloxone in promoting the counterregulatory responses to hypoglycemia. **Our ultimate goal is to develop an effective and feasible 'real world' approach to impact the pathogenesis of HAAF that will restore life-sustaining responses to hypoglycemia in patients with T1D.** In light of evidence that HAAF only develops in about 2/3 of normal subjects exposed to repeated episodes of experimentally-induced hypoglycemia, an important aspect of this proposal will be to first identify individuals who are susceptible to the development of HAAF.

SPECIFIC AIMS:

SPECIFIC AIM 1: (Subject Selection) To identify healthy subjects who develop HAAF as a result of recurrent episodes of hypoglycemia, and are therefore eligible for this experimental protocol, we will compare peak plasma epinephrine levels during the first hypoglycemic clamp vs. the third hypoglycemic clamp. On Day 1, each subject will undergo two 2-hour hypoglycemic clamps (target plasma glucose ~54 mg/dL) and on Day 2 they will have one identical 2-hour hypoglycemic clamp. As suggested in previously published studies (1), as well as in our preliminary data (See below), there is substantial inter-individual variability among individuals' responses to hypoglycemia and subsequently the development of HAAF. Indeed, it is likely that only ~2/3 of individuals will develop HAAF in response to recurrent hypoglycemia. In order to study interventions to prevent HAAF, it is necessary to first identify those subjects who are susceptible to developing HAAF, ie. who demonstrate a significant blunting in the epinephrine response to hypoglycemia between the first hypoglycemic clamp vs. the third hypoglycemic clamp. These eligible subjects will be sequentially enrolled in Aims 2 and 3.

SPECIFIC AIM 2: 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. Our recently published data indicate that pharmacologic activation of opioid receptors can experimentally recapitulate some features of HAAF. Having demonstrated that opioid antagonism with intravenous naloxone can reverse experimentally induced HAAF in healthy subjects, *we hypothesize that intranasal naloxone will be effective and feasible for preventing the development of HAAF in an outpatient setting.*

SPECIFIC AIM 3: To determine whether diazoxide has synergistic effects with naloxone to prevent HAAF in normal subjects. Given the relationship between K_{ATP} channels and μ-opioid receptors in glucose-sensing neurons, we propose that the K_{ATP} channel activator diazoxide would have synergistic effects with naloxone in promoting counterregulatory responses to hypoglycemia. *We hypothesize that these complementary approaches will augment hypoglycemia-associated counterregulation to a greater degree than either agent alone, and hence expect that HAAF will be prevented or ameliorated by their combined use.*

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 (2). 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 (3). The fear of hypoglycemia may lead to negative compensatory behaviors and poor compliance, undermining attempts at glycemic control (2). 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 (4). 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 (5). 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 (6). 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 (6).

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 (7). Increased GABAergic tone in the VMH mediates the development of HAAF in rats, which is modulated by KATP channels (8). Of note, naloxone inhibits all three isoforms of the μ-opioid receptor (7). 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 therapies to ameliorate this condition (9). We and others have demonstrated that intravenous naloxone, an opioid receptor antagonist, ameliorates HAAF (9). Intriguingly, while overnight administration of oral naltrexone increased epinephrine responses to hypoglycemia in T1D (10), twice daily oral naltrexone for one month had no effect on hypoglycemic symptoms or counterregulatory responses (11). Such chronic, low doses of naltrexone may up-regulate opiate binding sites

and lead to heightened sensitivity to endogenous beta-endorphins (12). **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 KATP channels in HAAF: It has been demonstrated in numerous rodent studies that hypothalamic KATP channels play an important role in detecting hypoglycemia (13). 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 (7). 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 KATP channel, collectively suggesting that activation of KATP channels improves counter-regulatory responses in T1D patients with established HAAF (14). 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 KATP channel activation by diazoxide, while others respond either to μ-opioid activation alone or to diazoxide alone (15). We have previously studied the impact of activating KATP 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 KATP 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 nalox-one offers the ability for patients to self-administer naloxone immediately at the onset of a hypoglycemic epi-sode, 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.

EXPERIMENTAL APPROACH AND METHODS:

Preliminary Data:

Inter-individual variability of HAAF: Previously published studies (1) as well as our preliminary data in n = 13 healthy subjects indicate that there may be significant variability among subjects' responses to hypoglycemia and the subsequent development of HAAF. Of the n = 13 subjects we studied, 11 subjects responded to hypoglycemia (defined as peak epinephrine level during the first or second episode of hypoglycemia, in placebo studies, higher than 400 pg/mL). Out of these 11 subjects, 8 subjects developed HAAF (defined by reduction in epinephrine levels of at least 30% in the third hypoglycemic clamp compared to the first and second hypoglycemic clamps).

Impact of opioid receptor activation on pathogenesis of HAAF: Our ***recently published data*** (16) 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 (16). 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$\pm$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 (at 60 mg/dl step; p=0.03, **Figure 4**).

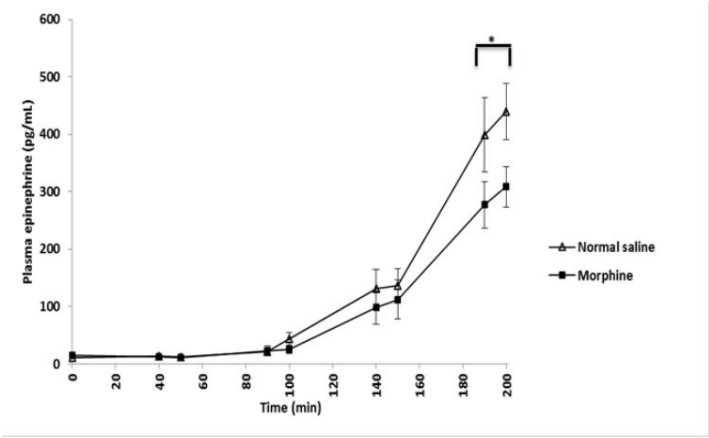


Figure 1 – Plasma epinephrine Concentrations

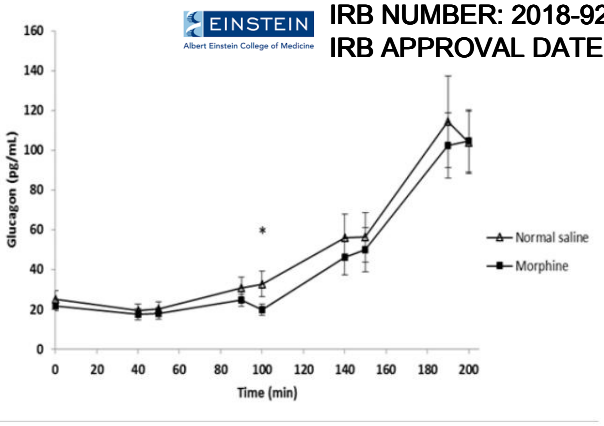


Figure 2- Plasma glucagon concentrations

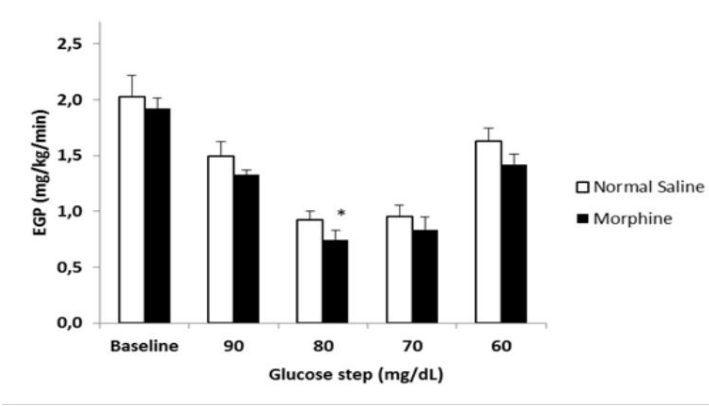


Figure 3- Endogenous Glucose Production

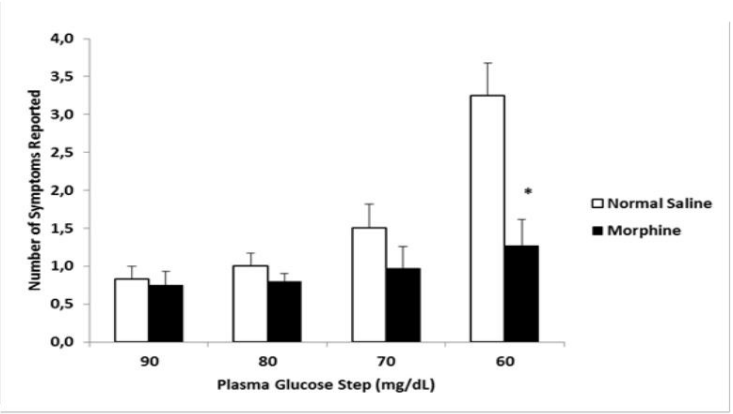


Figure 4- Hypoglycemia Symptoms Score

Opioid Receptor Blockade with Intranasal Naloxone Prevents the Development of Hypoglycemia-Associated Autonomic Failure: Having demonstrated that opioid antagonism with intravenous naloxone can prevent experimentally induced HAAF in healthy subjects, we hypothesized that acute self-administration of intranasal naloxone could be an effective and feasible 'real-world' approach to ameliorate HAAF in T1D. Furthermore, we hypothesize that intranasal naloxone could augment central blockade of opioid receptors by potentially bypassing the blood-brain barrier.

Using a randomized, double-blinded placebo-controlled study design, 7 healthy subjects (7M; age 43±3 yr; BMI 25.9±0.9 kg/m^2) previously demonstrated to develop HAAF participated twice (5 weeks apart) in paired two-day studies. Day 1 consisted of two 120-minute hypoglycemic (~54 mg/dl) hyperinsulinemic clamps, with hourly intranasal administration of either saline (placebo) or naloxone (4 mg per dose). On Day 2, all subjects underwent one 120-minute hypoglycemic clamp (~54 mg/dl). As demonstrated in Figure 5A, blood glucose levels were very tightly maintained at target levels for the final 75 minutes of the study.

As expected based upon their screening studies, HAAF was experimentally induced in the placebo studies, based on lower epinephrine levels (peak epinephrine: first hypoglycemic episode (mean±SEM) = 1375±182 vs. third episode = 858±235, pg/ml, p=0.004). With intranasal naloxone, epinephrine levels did not differ from the first to the third hypoglycemic episode (first episode = 942±190 vs. third episode = 857±134 pg/ml, p=0.4). Naloxone did not alter the responses of plasma glucagon, cortisol or growth hormone ,or hypoglycemic symptoms.

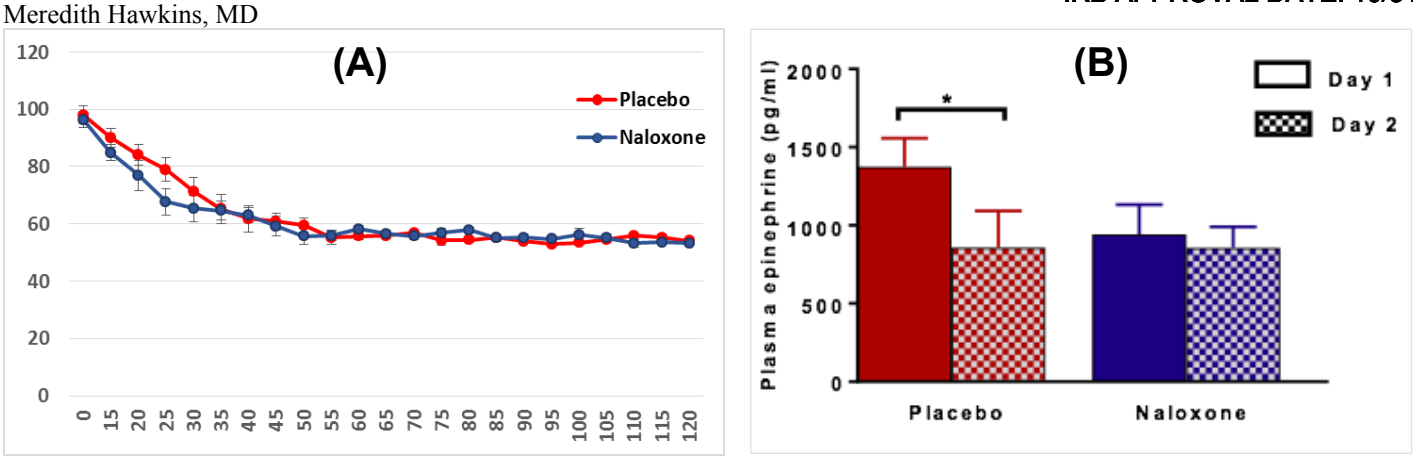


Figure 5. Average blood glucose levels during 2nd episode of hypoglycemia on Day 1 (A) Average epinephrine level during 1st episode of hypoglycemia on Day 1 vs. 3rd episode of hypoglycemia on Day 2 in both placebo and naloxone studies (B)

Collectively, these findings suggest that development of HAAF can be prevented by blockade of opioid receptors contemporaneous with hypoglycemia, presumably by inhibiting opioid receptor activation by circulating endorphins.

EXPERIMENTAL METHODOLOGIES:

Subject Recruitment: The subjects will be recruited by local and online advertising as well as from the clinical research center (CRC) database. All subjects will be screened for eligibility prior to enrollment into the study. 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 at the CRC suite at Albert Einstein College of Medicine, to allow for the clinical evaluation of the subjects, including history, physical examination, hematologic, lipid, urine, and chemistry screening, baseline EKG, and consent procedures. *Additional details of the inclusion and exclusion criteria for these studies are described under Human Subjects.*

General Experimental Conditions and Randomization:

Screening: This visit at the Einstein CRC will include a full history and physical examination, blood and urine analyses, baseline EKG, and consent procedures. A blood draw (~35 cc/7 tsp) and urine collection will be performed and the following labs will be sent to accessioning for all participants: CBC, LFT, lipid profile, basic metabolic panel, HbA1c, insulin, PT/PTT, urinalysis, urine drug screen. Additionally, a urine pregnancy test will be sent for all women of childbearing age. If recent documentation of these lab tests is available from the participant's primary care or other physician, the study doctor may decide to use these results, depending on when the previous labs were done, in place of those listed above and if participant consents to providing a copy of these for their study file. Urine drug tests and urine pregnancy tests will be repeated each morning of the study and the subject will be withdrawn from the study if either is positive (see below for indeterminate results). The subject will be notified by telephone or in writing of his/her eligibility status after his/her chart has undergone review by the P.I. or fellow.

Randomization: Each subject will receive the experimental agents in random order as determined by the study coordinator (using the envelope method). The subject and the other study investigators will be blinded as to which agent the subject is receiving.

Drug Storage: All medications received from both Weiler Pharmacy and from external pharmacies will be logged. Each study protocol will have its own drug binder with the medication log. Lot number, expiry date, and date of usage will be documented for all medications and additionally, date received and location of storage will be documented for medication obtained outside of Weiler pharmacy. Additionally, key personnel who prepared IV bags will be recorded for each clamp study.

Study Procedures:

We will employ a randomized, placebo controlled, cross-over, 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 three successive episodes of hypoglycemia in subjects who have been identified to be susceptible to developing HAAF.

Specific Aim 1. We will identify healthy subjects who develop HAAF as a result of antecedent hypoglycemia in our experimental protocol. We will study around 45 healthy non-diabetic subjects for the Subject Selection Aim (Aim 1) in order to attain 15 subjects for Aim 2 and 15 subjects for Aim 3, with the understanding that 66%-75% of subjects studied in Aim 1 are likely to develop HAAF and not all subjects recruited to Aims 2 and 3 will complete all studies. If the subjects studied in Aim 2 consent to participate in more studies, they may be studied again in Aim 3, in which case they will not undergo the Subject Selection Aim (Aim 1) again. Eligible subjects identified in Aim 1 will be recruited sequentially into Aims 2 and 3.

For Aim 1, the design of Day 1 and Day 2 is as in the other aims, with the exception that the subject will undergo only one 2-day study.

Specific Aim 2. We will determine whether administering intranasal naloxone will prevent the onset of HAAF in normal subjects during the third of three episodes of experimental hypoglycemia.

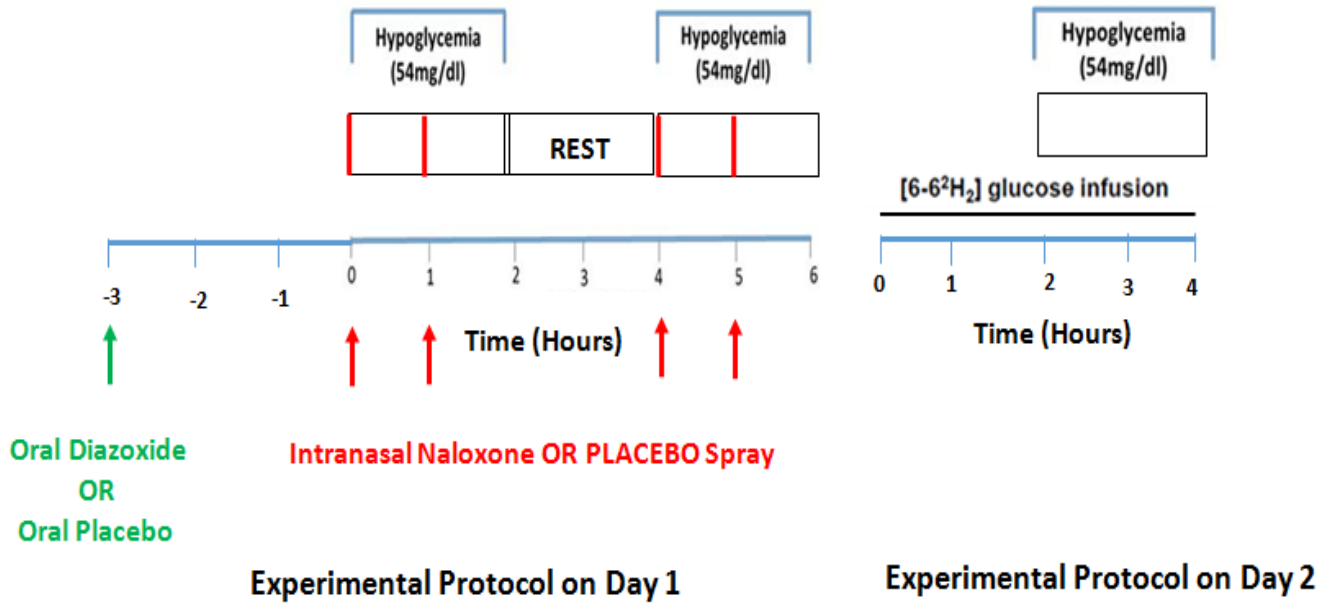
Specific Aim 3. We will determine whether administering diazoxide will have synergistic effects with intranasal naloxone in preventing the onset of HAAF in normal subjects during the third of three episodes of experimental hypoglycemia.

For all Aims, descriptions of Days 1 and 2 are as follows:

Each subject will undergo three hypoglycemic clamp studies over two days. Day 1 will involve the induction of two, 2-hour episodes of hypoglycemia, with a target blood glucose level of 54 mg/dL. There will be a two hour rest period between hypoglycemic clamps, during which the subject will receive a snack (12 grams of carbohydrate) and IV dextrose to normalize the blood glucose. In Aims 2 and 3, either intranasal naloxone or an intranasal placebo spray will be administered twice in a double blinded fashion during each hypoglycemia episode on Day 1, at the start of insulin administration and after one hour (see Figure 5). In Aim 3, oral diazoxide or placebo will also be administered in a double blinded fashion, three hours before the onset of the first hypoglycemic clamp. Day 2 will consist of an identical single two-hour hypoglycemic clamp, with no administration of medication or placebo. The order in which studies take place in Aims 2 and 3 will be randomized.

Hypoglycemic Clamp Procedures: At approximately 08:00 A.M. of each study day, all subjects will arrive fasting (from 10 pm the night before) to the CRC study room, will have their weight and vital signs checked and will undergo point-of-care pregnancy (for women in reproductive age) and urine drug testing. After that, one indwelling intravenous cannula will be inserted in each arm, and heparin (1000U) will be added to saline infusions (1000 cc) to keep intravenous lines patent. At approximately 9 A.M. (Aims 1 and 2) or at approximately 11:00 AM (Aim 3), a continuous infusion of insulin (100 U/1000 cc of normal saline which also includes 25 cc of human albumin) at a rate of 1.6-1.8 mU/kg/min will be initiated to rapidly lower the plasma glucose to 54 mg/dl and will be continued for a duration of two hours (See Figure 6). As noted below, the later start time in Aim 3 reflects the time of administration of diazoxide or matched placebo.

Figure 6: Experimental Protocol Day 1 & Day 2



The rate of insulin infusion will not vary unless the subject does not attain a nadir of 54 mg/dl, in which case the insulin infusion rate will be increased by increments of 0.2 mU/kg/min to a maximum infusion of 2.0 mU/kg/min. This is consistent with insulin infusion rates used in similar hypoglycemia studies in the literature (11). A variable infusion of 20% dextrose will be used as needed to prevent the plasma glucose concentration from falling below 54 mg/dl. As demonstrated in our preliminary data, our research team is highly experienced with the conduct of these studies and the range of blood glucose levels is usually tightly maintained in the range of 51-57 mg/dl (see Figure 5A) throughout the hypoglycemic phase of the studies. At the onset of the insulin infusion, subjects will receive oral potassium chloride powder packets dissolved in water in order to prevent hypokalemia during the insulin infusion, with a total daily dose of 80 mEq (split into two doses, 40 mEq at the start of each hypoglycemic episode) on Day 1 and 40 mEq on Day 2. Potassium chloride powder was chosen as the preferred form of oral potassium replacement since it is approved to be used in a single oral dose of 40mEq and it has a pleasant, sugar-free orange flavor. Blood glucose will be monitored every 5 minutes.

At the end of each 2-hour period of insulin infusion, 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 (12 grams of carbohydrate) and will rest for the next two hours. At that time, we will repeat the continuous infusion of insulin and variable infusion of 20% dextrose as above for an additional 2-hour period. Blood samples may be collected during both hypoglycemic clamps in the 15-min intervals for insulin, C-peptide, glucagon, growth hormone, epinephrine, norepinephrine, beta endorphins, free fatty acids, glycerol and lactate. Blood samples for glucose will be collected every 5 min, starting at 10-15 minutes after the initiation of insulin infusion. The total volume of blood drawn will be approximately 180 cc on each study day. After completion of the second 2h infusion, insulin will be discontinued, subjects will receive a meal along with dextrose tablets as needed, and they will be monitored until glucose concentration returns to 90 mg/dl. They will have their vital signs checked and will be evaluated by an MD. If stable, they will be allowed to return home. They will begin another overnight fast at 10 pm that evening in preparation for Day 2.

The following medications will be administered on **Day 1** in the three Aims:

- ***Aim 1.*** We will identify healthy subjects who respond to hypoglycemia and subsequently develop HAAF following three successive episodes over two days. This will follow the same study design as the subsequent aims, but **no medications will be administered**.

- ***Aim 2.*** 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 twice during each hypoglycemia episode; at the start of insulin administration in one nostril, and after one hour in the other nostril. 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 in the other nostril after 60 min. For Aims 2 and 3, studies will be spaced at least 4 weeks apart.

- ***Aim 3.*** We will determine whether administering intranasal naloxone, alone or together with oral diazoxide, will help to prevent HAAF. On Day 1, subjects will be given oral diazoxide alone (4-7 mg/kg orally) or an oral placebo three hours before the first hypoglycemic episode (t=-3h). Based on our previous studies (Protocol 06-414) investigating central regulation of glucose production with diazoxide, the effective dose was 4-6 mg/kg, and this dosage was just approved in Protocols 2018-9039, 9040. At the same time, there is published data on using diazoxide to restore counterregulation to hypoglycemia that reported safe use of a dose of 7 mg/kg dose (17). We will plan to start with a lower dose and increase as needed to up to 7 mg/kg, if we don't observe the expected response. The timing of oral diazoxide ingestion is based on previously published data (17) showing that its effects on glucose counterregulation started 3h after the administration with a duration of action ~3-12 hours. Additionally, the available literature indicates that its hypotensive and antihypoglycemic effect lasted ~3–12 h, with peak action at 5 h (18). We will then determine whether administering intranasal naloxone together with diazoxide during hypoglycemia on Day 1 will have synergistic effects on counterregulatory 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 (as above) during each hypoglycemia episode on Day 1, at the start of insulin administration and after one hour. *Oral diazoxide (4-7 mg/kg orally) or matched oral placebo will be administered three hours before* the first hypoglycemic episode on Day 1. *Hence, the study types in Aim 3 will be: oral diazoxide with placebo spray, oral diazoxide with naloxone spray, and* naloxone spray with *oral placebo.* Given the potential for diazoxide to lower BP (See Human Subjects), vital signs will be monitored hourly in Aim 3.

Day 2: All subjects will undergo a single two-hour hypoglycemic clamp study. At approximately 08:00 A.M. all subjects will arrive to the CRC study room and will have two indwelling cannulae inserted. At t= -120 min, a continuous infusion of 6,6–deuterated glucose (D2G) will be initiated with a bolus of 200 mg/m2 bolus for the first three minutes followed by continuous infusion of 2mg/m2/min for the entire period of study. At t= 0 min, a continuous infusion of insulin will be initiated at a rate of 1.6-1.8 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 goal 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. The enrichment of infused dextrose will be kept equivalent to plasma glucose enrichment by addition of D2G to the infused dextrose. At the beginning of the insulin infusion, we will administer oral potassium chloride powder packets (dissolved in water) in order to prevent hypokalemia during the insulin infusion with a total dose of 40 mEq. During hypoglycemic episodes, subjects will be evaluated for symptoms of hypoglycemia, and plasma samples for D2G, insulin, C-peptide, glucagon, epinephrine, norepinephrine, cortisol, growth hormone, free fatty acids, lactate and glycerol and β-endorphins may be obtained at 15-min intervals. Additionally, blood samples will be obtained in 5-min intervals for glucose measurement. At the end of the clamp (t = 120 min), all the infusions will be discontinued, and the subject will be given a meal along with dextrose tablets as needed, and be monitored until glucose concentration returns to 90 mg/dl. They will be evaluated by an MD and will have their vital signs checked and if stable they will be discharged from the CRC. The total amount of blood drawn on day 2 will be approximately 180 cc.

On certain occasions, a 2-day set of studies may be repeated if target glucose levels failed to consistently reach 54 mg/dl during the final hour of any of the studies, or if (for various reasons, such as loss of intravenous access) the subject was unable to complete the full 2-day study protocol.

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 (9). 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.

We will also measure levels of D2G tracer in order to be able to calculate endogenous glucose production and rate of glucose disappearance during the study. Additionally we will monitor level of awareness of hypoglycemia using a standardized questionnaire regarding eleven specific symptoms at predefined time points (18).

We will compare the effects of counterregulatory response to hypoglycemia and awareness to hypoglycemia in the same subjects after receiving opioid receptor blockade +/- diazoxide versus matched placebos during the antecedent two intervals of hypoglycemia.

For Aim 1, we will **define an individual to be susceptible to HAAF** if peak epinephrine levels in the 3rd clamp are $\geq$30% lower than during the first hypoglycemic clamp. Furthermore, in order to meet the definition of HAAF, eligible individuals must have normal physiologic epinephrine rises in response to hypoglycemia, ie. peak epinephrine levels >400 pg/ml during the first hypoglycemic clamp.

Following identification of eligible, HAAF-susceptible subjects in Aim 1, these subjects will be recruited successively to Aims 2 and 3. The purpose for this order is to first ensure that intranasal naloxone does impact HAAF prior to embarking on the third Aim, in which we examine potential synergy of naloxone and diazoxide. In the case of diazoxide, there is already established evidence (17) in humans that oral diazoxide improved hormonal counterregulatory responses to acute 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 Procedures:

Plasma glucose will be measured at the bedside during clamp studies by an Analox glucose analyzer (Analox GM9, UK), using the glucose oxidase method. Measurements of plasma insulin, C-peptide, glucagon and cortisol concentrations will be performed by radioimmunoassay in the Diabetes Research Center Hormone Assay Core, as previously reported (16). For the D2G 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 (16). 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 (Vanderbilt University, Nashville, TN). Beta-endorphins will be determined using enzyme immunoassay (Phoenix Pharmaceuticals, Burlingame, CA).

Steele's equation will be used for calculation of glucose turnover (19). Values for endogenous glucose production (EGP) and glucose rate of disappearance (Rd), will be obtained at 15-min intervals, and 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 ANALYSIS AND POWER CALCULATIONS:

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. The primary outcome will be the peak epinephrine levels during the first vs. the third hypoglycemic clamp. Paired t-tests comparing the difference in epinephrine between first and third clamps will be used in Aim 2, ie. naloxone vs. placebo. In Aim 3, we will use repeated measures ANOVA to compare the difference in epinephrine levels between the first and third hypoglycemic episode, to compare diazoxide, placebo, and diazoxide plus naloxone. In the event that the data for any of these analyses violate the normality assumptions of the t-test or ANOVA, appropriate 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.

Sample Size Calculations:

Aim 1. The primary outcome will be the peak epinephrine levels during the first vs. the third hypoglycemic clamp. A reduction of ≥30% in peak epinephrine levels between first and third episode will be considered to define HAAF. Our previous data indicate that we will need to study 13 subjects in order to identify 8 who develop HAAF. Similarly, published data (1) showed that 12 healthy subjects needed to be studied in order to define 8 with HAAF. Based on these findings, and based upon the intent to study 15 subjects in each of the two subsequent aims (see below), we expect to study up to 45 subjects in Aim 1.

Aim 2. The primary outcome will be the difference in peak epinephrine levels during the first vs. the third hypoglycemic clamp following intranasal naloxone vs. placebo administration. In our preliminary data (above), using intranasal naloxone vs. placebo, peak plasma epinephrine levels decreased by 517 pg/ml from the first to third hypoglycemic episode in the placebo group (consistent with development of HAAF) compared with an decrease of only 86 pg/ml in the naloxone group. A sample size calculation (SISA sample size calculator) based upon a paired comparison of the difference in epinephrine, setting a two-tailed alpha at 0.05 and power at 80%, yielded a required sample size of 8. We will plan to study 15 subjects in this aim to account for potential subject dropouts.

Aim 3. The primary outcome will be the difference in peak epinephrine levels during the first vs. the third hypoglycemic clamp following oral diazoxide +/- intranasal naloxone administration vs. matched placebos. Prior human studies showed a 37% increase in peak epinephrine levels after oral diazoxide compared with placebo (17). Since we do not yet have preliminary data (or literature) examining the combined effects of intranasal naloxone with oral diazoxide, we plan to study 15 subjects in this exploratory aim and will thereby generate data to perform a full power calculation.

HUMAN SUBJECTS:

Human Subject Inclusion and Exclusion Characteristics:

Inclusion: The study population will consist of around 45 healthy subjects aged 21-55 years. The following subjects will be specifically excluded: children, mentally disabled persons, prisoners, fetuses, subjects with a history of ethanol or drug or toxin exposure which could be associated with neuropathy, any subject deemed incapable of giving voluntary informed consent, and subjects with a history of a bleeding disorder or with a prolonged PT or PTT; significant anemia, renal disease, or liver impairment. Women in the child-bearing age-group will be allowed to participate, provided they have negative pregnancy tests.

Exclusion criteria:

- Age: Under 21 or over 55 years
- BMI: >35kg/m^2
- If BP > 150/90 or <90/60 on repeated measurements and on more than one occasion

- Uncontrolled hyperlipidemia defined as TG > 400 mg/dl and/or Total Cholesterol >300 mg/dl
- Clinically significant liver dysfunction: including thrombocytopenia (platelets <100,000/mcL), anemia (as below), hypoalbuminemia (<3.5 g/dL), coagulopathy (INR > 1.5), and/or liver enzymes more than 3 times the upper limit of normal
- Clinically significant kidney dysfunction, GFR: <60 mg/dL
- Clinically Significant Anemia: Prospective subjects with hemoglobin below the lower limit of 12 g/dl for for men and 11 g/dL for women will be assessed with history and physical exam to rule out clinically significant anemia, defined as an individual with symptoms (eg. fatigue, weakness, shortness of breath, palpitations), signs (pallor, brittle nails etc), or currently under treatment for anemia. In the absence of a documented hemoglobin decrease or iron deficiency, subjects will not be excluded.
- Clinically significant leukocytosis or leukopenia
- Clinically significant thrombocytopenia or thrombocytosis
- Coagulopathy
- Urine drug screen positive for any of the following: amphetamines, barbiturates, benzodiazepines, cocaine, methadone, opiates, oxycodone, PCP. As we are administering naloxone, to eliminate the possibility of a subject experiencing withdrawal symptoms from current/previous use of opiates, we will administer drug tests during the screening and on the morning of each study visit before beginning any procedures. As the drug test available in the CRC is a 7-drug panel, we cannot specifically choose which drugs are screened for. Additionally, in the interest of selecting patients on the basis of their reliability and dependability, we would like to exclude participants using illicit drugs. Occasional use of cannabis (not more than twice per week) is not an exclusion. If the test is read as “indeterminate” it will be repeated at the bedside and an additional sample will be sent to the lab. Decision to enroll subject that day prior to results from lab being available will be decided on a case by case basis, ie. when all previous drug testing had been negative and clinical suspicion is very low.
- Use of medications such as beta blockers that can affect counterregulatory responses to hypoglycemia
- Urinalysis: Clinically significant abnormalities
- Clinically significant electrolyte abnormalities
- Smoking >10 cig/day
- Alcohol: Men >14 drinks/wk or > 4 drinks/day, Women > 7 drinks/wk or > 3 drinks/day
- History of chronic liver disease, active hepatitis infection, HIV/AIDS, chronic kidney disease (stage 3 or greater), active cancer, cardiovascular disease or other heart disease, systemic rheumatologic conditions, seizures, bleeding disorders, muscle disease
- Surgeries that involve removal of endocrine glands except for thyroidectomy (if euthyroid on thyroid hormone replacement – if such history fT4 and TSH will be checked)
- Pregnant women
- Subject enrolled in another medication intervention study less than one month prior to their anticipated start date in this study besides those done by our group
- Family history in first degree relatives: diabetes or premature cardiac death
- Allergies to medication administered during study
- Uncontrolled psychiatric disorders
- Any condition which in the opinion of the PI makes the subject ill-suited for participation in the study

1. RISKS TO THE SUBJECTS

Human Subjects Involvement

Initial subject screening may be performed over the phone in response to recruitment advertising. An oral consent script and questions related to medical history is included.

Subject involvement will include a preliminary screening visit which will include informed consent procedures, followed by history, physical exam and lab testing to determine eligibility. Subjects determined to be eligible will participate in hypoglycemic clamp studies which will require infusion of insulin with frequent blood glucose monitoring. Potential risks to the subjects include the following:

1. **Blood withdrawal:** The total amount of blood sampled per visit will be approximately 198 ml, i.e. less than half of that donated by a blood donor.

2. **20% dextrose**: The infusion of 20% dextrose may be associated with local venous irritation. This will be minimized by use of a large-bore antecubital vein in which the glucose will be infused. The dextrose will be diluted by co-infusion of several other infusates, ie. insulin, tracer and saline, such that the final concentration of dextrose entering the vein would be about 5%.

3. **Insulin (*Novolin R*):** Intravenous low-dose insulin infusion in T2D subjects performed with simultaneous hourly blood glucose checks should not cause any harm or discomfort to the subject. Insulin is approved by the FDA.

4. **Intravenous Catheter** - The intravenous catheter is associated with a small risk of local bruising or infection.

5. **Stable Isotope-D2G (*Cambridge Isotope Laboratories, Cambridge, MA)*-** This D2G is not radioactive, is a naturally occurring stable isotope of glucose and has been safely used in many human studies. D2G is considered IND exempt.

6. **Diazoxide (*Proglycem)–*** The risk associated with higher doses of diazoxide therapy is hypotension. Symptoms of low blood pressure are dizziness and light-headedness. During the study, there will be frequent monitoring of blood pressure. Please note that the amount of diazoxide given in this study will be much lower than the doses which cause lowering of blood pressure. Of note, the oral doses the subjects will receive have been used safely in human subjects in our previous studies without lowering blood pressure or any other side effects. Diazoxide is approved by the FDA and its use in this study meets the criteria for being IND exempt.

7. **Naloxone (*Narcan)* –** Much higher doses may cause nausea and vomiting, hypertension, and cardiac arrhythmias. The abrupt reversal of narcotic depression may result in nausea, vomiting, sweating, tachycardia, increased blood pressure, and tremulousness. Intranasal naloxone may be associated with nasal dryness, nasal edema, nasal congestion and nasal inflammation. All subjects will be screened for opiate usage in order to eliminate the possibility of reversal of narcotic effect. Naloxone is approved by the FDA and its use in this study meets the criteria for being IND exempt.

8. **Hypoglycemia (low blood sugar)** – During the hypoglycemic clamp studies, as described above, subjects' blood glucose will be lowered to the goal of 54 mg/dL. Symptoms of low blood sugar include palpitations, sweating, hunger, dizziness, light-headedness, confusion, and fainting. The subject will be receiving intravenous glucose during the study and their blood glucose levels will be checked every 5 minutes (starting 10-15 min after the initiation of insulin infusion) in order to prevent blood glucose levels from falling below the target of ~54 mg/dl. Their symptoms will be monitored in the pre-specified regular intervals with the hypoglycemic questionnaire. In addition, a fellow (an MD) will be present during the entire study in order to address any signs or symptoms the subject may experience. If the subject finds symptoms of hypoglycemia intolerable, the study will be stopped and the blood glucose will be normalized with IV dextrose infusion and oral carbohydrates. In our extensive experience performing hypoglycemic clamp studies, including the ones targeting blood glucose of ~54 mg/dL, the vast majority of the subjects experienced mild and tolerable symptoms of hypoglycemia.

While our glucose target is 54+/-5 mg/dl, there is a substantial precedent of hypoglycemic clamps in with plasma glucose levels are maintained at 45 mg/dl for at least one hour (20, 21, 22, 23). Thus, we would report a plasma glucose level sustained at less than 45 mg/dl for two consecutive 5 minute intervals, regardless of symptoms, as an adverse event.

9. **Potassium chloride oral powder (*Klor-Con 20mEq)–*** The most common adverse reactions with oral potassium chloride salts are nausea, vomiting, flatulence, abdominal pain/discomfort, and diarrhea Potassium chloride is approved for use by the FDA.

10. **Heparin (Heparin sodium 1000 units/L in normal saline:** Risks associated with heparin use include hemorrhage, thrombocytopenia, heparin-induced thrombocytopenia and hypersensitivity. However, the dose of heparin used in this study (maximum 1500 units) is significantly lower than the initial dose used for therapeutic anticoagulation or prevention of thromboembolism (5000-10,000 units). Therefore, any adverse events are very unlikely in these subjects in a good general health and with very low doses of heparin used in the study.

11. **Human albumin (Albutein 5% solution, 12.5 g/250 mL):** The amount of albumin used for this study (~2g) is significantly lower than the doses commonly used for other indications (for which initial dose used is up to 100 g) therefore we don't expect any adverse events such as increased blood volume in these participants in good health who are receiving a minimal dose of albumin. There is a very unlikely possibility of anaphylactoid/anaphylactic type reactions.

Of note, in over 20 years of experience from our lab, as well as from other groups at Einstein, there have been no significant adverse events with administration of heparin or human albumin in this setting.

*All drugs are dispensed and obtained through Weiler Pharmacy unless otherwise stated.

2. ADEQUACY OF PROTECTION AGAINST RISKS

Recruitment and Informed Consent Procedures:

We will recruit at least 45 non-diabetic subjects by local advertising. Oral consent will be initially obtained during telephone screening performed by a research fellow or the study coordinator to discuss their medical history. Formal consent procedures which adhere to policies of the Institutional Review Board of the Albert Einstein College of Medicine will be followed. Specifically, each subject will be verbally informed in layman's language of the purpose, benefits and possible risks of the studies. They will then read the written consent form in the presence of a member of the research team. A physician will be available to answer any further questions. The subject and witness shall be asked to sign the consent form which will be kept in the subject's chart. Each subject's potential participation in related experiments (for example, when each subject is asked to perform repeat studies) will be explicitly stated.

All clinical data on the subjects will be confidential. Data generated in these studies will be considered only relevant for research purposes and will not be included in any clinical databases or the subject's chart. Since the CRC databases are coded, the study data will not be available on any clinical database system.

Protection Against Risks:

Procedures employed to protect subjects from undue risks include the following:

1) **Blood withdrawal:** Blood withdrawal during any single study will be limited to approximately 180 cc and subsequent studies will be separated by at least a four-week interval. The infusion and withdrawal catheters may produce infection or local hematoma, but strict aseptic technique will be observed by an experienced RN or physician performing the procedure.

2) **20% dextrose:** Venous irritation with 20% dextrose infusion will be prevented by use of a large-bore antecubital vein.

3) **6,6-deuterated glucose (D2G-glucose):** 6,6–deuterated glucose for infusion will be freshly prepared using sterile technique and following good clinical practice. Being at a low concentration (in saline or 20% dextrose), 6,6-deuterated glucose itself is not expected to cause any type of local venous irritation.

4) **Hypoglycemia:** All of these studies will be performed under the supervision of a physician. The subject will receive intravenous glucose during the study and their blood glucose levels will be checked every 5 minutes (starting 10-15 min after the initiation of insulin infusion) in order to assure that blood glucose levels do not go below the target of ~54+/-5 mg/dL. Their symptoms will be monitored in the pre-specified regular intervals with

the hypoglycemic questionnaire. In addition, a fellow (an MD) will be present during the entire study in order to address any signs or symptoms the subject may experience. If the subject finds symptoms of hypoglycemia intolerable, the study will be stopped and the blood glucose will be normalized with IV dextrose infusion and oral carbohydrates. As noted above, most subjects in our previous studies had only mild and tolerable symptoms of hypoglycemia.

5) **Diazoxide (*Proglycem*):** As noted above, vital signs will be carefully monitored throughout the studies. An intravenous line will be established prior to the administration of diazoxide or placebo. In the very unlikely event that the blood pressure drops below 90/60, the patient will be maintained in a supine position and intravenous saline will be administered at a rate sufficient to correct any significant decrease in blood pressure. As noted above, it is highly unlikely that there will be any reduction in blood pressure.

6) **Intranasal Naloxone (*Narcan*):** This route of administration would be expected to reduce the risks relative to intravenous administration of this agent.

7) **Potassium Chloride oral powder (*Klor-Con 20mEq*)**: Potassium chloride will be given to prevent any significant drop in potassium with insulin infusion. An oral dose of 40 mEq of potassium chloride (oral powder dissolved in water) is in concordance with FDA-recommended dosing for oral powder. The total dose of up to 80 mEq that subjects receive is within the limit approved by the FDA (up to 100 mEq/day). The powder will be given while the subject is sitting up and with a plenty of water. This is to prevent any gastrointestinal distress. Potassium chloride will be given to prevent any significant drop in potassium with insulin infusion.

8) **Heparin:** In an unlikely event of occurrence of signs/symptoms suggestive of hemorrhage, infusion of heparin will be stopped immediately, vital signs and laboratory tests will be obtained (complete blood count, PT, PTT), the subject will be evaluated by the physician and RN (during the course of the study), and if indicated will be transferred to the emergency room for further treatment. In an unlikely event that symptoms/signs of hypersensitivity reaction do occur, the infusions will be stopped immediately, the subject will be evaluated by the physician and RN (during the course of the study), if indicated epinephrine injection will be administered (available in the study room) and if needed the subject will be transferred to the emergency room for further treatment.

11) **Human Albumin:** As noted above, the total dose of albumin used for the study will be extremely low (~2g) compared to common clinical indications for its use (up to 100 g). In the very unlikely event that symptoms/signs of an allergic reaction do occur, the infusions will be stopped immediately, the subject will be evaluated by the physician and RN (both being at the bedside during the course of the study). If indicated, epinephrine injection will be administered (available in the study room) and if needed the subject will be transferred to the emergency room for further treatment. This subject would not be studied further in any protocols involving use of human albumin.

3. POTENTIAL BENEFITS OF THE PROPOSED RESEARCH TO THE SUBJECTS AND OTHERS

The study will be of no direct benefit to the subject, although subjects may gain a better understanding of their own metabolic processes and contribute to generalizable knowledge by their participation.

4. IMPORTANCE OF THE KNOWLEDGE TO BE GAINED (Risk: Benefit Ratio):

Based upon the Specific Aims proposed above, we anticipate that the administration of intranasal naloxone will ameliorate HAAF. Furthermore, combined administration of intranasal naloxone and oral diaxozide are anticipated to 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.

5. DATA SAFETY AND MONITORING PLAN (DSMP):

All human subject research being conducted at the Einstein CRC must have a DSMP. The guidelines for Data and Safety Monitoring have been established by the CRC Research Subject Advocate (RSA), and approved by the Assistant Dean for Compliance who oversees human research subject safety at the Albert Einstein College of Medicine. http://gcrcweb.aecom.yu.edu/gcrc/dsm.htm

The PI and research team will regularly review study progress and any adverse events (see Monitoring Plan below). Reports of any adverse events will be transmitted to the Einstein IRB.

Monitoring Plan: Study progress, including all adverse events, will be reviewed at bi-monthly research group meetings. All abnormal findings from history and physical examination will be documented in the research chart and reviewed by the PI or Co-Investigators. Clinical and laboratory data will be reviewed within 24 hours of receipt and any adverse events will be reported according to Einstein IRB policy. The investigators will periodically assess and review data collection and storage procedures to maintain confidentiality. In addition, annual reports summarizing study progress (including recruitment progress, interim data analysis and data quality, adverse events and protocol changes) will be prepared and submitted to the Einstein IRB.

Safety Officer: Sofiya Milman, MD, MS, Director of the Human Longevity Studies for the Einstein Aging Center, will serve as the Safety Officer for this project. She is a highly experienced patient-oriented investigator who is not involved in this research protocol, but is very familiar with all of the proposed methodologies and the potential risks to human subjects, having performed many hypoglycemic clamp studies in human subjects. She will routinely review the safety information related to this project at yearly intervals, and will be contacted immediately should a serious adverse event occur.

Adverse Event Monitoring:
Process: Data and safety monitoring for this study will be performed by the principal investigator on an ongoing basis. All potential subjects will be seen and assessed by the P.I., fellow or research staff. Laboratory data will be reviewed by the P.I. or the fellow within 24 hours of receipt.
Reporting: All adverse events will be compiled, and reported in summary form, on an annual basis to the IRB, and at the conclusion of the study. Unanticipated (non-serious) adverse events will be reported to the IRB within 30 days and serious adverse events will be reported to the IRB within 48 hours by phone, email or fax

Recruitment Monitoring:
Process: The PI and Co-Investigator will assess the recruitment and retention of study subjects on an ongoing basis. The recruitment goal for this protocol is for 30 subjects to complete ALL of the proposed studies, with a planned enrollment of around 45 subjects (considering variability in development of HAAF, and subject drop out).
Reporting: Summary statistics regarding recruitment and retention of study subjects will be reported to the IRB on an annual basis, and at the conclusion of the study.

Early Study Termination:
Process: The PI and fellow will determine if the study is to be terminated prior the scheduled study conclusion. Early study termination will be considered in the event of an unanticipated serious adverse event determined to be possibly, probably or definitely related to the study.

Reporting: The P.I. will report the decision to terminate the study to the IRB within 48 hours of this determination. The P.I. will submit a narrative description of the reasons for early termination of the study within 10 days

6. DATA SHARING
We respect that the rights and privacy of people who participate in NIH-sponsored research must be protected at all times. Thus, data intended for broader use will be free of identifiers that would permit linkages to individual research participants. We and our collaborators will make the data and associated documentation available to users only if they provide: (1) a commitment to using the data only for research purposes and not to identify any individual participant; (2) a commitment to securing the data using appropriate computer technology; and (3) a commitment to destroying or returning the data after analyses are completed. We expect that the results of this study will be presented at national scientific meetings and published in peer reviewed journals. Investigators from Vanderbilt University are not research collaborators, and are solely providing a service in the form of catecholamine assays. Vanderbilt University will only receive de-identified study IDs, which cannot be linked to any patient records.

7. COST AND COMPENSATION TO SUBJECTS: There will be no costs to the participants in the study. Subjects will receive payment by Clincard Greenphire Payment for time and inconvenience associated with the study. Subjects will be asked to provide their social security numbers to receive monetary compensation, which

will be loaded within 24-48 business hours from the day of their study. Their social security numbers will be kept confidential. For completion of each study visit (which consists of Day 1 and Day 2), subjects will receive $400 per 2-day study visit. Therefore, a subject who completes only Aim 1 will receive $400 total (for one two-day study); if they complete both Aim 1 and Aim 2, they will receive $1200 total ($400 for one 2-day study in Aim 1 and additional $800 for two 2-day studies in Aim 2); if they complete all three aims of the study they will receive $2400 ($400 for one 2-day study in Aim 1, $800 for two 2-day studies in Aim 2 and $1200 for three 2-day studies in Aim 3). If subjects choose to withdraw or cannot complete the study, the compensation will be prorated based on the time spent. All subjects will be drug-tested upon the arrival to the Weiler hospital utilizing an instant result drug panel (*Alere iCup Dx14*). If the test is positive, the subject will be withdrawn from the study based on our exclusion criteria. If the test is read as “indeterminate” it will be repeated at the bedside and an additional sample will be sent to the lab. Decision to enroll subject that day prior to results from lab being available will only be in cases where all previous drug testing had been negative and clinical suspicion is very low.

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