Partial blockade of nicotinic acetylcholine receptors improves the counterregulatory response to hypoglycemia in recurrently hypoglycemic rats

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Recurrent exposure to hypoglycemia can impair the normal counterregulatory hormonal responses that guard against hypoglycemia, leading to hypoglycemia-unawareness. This pathological condition, known as hypoglycemia-associated autonomic failure (HAAF) is the main adverse consequence that prevents individuals with Type 1 diabetes mellitus from attaining the long-term health benefits of tight glycemic control. The underlying molecular mechanisms responsible for the progressive loss of the epinephrine response to subsequent bouts of hypoglycemia, a hallmark sign of HAAF, are largely unknown. Normally, hypoglycemia triggers both the release and biosynthesis of epinephrine through activation of nicotinic acetylcholine receptors (nAChR) on the adrenal glands. We hypothesize that excessive cholinergic stimulation may contribute to impaired counterregulation. Here, we tested whether administration of the nAChR partial agonist, cytisine, to reduce post-ganglionic synaptic activity can preserve the counterregulatory hormone responses in an animal model of HAAF. Compared to nicotine, cytisine has limited efficacy to activate nAChRs and stimulate epinephrine release and synthesis. We evaluated adrenal catecholamine production and secretion in non-diabetic rats subjected to two daily episodes of hypoglycemia for 3 days followed by a hyperinsulinemic-hypoglycemic clamp on day 4. Recurrent hypoglycemia decreased epinephrine responses and this was associated with suppressed TH mRNA induction (a measure of adrenal catecholamine synthetic capacity). Treatment with cytisine improved glucagon responses as well as epinephrine release and production in recurrently hypoglycemic animals. These data suggest that pharmacological manipulation of
ganglionic nAChRs may be promising as a translational adjunctive therapy to avoid HAAF in Type 1 diabetes mellitus.
INTRODUCTION

An attenuated sympathoadrenal response following recurrent exposure to hypoglycemia results in a pathological inability to recover from low blood glucose levels, contributes to patient unawareness of hypoglycemia, and is associated with increased mortality in Type 1 and possibly in Type 2 diabetic patients; a condition known as hypoglycemia-associated autonomic failure (HAAF; (11, 1). HAAF is recognized as a major public health problem for patients with diabetes. It can also be induced in healthy subjects, in infants and in animal models suggesting it may involve a maladaptive response to repeated stress (20, 9, 12, 14).

Despite the well-established role of hypoglycemia per se in the development of HAAF, the mechanism(s) underlying the key feature of the pathogenesis of HAAF in diabetes – the attenuated sympathoadrenal epinephrine response to falling plasma glucose levels in a background of imperfect insulin replacement and impaired glucagon responses (10) remains elusive. The reduced sympathoadrenal response in HAAF could result from adaptations/maladaptations within central or peripheral neural-humoral networks that regulate glucose homeostasis (7) or from alterations at the level of the afferent or efferent components of the sympathoadrenal system. While central mechanisms contributing to HAAF have been intensively studied in humans and animal models (11), the capacities of efferent neural outputs has received less attention.

Nicotinic acetylcholine receptors play a critical role in this process since ganglionic blockers and/or surgical ablation of autonomic inputs markedly impair autonomic activation during insulin-induced hypoglycemia in animal models and in humans (rev. in 42). Given that under HAAF conditions, adrenal sympathetic nerve
impulse activity remains elevated for 24 hours, (40, 21), the attenuated plasma
epinephrine response and the accompanying reduction in adrenomedullary catecholamine
content (15) likely arise from factors that alter the capacity of chromaffin cells to
maintain the releasable pool of catecholamines separate from a possible failure of
sustained presynaptic input or a putative deficiency in chromaffin cellular secretory
mechanisms.

Since low intensity nicotinic receptor activation induces catecholamine
biosynthesis *in situ* for prolonged periods (48), and this result does not appear to be
happening *in vivo* during HAAF, we hypothesized that a partial reduction in high
intensity synaptic transmission (rather than complete ganglionic blockade) may preserve
the capacity to synthesize and release adrenal epinephrine during protracted stress in the
intact animal. To test this hypothesis, we used the nicotinic receptor partial agonist
cytisine to reduce peripheral autonomic neurotransmission in a well characterized animal
model of HAAF (39) known to have sustained transsynaptic adrenal nerve activity (40,
21). Cytisine was chosen because of its limited brain penetration, lack of reported adverse
effects *in vivo* (45) and adequate pharmacokinetics. At an intravenous dose of 1 mg/kg,
the plasma clearance of cytisine in rats is ~35 mL min\(^{-1}\) kg\(^{-1}\) and its half-life is 1.5h (36).
Cytisine binds with differing affinities to several subtypes of nAChR and exhibits lower
efficacy (based on electrophysiological assays) compared to endogenous ligands and/or
nicotine (36).

We demonstrated that alone, cytisine acts as a typical partial agonist *in vivo*
inducing modest catecholamine secretion and *de novo* catecholamine synthesis, but is
significantly less effective than an exogenous (i.e. nicotine) or endogenous (insulin-
induced release of acetylcholine) full agonist. More importantly, treatment with cytisine significantly improved counterregulatory responses in recurrently hypoglycemic animals, showing both enhanced catecholamine release and greater expression of adrenal tyrosine hydroxylase (TH) mRNA - the rate limiting enzyme in catecholamine biosynthesis.

**MATERIALS AND METHODS**

*Animals:* Adult, male Sprague-Dawley rats weighing 285–320g with carotid artery (CA) and jugular vein (JV) cannulation (Harlan Labs, Inc. Indianapolis, IN) were individually housed in temperature (21°C), humidity (35%) and light controlled (12 hour light: dark cycle) rooms. Regular rat chow (Agway Prolab 3,000; Syracuse, NY) and water *ad libitum* were provided to the animals unless otherwise stated. Experimental protocols were approved by the Institutional Animal Care and Use Committee at New York Medical College and Yale University.

*Drugs:* Cytisine and nicotine di-tartrate (Sigma Chemical, St. Louis, MO, U.S.A.) were dissolved in sterile saline and injected intraperitoneally (i.p.) at the doses and times indicated in the figures. Nicotine doses were calculated as that of the base, while cytisine doses are expressed as that of the salt. Regular human insulin (Humulin R, Eli Lilly, Indianapolis, IN) at a dose of 2 IU/kg was used to induce hypoglycemia during the antecedent hypoglycemia treatment (39).

*Experimental design:*

1. **Effect of cytisine on adrenal epinephrine secretion and production in vivo - dose-response study:** In PC12 cells (a rat pheochromocytoma tissue culture model of adrenal chromaffin cell functions) we previously showed that cytisine exhibits typical partial agonist properties at native co-existing α3β4 and α7 nAChR with regard to its effects on
catecholamine secretion and synthesis (44). In the present report we aimed to compare the effects of cytisine and the full agonist, nicotine, on epinephrine secretion and their ability to alter TH mRNA levels in vivo. Cytisine doses ranging from 0.3 to 3 mg/kg or nicotine (1mg/kg) were given i.p. to chronically catheterized animals twice daily (9 AM and 1 PM) for 3 consecutive days to mimic the HAAF-model animal protocol (39). Control animals received an equal volume saline injection under similar conditions. On day 4, the catheters were extended outside of the cages for stress-free blood sampling. Two hours was allowed for the animals to recover from handling stress before the final AM dose of cytisine, nicotine or vehicle was given through the JV catheter. Arterial blood samples were collected before (baseline) and every 30 min after drug administration for the ensuing 2 hours. After the collection of each blood sample, the erythrocytes were re-suspended in an equivalent volume of artificial plasma and re-infused back into the animal to prevent volume depletion and anemia (8, 28). Animals were sacrificed 5 h after the AM treatment (the time point at which maximal induction of TH mRNA occurs in response to hypoglycemia (46) and the adrenal medullae were harvested and then immediately frozen at -80°C for TH mRNA analyses.

2. Effect of cytisine treatment on the epinephrine response to hypoglycemia in recurrently hypoglycemic rats: By definition, partial agonists have dual functions: when endogenous ligand concentrations are low or absent, they can act as agonists with a smaller maximal effect at full receptor occupancy than the endogenous ligand (full agonist). However, since they are less effective than the endogenous ligand, partial agonists can also act as antagonists by suppressing the effects of high concentration of endogenous ligand (35, 36, 32). To test whether treatment with cytisine can improve the epinephrine response in
recurrently hypoglycemic animals, we randomly assigned chronically catheterized rats to one of four experimental groups: recurrent saline (RS), recurrent cytisine (RC), recurrent hypoglycemia (RH) and recurrent hypoglycemia + cytisine (CRH). Animals were administered the study drug i.p. twice daily at 9 AM and 1 PM with either saline, 1mg/kg cytisine, 2IU/kg insulin or a combination of cytisine and insulin (CRH group) with cytisine given 30 min before the insulin (26, 36). Animal chow was removed in all groups for 3 hours during each antecedent drug protocol treatment. Blood glucose was monitored from tail nick samples using handheld glucometers (AlphaTrak, Abbott Laboratories, Chicago, IL) every 30-min throughout each hypoglycemic episode and if needed, additional insulin was given in order to maintain glucose levels between 40 - 50 mg/dL (Figure 3) in the RH and CRH groups. Animals were rescued with food or dextrose per os if blood glucose levels dropped below target values. Control groups (RS and RC) underwent the same procedure to ensure uniform exposure to handling stress for all groups. All antecedent treatments were for three consecutive days as illustrated in Figure 2. On day 3, only the morning treatment was given to allow the animals to fully recover before being fasted overnight prior to the clamp procedure on day 4. On day 4, all four groups underwent a 90 min hyperinsulinemic-hypoglycemic glucose clamp (22, 23). Animal weights were monitored on a daily basis to ensure wellbeing.

Hyperinsulinemic-hypoglycemic clamp: Vascular catheters were extended outside of the cages for stress free blood sampling (carotid artery) and glucose/insulin infusion (jugular vein) and animals were rested at least 2 hrs prior to the start of the clamp. Animals treated with cytisine (RC and CRH groups) received a final dose of cytisine via the jugular vein 30 min before the clamp. A constant infusion of regular human insulin
(50mU/kg/min; Eli Lilly, Indianapolis, IN) and a variable 20% dextrose infusion were started and plasma glucose levels were monitored every five minutes to guide dextrose infusion and maintain target glucose levels at around 45 mg/dL for 90 min (Figure 4; ref. 22, 23). Blood samples were collected at 30 min intervals throughout the study for subsequent measurement of plasma glucagon, catecholamine, and corticosterone responses. For measuring plasma insulin levels blood samples were collected at the beginning and at the end of the glucose clamp. At the end of the study, animals were provided with food to recover from the hypoglycemic episode and were then sacrificed 5 hrs after initiation of the hyperinsulinemic-hypoglycemic clamp with an overdose of i.v. sodium pentobarbital followed by decapitation. The adrenal medullae were then dissected, immediately frozen and stored at -80°C degrees until further analyses.

To determine whether there was a separate effect of re-feeding on TH mRNA expression, we conducted a parallel set of studies in a smaller cohort of animals (n=3 for each of the four treatment groups described above) whereby we maintained the hypoglycemic clamp for the entire 5 hour period before sacrificing the animals (Fig. 6 B).

**Analytical methods:**

**Hormone analyses:** Plasma glucagon, insulin and corticosterone were determined using commercially available radioimmunoassay kits from Linco Research, St. Charles, MO and Diagnostic Products, Los Angeles, CA (8). Plasma epinephrine and norepinephrine concentrations were determined using a competitive enzyme immunoassay (Rocky Mountain Diagnostics, Colorado Springs, CO) as described (44).

**Isolation of RNA and Northern blot analyses:** Each left adrenal medulla was used for Northern blot analyses (31). The blots were hybridized consequently to labeled probes
for TH and 18S rRNA. X-ray films were scanned and analyzed by Quantity One Software using BioRad GS 800 densitometer. The integrated optical density for each mRNA was normalized for the densities obtained for 18S rRNA levels in the same samples, on the same blot.

Statistics: Statistical analysis was performed with Sigma STAT/Plot software; version 12 (San Jose, CA). All data were expressed as means ± standard error of the mean (SEM). Significance was assumed at p < 0.05. Comparisons of basal and hypoglycemic responses were made using the one way analysis of variance (ANOVA) followed by a Neuman-Keuls post hoc analysis. Sequential counterregulatory hormonal responses to hypoglycemia and glucose parameters during hypoglycemia were compared using repeated-measures ANOVA. Correlation analysis between peak plasma epinephrine levels and the observed adrenal TH mRNA levels was also performed.

RESULTS

Cytisine modestly stimulates epinephrine secretion and elevates adrenal TH gene expression in vivo: Compared to saline, cytisine (0.3 to 3mg/kg, i.p., ref. 26, 36) induced modest (but significant) increases in plasma epinephrine levels in a dose-dependent manner (Figure 1A). This rise was associated with a dose – dependent increase in adrenomedullary TH mRNA levels (Figure 1B) indicating cytisine has the capacity to enhance both secretion and de novo synthesis of epinephrine. These effects of cytisine (3mg/kg) were significantly less when compared to nicotine (1mg/kg) or insulin-induced release of acetylcholine during hypoglycemia (peak epinephrine values of 110±10 pg/ml, 1200±122 pg/ml and 3310±140 pg/ml, respectively) which is consistent with its limited
potency to activate nicotinic receptor-mediated functions (36). Together, our data suggests that cytisine acts as a partial agonist on adrenal postganglionic nicotinic receptors in vivo serving two biologically important functions - to enhance the secretion and de novo synthesis of epinephrine. As the effects of cytisine appeared to plateau at a dose of 1mg/kg, this dose was used in the remaining studies.

Can cytisine improve the counterregulatory defect caused by insulin-induced recurrent hypoglycemia? Daily plasma glucose concentrations during the recurrent periods of treatment for all experimental groups are summarized in Fig. 3. There were no significant differences in the level of hypoglycemia that was achieved between the RH and CRH groups. Similar plasma glucose levels were also obtained for the control groups – RS and RC. It should be mentioned that animals from all experimental groups displayed similar weight changes throughout treatments (data not shown).

Plasma glucose and insulin levels during the hypoglycemic clamp: Target blood glucose levels (50 mg/dL) were achieved by 30 minutes and were similarly maintained at this level in all groups (Figure 4A). Also, there was no significant difference in plasma insulin levels at baseline or after the clamp between treatment groups during the study (Table 1). These observations indicated that all animals were exposed to identical glucose and insulin stimuli during the glucose clamp portion of the protocol, differing only by their antecedent drug intervention history in the preceding 3 days. The glucose infusion rates are shown on Fig. 4B. Area under the curve (AUC) was calculated according to (43). Total exogenous glucose requirements in the RH group (AUC value of 772±85.79 mg/kg) were significantly higher compared to controls (calculated AUC for both, RS and RC being 290±37.24 and 357.59±52.14 resp., p <0.05). With cytisine treatment before
each bout of hypoglycemia (CRH group) the total amount of glucose administered (AUC value of 578.1±30.9) was lowered compared to RH group.

Effect of cytisine on counterregulatory hormone release following insulin-induced recurrent hypoglycemia: There were no statistically significant differences in baseline blood levels of counterregulatory hormones between the four treatment groups (Figure 5). In response to hypoglycemia, plasma concentrations of epinephrine (Figure 5A) and glucagon (Figure 5C) increased significantly from euglycemic values in all groups. Despite similar plasma glucose (Figure 4) and insulin levels during the hypoglycemic clamp (Table 1), the rise in epinephrine (at all time points) was attenuated by nearly 50% in the recurrent hypoglycemia group (RH) compared to the saline treated (RS) group (Fig.5A, **p < 0.007). This is consistent with previously reported data for this model of HAAF (39). The epinephrine response was reduced in the cytisine alone group (RC) compared to saline treated animals (RS, p < 0.007 at 60 min), suggesting that cytisine may compete with acetylcholine for receptor occupancy and partially block it’s effects. On the other hand, cytisine treatment significantly improved the epinephrine response in animals exposed to recurrent hypoglycemia (Fig. 5, RH vs. CRH, ^\textsuperscript{xx}p = 0.00173).

Recurrently hypoglycemic animals (RH) had a significantly attenuated glucagon response and this response was completely restored with cytisine treatment (CRH group). Cytisine treatment alone in the absence of recurrent hypoglycemia (RC group) did not affect the glucagon response (Fig. 5C).

Baseline norepinephrine values and its increased increments during the hypoglycemic clamp were not significantly different between the four experimental groups (Fig. 5B) and were consistent with previously published results for RS and RH,
These results suggest that sympathetic postganglionic outflow was similar in all experimental conditions and was not significantly affected by cytisine.

Similarly, baseline corticosterone values in all groups were not different from one another and showed the expected normal diurnal levels for that time of day (~100 ng/ml, see Figure 5D). During the hypoglycemic clamp, plasma corticosterone levels increased in all groups (p < 0.05 vs. corresponding baseline) but again, the responses were not different between treatment groups.

**Relative adrenal TH mRNA levels parallel the magnitude of the epinephrine response:** The release of catecholamines from the adrenal medulla in response to stress is accompanied by compensatory increases in their biosynthesis via mechanisms that result in increased TH mRNA, TH protein and enzyme activity in order to maintain the releasable pool of cellular catecholamines at constant levels (24). Adrenal TH mRNA levels increased significantly in all animal groups that underwent the hypoglycemic clamp, compared to non-manipulated controls (CON). However, the rise in TH mRNA was significantly less in the group exposed to twice daily episodes of antecedent hypoglycemia (RH vs. RS; **p < 0.05). The observed changes in TH mRNA levels in response to acute and recurrent hypoglycemia in the presence of cytisine (RC and CRH) were not significantly different from RS group and correlated with peak plasma epinephrine responses (Pearson’s coefficient of 0.9835). Importantly, the CRH group showed an improving trend compared to the RH group (p=0.06, Figure 6A).

In a control experiment, animals were sacrificed at the desired time point for tissue collection (5 hours) but without the recovery and re-feeding interventions (6B). A similar pattern was obtained for all experimental groups indicating that the observed
responses were in fact due to differences arising from the antecedent exposure rather than during the period of recovery following the hypoglycemic clamp.

**DISCUSSION**

Our data supports the hypothesis that excessive adrenal nerve stimulation of nicotinic acetylcholine receptors of the adrenal chromaffin cells contributes to the development of HAAF. Consistent with all prior work (16), rev. in (24), our results confirmed that the maximal epinephrine response to hypoglycemia (Figure 5) is directly associated with a parallel increase in TH mRNA levels (RS, Fig. 6A). However, for the first time, we also demonstrated quantitatively that steady-state levels of adrenal TH mRNA, like epinephrine blood levels, are attenuated by ~50% during HAAF. We interpret this reduced responsiveness as arising from excessive pre-synaptic adrenal nerve cholinergic stimulation of adrenal chromaffin cells since partial pharmacological inhibition of nAChRs (before each bout of hypoglycemia) significantly improved the impaired epinephrine and glucagon responses in this animal model of HAAF. Our results are intriguing and offer a proof-of-concept in the peripheral nervous system that parallels the previously recognized effects of nicotinic receptor partial agonists on central catecholaminergic cell responses (11, 36, 37, 29, 30).

*Transsynaptic Modulation:* Cytisine has been used since the 1960s as an aid for smoking cessation in eastern and central European countries (17), but there is only a small amount of data on its efficacy since its clinical utility is limited by its affinity for other nAChR subtypes and limited blood-brain barrier penetration (36). Its potential effects on peripheral nAChR function *in vivo* during whole animal responses have not been previously reported. Our data are consistent with cytisine functioning as a typical
partial agonist at postsynaptic nicotinic receptors of the adrenal medulla which can
induce catecholamine release (epinephrine) and *de novo* catecholamine synthesis
(increased adrenal TH mRNA levels). In addition, as expected, the magnitude of the
response is significantly lower than that of a full agonist(s) compare to either,
exogenously administered nicotine (Figure 1) or insulin induced acetylcholine release
(Figures 5A, 6).

Although binding and antagonism tests were not conducted in our studies, other
reports have shown that cytisine has similar binding affinity for adrenal α3β4 receptors
(27) as nicotine and no affinity for muscarinic or histamine receptors (2, 27).
Furthermore, in PC12 cells (44) and in perfused adrenal glands, cytisine stimulates
catecholamine release in a dose-, time- and Ca^{2+}-dependent manner (27, 49) and blocks
the actions of acetylcholine on nicotinic receptors through a competitive mechanism (32).

Our *in vivo* data shows that recurrent exposure to cytisine produces a modest
reduction in epinephrine secretion in response to acute hypoglycemia (as observed in the
RC experimental group, Figure 5), consistent with it’s partial agonist properties. When
animals were pre-treated with cytisine before each hypoglycemic episode in an animal
model of HAAF, the circulating epinephrine responses to subsequent bouts of
hypoglycemia were significantly improved (CRH vs. RH group, Fig. 5). Given that
ganglionic blockers and/or surgical ablation of autonomic sympathetic nerve inputs
markedly impair autonomic activation of the adrenal medulla during insulin-induced
hypoglycemia in both animal models and in humans (42), it is reasonable to conclude,
that cytisine most likely acts on post-ganglionic neuronal nAChR to modulate their
activation by the endogenously released pre-synaptic acetylcholine.
Insulin-induced hypoglycemia is a potent activator of the adrenomedullary hormonal system (19). This activation is mediated by indirect reflex excitation of the splanchnic nerve, which evokes large increases in catecholamine secretion (resulting in up to 70% of epinephrine content depletion (47), accompanied by activation of TH enzyme and compensatory catecholamine biosynthesis in the adrenal medulla to maintain cellular catecholamine levels constant (41). As an index of catecholamine biosynthesis, we quantified changes in adrenal TH mRNA (25). After the hypoglycemic clamp, TH mRNA levels increased in all treatment groups compared to non-manipulated controls. However, the rise in TH mRNA was significantly less in the RH group. This is consistent with reports showing reduced in situ staining of TH and PNMT mRNA levels in the adrenal medullas of diabetic rats that underwent the same recurrent hypoglycemia paradigm (HAAF) as our model (22, 23). Our quantitative TH mRNA data suggests that following recurrent exposure to hypoglycemia, a molecular mechanism may exist that can limit the capacity to replenish the adrenal pool of epinephrine. The possible contribution of non-cholinergic receptor activation by presynaptic neuropeptides that are co-localized and co-released with acetylcholine (24, 41) is another outstanding question that needs to be addressed in future studies. Importantly, the effect of cytisine on TH mRNA levels during acute (RC) or recurrent (CRH) hypoglycemia correlated with the corresponding rates of epinephrine secretion and it was consistent with partial agonist properties of the drug suggesting future research may prove instructive as a potential therapy.

Nicotinic receptor tachyphylaxis is an unlikely mechanism to explain these results for several reasons: First, it is known that α3β4 nAchRs are only moderately susceptible
to desensitization (34). Secondly, if desensitization had occurred, one would expect the opposite effect (or no effect) in the CRH group. Thirdly, using intact adrenal glands in situ, reduced responsiveness was not observed – i.e. perfused rat adrenal glands had the capacity to sustain biosynthesis (increase TH mRNA, TH protein and TH activity) and epinephrine release for prolonged periods of time via transsynaptic mechanisms (48). Taken together, sustained release in situ yet failure in vivo, implicates other control signals are operative during HAAF in an intact animal. For example, circulating factors may also exist (e.g. hormones, free fatty acids or other substances) that are increased during recent hypoglycemia, which contribute to the inability of the adrenal medulla to sustain epinephrine biosynthesis and release (33). Future research is necessary to ascertain whether any novel humoral factors contribute to the reduced adrenal capacity to produce catecholamines during HAAF.

Hormonal pathways: Plasma glucagon levels were attenuated during HAAF as previously reported (4); while in contrast, similar increments in glucagon were obtained in the remaining experimental groups. Notably, treatment with cytisine substantially improved the circulating glucagon responses in recurrently hypoglycemic animals to nearly the same levels as seen in the saline group. Glucagon release from pancreatic α-cells is regulated by a number of factors including glucose, insulin and somatostatin secreted from neighboring β- and delta-cells, as well as by redundant autonomic inputs from sympathetic, parasympathetic and sympathoadrenal neurotransmitters and neuropeptides (rev. in (42). How cytisine treatment influences the relative contribution from each of these inputs remains to be elucidated.
No differences in plasma corticosterone concentrations were observed between the four treatment groups suggesting similar levels of stress exposure and responsiveness of the HPA axis under all experimental conditions. Although several studies in non-diabetic humans (13) and normal rats (38) showed that elevated plasma glucocorticoids during antecedent hypoglycemia contribute to the development of HAAF (18), we did not observe elevated baseline corticosterone levels in our model of HAAF.

A limitation of our approach is that our current data cannot exclude a direct or indirect effect of cytisine working through central nervous system nAChR’s; although it has been reported that cytisine has limited brain penetrance (36). Cholinergic receptors are crucial for acetylcholine neurotransmission in the CNS, and increased expression of α7 nAChR has been observed in the cerebellum of diabetic and control rats during hypoglycemia (3). We are not aware of any data supporting nAChR-mediated regulation of glucose sensing neurons, but nAChRs in general can modulate neuronal activity at the microcircuit synaptic level, by altering the cell processing of information and by influencing the velocity of action potentials or the synchrony of communication between brain areas (5). Further studies are needed to evaluate the potential peripheral versus central effects of cytisine, pertinent to glucose counterregulation. It should be noted that in some human studies a direct muscarinic cholinergic inhibition of hepatic glucose production has been observed (6) but our current data reveal that reducing cholinergic neurotransmission at nicotinic receptors improves counterregulatory responses and perhaps also hepatic glucose production, although the latter was not evaluated.

In summary, we showed that activation of nAChRs during prior, antecedent bouts of hypoglycemia can reduce the capacity of the adrenal medulla to replenish the
releasable pool of catecholamines (compare TH mRNA in RH vs RS group, Fig. 6A)
which in turn, can lead to impairment of catecholamine secretion during subsequent bouts
of hypoglycemia. This novel mechanism may represent a significant contribution to the
neurogenic component of the clinical syndrome of HAAF. We speculate that modulating
the nicotinic signal at the splanchnic adrenal nerve-chromaffin cell synapse may afford
clinicians a new opportunity to help improve sympathoadrenal responses in compromised
patients.

Acknowledgements:
The authors appreciate the encouragement of Mladen Vranic, MD and the helpful
suggestions of Esther Sabban, PhD, Lidia Serova, PhD and Gad Alpan, MD, MBA during
the progression of this work.

Grants:
We would like to recognize the contributions of the Juvenile Diabetes Foundation
(to EFL and BN), Yale Diabetes Research Center (DK-45735), the Empire Clinical
Research Investigator Program (ECRIP) of New York State (to EFL), the Children’s and
Women’s Physicians of Westchester, LLP (CWPW) grant program and the Children’s
Health and Research Foundation for their economic support.

Disclosures: The authors declare no conflict of interest, financial or otherwise.
REFERENCES


**FIGURE LEGENDS**

**Figure 1:** Effect of cytisine on Epinephrine release and adrenal TH mRNA levels: Dose response. Experimental groups: Saline; 0.3Cyt – animals injected i.p. with 0.3 mg/kg Cytisine; 1Cyt – 1mg/kg cytisine; 3Cyt – 3mg/kg Cytisine; 1N – 1mg/kg Nicotine. Each dose/drug was given twice daily, total of 7 episodes - to mimic the HAAF protocol. On day 4 the catheters were extended out of the cages and arterial blood samples were collected stress-free before and every 30 min after last drug application (i.v.) for total of 2 hours. The results (A, Plasma epinephrine values) are from two independent experiments (n=6) and are presented as mean ± SE, *p≤0.05 Cyt vs. Sal; **P≤0.002 Nic vs. Cyt. For RNA analyses (B) animals were sacrificed 5 hrs after treatment and changes in adrenal medullary TH gene expression analyzed by Northern blots. The results are calculated as fold induction from control (Sal – saline injected animals).

**Figure 2:** Schematic diagram depicting the main protocol used in these experiments, designed to determine the effect of cytisine given before each insulin treatment. JV - jugular vein; CA – carotid artery. ON- overnight

**Figure 3:** Plasma glucose levels during each antecedent treatment period

Experimental groups: recurrent saline controls (RS), recurrently hypoglycemic animals (RH), recurrent cytisine controls (RC) and animals who received cytisine before each insulin treatment (CRH). Results are presented as mean ± SE, n= 9.

**Figure 4:** A: Plasma glucose concentrations during the hyperinsulinemic-hypoglycemic glucose clamp, Experimental groups: recurrent saline (RS), recurrent hypoglycemia (RH), recurrent cytisine (RC) and recurrently hypoglycemic animals pre-treated with
cytisine (CRH). B: Glucose infusion rates during the clamp. Results are presented as mean ± SE, n ≤ 9.

**Figure 5:** Effect of cytisine pretreatment on counter regulatory hormone release during insulin-induced recurrent hypoglycemia.

Plasma epinephrine (A), norepinephrine (B), glucagon (C) and corticosterone (D) responses during hypoglycemic clamp in recurrent saline (RS, maximal response group), recurrently hypoglycemic animals (RH – HAAF group); animals receiving cytisine alone (RC) and recurrently hypoglycemic animals given cytisine before each insulin treatment (CRH). The results are summarized from three independent experiments (n=9) and are presented as mean ± SE. *p ≤ 0.05; **p ≤ 0.002 RH vs. RS; *P ≤ 0.05 and **P ≤ 0.002 RH vs. CRH.

**Figure 6:** Relative adrenal TH mRNA levels correlate with the magnitude of the epinephrine response. A) After the hypoglycemic clamp the animals were provided food and sacrificed 3.5 hrs later. The results (Northern blot) are summarized from two independent experiments (n=6) and are calculated as fold induction from CON (absolute control – not treated animals) and are given as mean ± SE, *p ≤ 0.05. B) Animals (n=3 for each group) were maintained in hypoglycemic state until sacrifice (5 hrs after the initiation of hypoglycemic clamp).

**Table 1:** Average plasma insulin concentrations on day 4 before (baseline) and after hyperinsulinemic-hypoglycemic clamp.

Data are summarized from three independent experiments (n ≥ 6 per group); units: uIU/ml; values are mean ± SE.
Figure 1 rev.

A

Epinephrine pg/ml

0 30 60 90 120 min

3Cyt 1Cyt 0.3Cyt

Sal Nic 0.3Cyt 1Cyt 3Cyt

B

Relative TH mRNA levels

Sal Nic 0.3Cyt 1Cyt 3Cyt

*** ** *
Day 4: 90 min hyperinsulinemic-hypoglycemic clamp after ON fast (glucose target 40-50 mg/dL), all

*Cytisine given 30 min before insulin infusion for RC and CRH groups
Figure 3 rev.

Insulin/Saline/Cytisine

Day 1 AM

Day 1 PM

Day 2 AM

Day 2 PM

Day 3 AM

Plasma glucose (mg/dl)

Treatment:

RS
RH
RC
CRH

(min)
Figure 4 A rev.

**Antecedent treatment:**
- RS
- RH
- RC
- CRH

**Insulin**

**Plasma glucose (mg/dL)**

**Time (min):** 0 10 20 30 40 50 60 70 80 90
Figure 4: B new

Glucose infusion rate (mg/kg/min)

Insulin

Antecedent treatment:
- RS
- RH
- CR
- CRH

0 10 20 30 40 50 60 70 80 90 (min)
Figure 5 rev.

Antecedent treatment:
- RS
- RH
- RC
- CRH

Graphs A, B, C, and D show the concentration of various hormones over time:
- A: Epinephrine ng/ml
- B: Norepinephrine ng/ml
- C: Glucagon pg/ml
- D: Corticosterone ng/ml

The graphs illustrate the response to antecedent treatments (RS, RH, RC, CRH) with time points at 0, 30, 60, and 90 minutes.
Figure 6 rev.
Table 1: Plasma Insulin

<table>
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<tr>
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<th>RS</th>
<th>RH</th>
<th>RC</th>
<th>CRH</th>
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<td>Baseline:</td>
<td>3.2 ± 0.85</td>
<td>4.01 ± 0.98</td>
<td>4.36 ± 1.25</td>
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</tr>
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<td>Hypoglycemia:</td>
<td>1932 ± 120</td>
<td>2230 ± 252</td>
<td>2021 ± 323</td>
<td>2032 ± 312</td>
</tr>
</tbody>
</table>