

Classical Conditioning of Insulin Effects in Healthy Humans

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Objective: Classical conditioning of insulin effects was examined in healthy humans using a placebo-controlled design. This study examined whether subjects who experienced a conditioned stimulus (CS) paired with insulin in the acquisition phase of a conditioning protocol would show a conditioned decrease of blood glucose when receiving the CS with a placebo injection in the test phase. **Methods:** Twenty healthy male students were assigned either to group 1, which received insulin (0.035 IU/kg IV), or to group 2, which received IV saline on 4 consecutive days (acquisition). On day 5 (test), both groups were injected with saline. The CS was an olfactory stimulus. Blood glucose, serum insulin, plasma glucagon, plasma catecholamines, serum cortisol, and symptoms were repeatedly measured during each session. **Results:** In the test phase, group 1 reacted with a significantly larger decrease of blood glucose after presentation of the CS than group 2. Within group 1, a larger conditioned blood glucose decrease was associated with features that enhance classical conditioning (ie, intensity of the unconditioned response and intensity of the CS). Furthermore, in group 1, there was an increase of baseline insulin from day 1 to day 5 and a tendency for insulin reduction after CS presentation. Groups also tended to differ in cortisol and neuroglycopenic symptoms after CS presentation. **Conclusions:** Conditioned effects in blood glucose are in accordance with the predictions. As a result of the exploratory analyses, our data also provide hints about conditioned changes in insulin, counterregulatory hormones, and symptoms. **Key words:** insulin, classical conditioning, humans, blood glucose, counterregulatory hormones, symptoms.

CNS = central nervous system; COND = conditionable; CR = conditioned response; CS = conditioned stimulus; CV = coefficient of variation; EDTA = ethylene diamine tetraacetic acid; EGTA = ethylene glycol-bis(beta-aminoethyl ether)N,N-tetraacetic acid; HPLC = high-pressure liquid chromatography; IU = international unit; IV = intravenous; MEIA = micro-particle enzyme immunoassay; NA = noradrenaline; RIA = radio immunoassay; SEM = standard error of mean; UR = unconditioned response; US = unconditioned stimulus.

INTRODUCTION

Interest in classical conditioning of drug effects has recently increased (eg, Refs. 1 and 2). Insulin is a peptide hormone produced by the B-cells of the endocrine pancreas. Its main functions are to increase uptake of glucose into muscle and adipose cells and to guarantee its storage as glycogen in liver and muscle, thus inducing a reduction in blood glucose. In a fasting healthy organism, glucose concentration of venous blood ranges from 70 to 100 mg/dl. Hypoglycemia is

biochemically defined as a blood glucose level below 50 mg/dl. Because classical conditioning always requires the CNS, insulin raises special interest for several reasons. First, insulin secretion is neurally mediated (3, 4). Second, insulin reaches the CNS by a saturable transport system from blood to the brain (5), where insulin receptors are localized, predominantly within the olfactory bulbs, the hippocampus, and those areas of the hypothalamus and lower brainstem that are involved in the regulation of food intake and body weight (4). Third, the brain detects hypoglycemia and initiates counterregulation (2, 6).

Until now, conditioning of insulin effects has been studied mainly in animals, with a focus on blood glucose as the dependent variable. The first data came from Russian laboratories (7), but control groups were lacking. Controlled experiments of classical conditioning of blood glucose in rats were initiated by Woods et al. (8). In most of their studies (reviewed in Ref. 4), a relatively high dose of insulin (50 IU/kg) was used. Rats were either exposed to CS–insulin pairings (experimental group) or to CS–saline pairings (control group). When receiving the CS, typically an olfactory stimulus (9), and a saline injection in the test, subjects in the experimental group with the previous CS–insulin experience responded to the CS with a reduction of blood glucose, defined as conditioned hypoglycemia (eg, Ref. 10). Several experiments supported the interpretation as a conditioning phenomenon (11, 12) and also revealed its vagal mediation: Conditioned hypoglycemia was abolished by vagotomy, atropine (13, 14), and streptozotocin, a drug that destroys pancreatic B cells (15). Conditioned hyperglycemia instead of conditioned hypoglycemia, was obtained when a novel environment activating the pituitary–

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adrenal–sympathetic stress system was used as the CS paired with insulin (13, 16–19). Corresponding to the vagal mediation, Woods et al. (15, experiment 2) showed that conditioned blood glucose reduction is based on a (neurally mediated) conditioned release of insulin. However, so far only two animal conditioning studies have simultaneously analyzed blood glucose and insulin (20, 21); however, in these studies, tolbutamide (20) and food (21), not insulin, were used as the nominal US. Thus, more data on insulin secretion in a classical conditioning protocol using insulin need to be obtained.

Classical conditioning of insulin effects in humans has rarely been studied. The first hints of conditioning effects were obtained from schizophrenic patients treated with insulin shock therapy (7): Blood glucose decreased and hypoglycemic symptoms (eg, drowsiness and sweating) occurred under saline after previous insulin injections in the same setting. However, methodological shortcomings (missing control groups) are obvious. More than 30 years later, experiments with healthy humans started. Fehm-Wolfsdorf et al. (22) initially used a differential conditioning design. In the test session, when the CS+ and the CS– were both presented with saline, a conditioned blood glucose reduction to the CS+ could not be detected. But in a second experiment using a two-group design (23) with two types of CS (a stimulus compound versus the injection procedure per se) functioning as the independent variable, substantial deviations (>10 mg/dl) from the baseline blood glucose level were found in 11 of 25 subjects (44%). Bidirectional changes occurred, with the majority of subjects ($N = 8$) responding to the CS with a glucose decrease, two with a rise, and one with fluctuations of 20 mg/dl. Salivary cortisol was measured, but a CR was not found.

Up to now, insulin conditioning experiments in healthy humans have not included a control group receiving placebo on all days of the conditioning protocol. Consequently, we used a placebo-controlled, two-group design. In addition to examining classical conditioning of the blood glucose response, we also wanted to obtain pilot data on the mediation of conditioned blood glucose decrease by conditioned insulin secretion and on the conditionability of counterregulatory hormones and symptoms, thus extending the scope of variables. These additional questions constitute the pilot part of our study. As pointed out above, even in animal conditioning studies, insulin was seldom measured. Furthermore, because level of blood glucose is not a simple UR to insulin but an integral result of several ongoing endocrine processes, measuring counterregulatory hormones is important. As Ramsay and Woods (2) emphasize, the centrally

detectable effect of the drug, not the drug itself, constitutes the US. In the case of exogenously injected insulin, the US is the insulin detected by the insulin receptors in the CNS (4, 10). The actions the organism generates via the CNS constitute the true UR. The brain detects the fall of glucose (2, 24), initiates the secretion of counterregulatory hormones (glucagon and catecholamines in a first line of defense and then growth hormone and cortisol) (6, 25), and directly innervates organs (eg, pancreas and liver), which directly increase blood glucose levels (2). Because of its CNS-initiated release and neural control (3), the secretion of counterregulatory hormones should be conditionable. We know of only one animal study in which corticosterone was measured in addition to blood glucose (19); results revealed a trend for a conditioned corticosterone increase. A placebo-controlled experiment in which blood glucose, insulin, and counterregulatory hormones are all analyzed has not been published until now.

We hypothesized that human subjects who previously received CS–insulin pairings (experimental group) would show a larger decrease of blood glucose after CS presentation in the placebo test session than subjects who received the placebo in all sessions (control group). For exploratory purposes, we also addressed the mediation of conditioned hypoglycemia (13–15) by insulin and the conditionability of counterregulatory hormones and symptoms. We expected that the experimental group would react with a higher increase of insulin secretion in the test session than the control group. Based on the CNS-mediation of counterregulation, we expected a conditioned increase in glucagon, catecholamines, and cortisol and an increase in hypoglycemic symptoms in the experimental group as compared with the control group.

METHODS

Design

A two-group design was used. Groups differed in the injection substance at day 1 to 4 (acquisition phase) of the conditioning protocol. Subjects in group 1 (CS–INS) received an IV bolus injection of insulin (human insulin, Actrapid, NovoNordisk, Mainz, Germany) at a dose of 0.035 IU/kg body weight, diluted with NaCl (0.9%, Braun, Melsungen, Germany) to a final volume of 1 ml. Subjects in group 2 (CS–NaCl) were given an IV injection of 1 ml of physiological saline (0.9% NaCl, Braun). Day 5 constituted the test phase. Subjects in both groups received the CS and were injected with saline. The experiment was conducted under double-blind conditions. The protocol was realized on 5 consecutive days (Monday to Friday). Sessions were separated by 24 hours. The design was approved by the Ethics Committee of the Medical Faculty of Heinrich-Heine-University, Düsseldorf, Germany. Subjects gave written consent.

Based on the localization of insulin receptors in the olfactory

bulbs (eg, Ref. 26), an olfactory stimulus served as the CS. It consisted of a mixture of 2.1 ml of rosewood oil (*Oleum Rosea Ligni Brasil*, Caelo, Caesar and Loretz, Hilden, Germany) and 1.4 ml of peppermint oil (*Oleum Mentae Piperitae*, 10%, Caelo, Caesar and Loretz) mixed in 50 ml of water (see below for application). The smell had been pretested for sufficient novelty, intensity, and pleasantness in an independent sample of healthy subjects. The CS was applied by a special device, which guaranteed that the smell was discretely applied during a predefined time interval and that the air in the room was not affected by the smell. Air flow was produced by a compressed-air tank equipped with a two-way magnet valve. Flow rate was controlled by a rotameter and set at 37.6 ml/second. This air flow was aromatized using the rosewood/peppermint oil emulsion, which was filled in a bubbling flask equipped with an asperger. Silicon tubes, fixed by nose goggles, conducted the air flow to the subjects' nostrils. One minute before injection, air flow was initialized using an electronic timer and automatically ended 6 minutes later.

Subjects

Subjects were 20 healthy male students aged 20 to 30 years who were randomly assigned to group 1 (CS-INS) or group 2 (CS-NaCl). They were recruited on campus by an announcement for healthy, nonsmoking male students of normal body weight. Mean age (\pm SEM) was 25.2 ± 0.7 years in group 1 and 24.3 ± 0.7 years in group 2. Body mass index was within the normal range in both groups (mean = 22.7 ± 0.4 kg/m² in group 1 and 23.1 ± 0.5 kg/m² in group 2). In addition to smokers (27), athletes (28) were excluded. Medical exclusion criteria were diabetes (also in parents or siblings), endocrinological and neurological diseases, cardiovascular disorders, and medication with glucocorticoids. Eligibility was determined during an interview and a physical examination. Before participation, subjects were informed about the study, in particular about the schedule, amount of blood loss expected during each session, and possible risks.

All subjects (future insulin and future saline) took part in a one-session prestudy in which they were injected with insulin (0.035 IU/kg) but did not receive any olfactory CS. The rationale for the prescreening with insulin was to identify those subjects that (1) do not reach a blood glucose minimum of at least 50 mg/dl (ie, hypoglycemia) under the insulin dose and/or (2) refuse to participate in the five sessions of the conditioning study because of side effects (eg, extreme hypoglycemia or venipuncture problems) or personal reasons. Of the 33 subjects approached in the prestudy, nine did not enter the conditioning study because of the first reason and three did not enter because of the second reason. One additional subject dropped out during session 2 of the conditioning protocol after experiencing extreme side effects. The mean interval between the prescreening and conditioning sessions was 76.4 days (SEM = 8.9 days). Degree of hypoglycemia at the time of maximum hypoglycemia (24 minutes after injection) during prescreening tended to be correlated with degree of hypoglycemia (minute 24) on day 1 of the acquisition phase (day 1, minute 24; $r = 0.51, p < .10$) in group 1 (CS-INS). The correlation attenuated on days 2 to 4, possibly indicating a separate influence of the new conditioning experience.

Subjects were instructed to fast overnight (for at least 8 hours with no food or beverages except water or unsweetened herbal tea), not to drink any alcohol during the total 5 days starting the evening before the first session, and not to exercise or participate in sports on the morning of the experiment. The subjects received a total payment of 300,00 Deutsche Marks (equivalent to about \$170 US) at the end of the last session.

Experimental Procedure

Sessions were scheduled at 7:45 AM on 5 consecutive days. The time line of each session is summarized in Figure 1.

Before the start of each session, an indwelling catheter (Vasofix, 20 gauge, 10/10 mm, ϕ 1 mm, Braun) was inserted into the antecubital vein of the nondominant arm. When local vein irritation occurred, the dominant arm was used the next day. The experimental

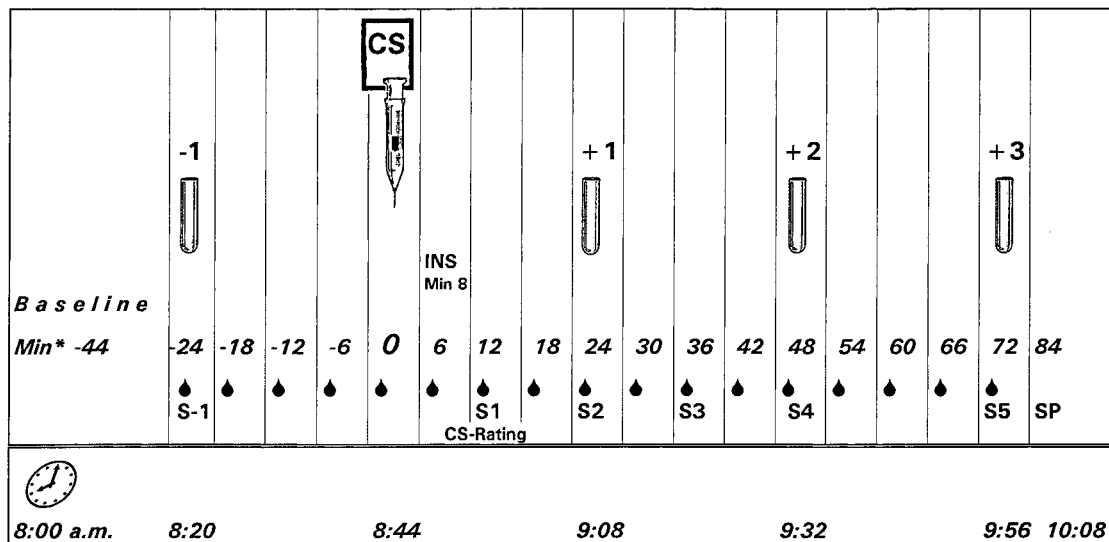


Fig. 1. Time schedule of each experimental session on 5 consecutive days. indicates times at which 20 ml of blood was drawn to measure levels of insulin (INS), glucagon, adrenaline, noradrenaline, and cortisol at minutes -24, 24, 48, and 72. indicates times at which 20 μ l of blood was drawn for measurement of blood glucose. CS = conditioned stimulus; S = symptom list; SP = substance perception; Min* = minutes relative to injection.

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procedure started at 8:00 AM. Two subjects were run in the same session with a 3-minute lag. To increase standardization and to guarantee the time schedule, the sequence of events during each session was presented on a computer screen. The person who performed the experiment completed each task and indicated its completion by responding on the computer keyboard, thus initiating the announcement of the subsequent task. Each session lasted 128 minutes and was divided into a preinjection phase (44 minutes, indicated as minute -44 to minute 0 in Figure 1) and a postinjection phase (84 minutes after injection). The first 20 minutes of the preinjection phase constituted the resting period, during which time subjects were allowed to read magazines. Twenty-four minutes before injection, a blood sample for analysis of blood glucose, insulin, glucagon, catecholamines, and cortisol was taken. Blood samples for glucose analyses were drawn from venous blood every 6 minutes. A total of 17 blood glucose samplings were obtained, four before (minutes -24, -18, -12, and -6) and 13 after application of the CS (starting with minute 0 immediately preceding the injection until minute 72). Samples for insulin and counterregulatory hormones were subsequently drawn every 24 minutes after injection (ie, at minutes 24, 48, and 72). Another blood sample for insulin determination was also obtained 8 minutes after injection (minute 8). Symptoms were recorded at six time points (minutes -24, 12, 24, 36, 48, and 72) using symptom lists. Each symptom list was presented in a printed version and contained 19 symptoms, including typical symptoms of hypoglycemia that had to be identified and rated by the subject (see Dependent Variables for details). Furthermore, 3 minutes after the smell was terminated (minute 8), subjects were given a questionnaire on the features of the CS smell and had to rate its pleasantness, novelty, and intensity on a nine-point rating scale. Finally (minute 81), subjects completed a questionnaire (substance perception) in which they indicated which substance they assumed they had received (insulin or saline).

Dependent Variables

Dependent variables were blood glucose, serum insulin, plasma glucagon, plasma adrenaline and noradrenaline, serum cortisol, and symptoms.

Blood glucose was determined from venous hemolysated blood samples (each 20 μ l, filled into a 1.5-ml plastic container containing a hemolysis inhibitor) by an Eppendorf EPOS analyzer 5040 using an enzymatic hexokinase method. Each blood glucose analysis was done in duplicate. The result was expressed as the mean of the duplicate measurement. In case of a difference of ≥ 6 mg/dl, a third measurement was performed, and the two less distant values were used to calculate the mean. The mean coefficient of retest reliability (averaged over the 17 blood samples for each group) ranged from 0.80 to 0.95 for days 1 to 5. Standard measurement error per day reached mean values between 1.4 and 1.8 mg/dl for the 5 days of the experiment. Samples for the later determination of insulin and counterregulatory hormones (glucagon, catecholamines, and cortisol) were stored on ice during each session. After the end of a session, the samples were immediately centrifuged (catecholamines: 10 minutes, 4°C; all other hormones: 10 minutes, room temperature) and frozen at -20°C until assays were performed. Serum insulin was assayed with a commercially available MEIA (Abbott, Wiesbaden, Germany), measuring biologically active immunoreactive human insulin. The CV of the method is 6.0% for a mean insulin level of 8.3 mU/liter, corresponding to the fasting insulin level, and 4.4% for mean insulin levels of 40.4 and 121.7 mU/liter. Glucagon was measured in Trasylol-EDTA plasma (1000 U/ml blood, Trasylol, Bayer, Leverkusen, Germany) using a RIA developed by Drost (29). This method has an interassay CV of 7.3% for mean glucagon values of

96 \pm 7 pg/ml and 7.9% for mean glucagon values of 441 \pm 35 pg/ml. Plasma catecholamines (adrenaline and noradrenaline) were analyzed by HPLC in EGTA-plasma using a Beckman System Gold Autosampler 507, a Pharmacia Beckman electrochemical detector, and commercial kits (Chromsystems, Munich, Germany). The method has an interassay CV for adrenaline of 5.6% for mean plasma adrenaline values of 64 and 5.8% for mean adrenaline values of 398. The interassay CV for noradrenaline is 6.1% for mean plasma noradrenaline values of 236 and 3.9% for mean noradrenaline values of 1315 pg/ml. Serum cortisol was measured using a commercially available RIA (Biermann, Bad Nauheim, Germany) with an interassay CV of ≤ 6.4 for mean cortisol values ranging from 3.3 to 36.0 μ g/dl.

The symptom list covered 19 symptoms: headache, palpitation, need to urinate, thirst, hunger, weakness, sweating, fatigue, dizziness, irritation, reduced concentration, anxiety, nervousness, trembling, blurred vision, nausea, salivation, breathing difficulty, and tingling around lips. Symptoms were identified and rated by the subject. Subjects were instructed to mark the actual intensity of each symptom by crossing a number on a five-point, numerically graded rating scale. The scale was also verbally anchored (0 = not at all, 1 = hardly, 2 = moderately, 3 = strong, and 4 = very strong). On the basis of results of hypoglycemia studies (eg, Refs. 30 and 31), symptoms were divided into neuroglycopenic and autonomic symptoms. The neuroglycopenic symptom score consisted of the symptoms weakness, fatigue, dizziness, blurred vision, and reduced concentration (see Ref. 31 for an analogous operationalization), which suggest a reduced glucose supply within the brain. The autonomic score consisted of sweating, tremor, palpitation, hunger, anxiety, and nervousness (cf. Ref. 31), indicating hypoglycemia-induced activation of the autonomic nervous system. The subjects' responses on the symptom list were dichotomized into the values 0 (no) if the symptom intensity was qualified as "not at all" and 1 (yes) for intensity 1 to 4. Thus, the neuroglycopenic score, consisting of five symptoms, reached values between 0 and 5; the autonomic score (six symptoms) ranged from 0 to 6. In addition, all symptoms were added for a total symptom score (range, 0-19).

Data Analysis

Data are expressed as mean \pm SEM. Because of a large variation between subjects in the baseline values of most of the endocrine parameters, differences from baseline values were calculated. Intergroup comparisons were made using the change scores. Inferences were made only for the cumulative blood glucose change on day 5 (test phase). Because of the small sample size, and to reduce the impact of outliers, comparisons were calculated with nonparametric tests for rank-scaled data. Accordingly, intergroup comparisons were calculated using the Mann-Whitney *U* test. Analyses were conducted with the SPSS statistical package. Because of the sample size (10 subjects per group), there were fewer than 30 different values in the combined sample; thus, reported *p* values are based on the calculation of exact tests. For intragroup comparisons, the Lam-Longnecker test for paired data (32) was used. Data analyses for insulin, counterregulatory hormones, and subjective symptoms are considered exploratory, not confirmatory, and were done for descriptive purpose only to address the pilot questions of the study (see Introduction). Accordingly, the latter *p* values were not corrected for multiple comparisons. Furthermore, we also report results reaching a *p* value of $<.10$ in the test session. The rationale is twofold. First, this is the first insulin conditioning study in which blood glucose, insulin, counterregulatory hormones, and symptoms were simultaneously measured. Thus, exploratory data must be gathered to identify parameters that are sensitive to conditioning.

Second, even in the URs, intergroup differences did not attain the 5% but only the 10% level on some days during the acquisition phase. This is the case for cortisol and neuroglycopenic symptoms. It is reasonable to expect that the effects seen in the CR do not exceed those seen in the UR.

RESULTS

All endocrine and subjective parameters of the acquisition phase are described first, followed by the data of the test (day 5). Inference statistics were calculated only for the cumulative blood glucose change in the test. All other comparisons are considered to be descriptive only and address the pilot questions of our study.

Blood Glucose

Figure 2 summarizes the blood glucose data during the acquisition phase (days 1–4) for group 1 (CS-INS) and group 2 (CS-NaCl). The blood glucose minimum in group 1 was obtained 24 minutes after injection of insulin and reached the level of hypoglycemia (≤ 50 mg/dl) on all 4 days (mean = 43.0 ± 3.1 , 41.8 ± 3.3 , 40.4 ± 2.6 , and 43.4 ± 2.5 mg/dl on days 1–4, respec-

tively). Group 2 reached mean values of about 75 mg/dl in the corresponding measurement point (minute 24) on all 4 days (mean = 75.5 ± 1.4 , 76.3 ± 2.0 , 75.0 ± 1.5 , and 76.7 ± 1.2 mg/dl on days 1–4, respectively).

Inferential Statistics

Corresponding to analyses in animal experiments (eg, Refs. 8, 10, 14, and 33), the results of day 5 (test phase) were expressed as deviation from baseline blood glucose level. Baseline blood glucose was calculated as the mean blood glucose level in the measurement points before CS presentation (ie, minutes -24, -18, -12, and -6). Baseline blood glucose (see Figure 3) did not differ between groups 1 and 2 ($U = 44$, $p = .684$, two-sided). For each subject, the difference between blood glucose in the actual measurement point and the subject's baseline level was calculated. The blood glucose change after CS presentation was expressed as the cumulative change, that is, the sum of blood glucose changes over the 13 measurement points after CS presentation (minutes 0–72). The mean cumulative blood glucose decrease was significantly larger

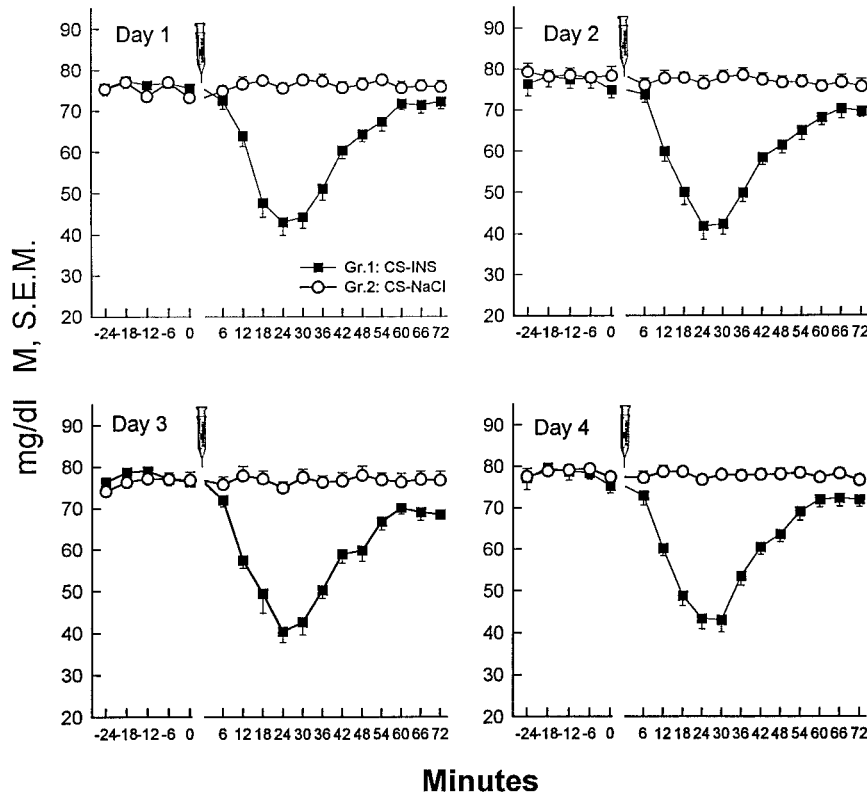


Fig. 2. Blood glucose (mg/dl) on days 1 to 4 of the acquisition phase. Mean (M) and SEM values are shown for groups 1 and 2. Injection was given immediately after blood sampling at minute 0.

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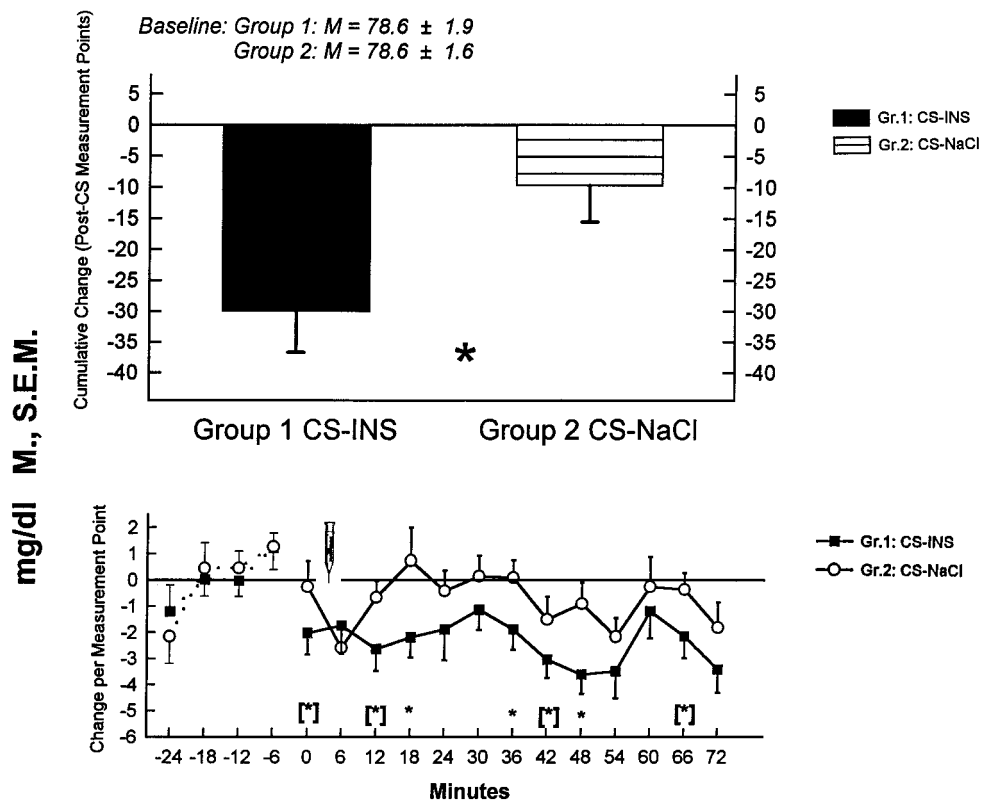


Fig. 3. Blood glucose change (mg/dl) relative to mean (M) baseline level during the test phase (day 5) under CS-saline in groups 1 and 2. *Top*, Cumulative change over the 13 post-CS measurement points (mean \pm SEM). Baseline level is also shown. *Bottom*, Change per measurement point. Pre-CS measurements (minutes -24 to -6) are interconnected by dotted lines; post-CS measurements are interconnected by solid lines. Results of descriptively conducted Mann-Whitney U tests: [*] = $p < .10$; * = $p < .05$.

in group 1 (-30.0 ± 6.7 mg/dl) than in group 2 (-9.8 ± 5.8 mg/dl; $U = 23$, $p = .022$) (Figure 3, *top*).

Exploratory Analyses

Blood glucose during test phase. Time course. To describe the time course of blood glucose changes, U tests were performed for the difference from baseline for each measurement point. There was no hint of any group difference in the four pre-CS measurement points (minutes -24, -18, -12, and -6). But after CS presentation, group 1 reached lower values in 12 of the 13 post-CS measurement points, with $p < .05$ in three comparisons (minutes 18, 36, and 48) and $p < .10$ in four comparisons (minutes 0, 12, 42, and 66), indicating a systematic effect in the single measurement points (Figure 3, *bottom*).

Features of conditionability. To explore whether a larger blood glucose decrease during the test is associated with variables that facilitate classical conditioning per se (ie, intensity of the CS and UR) (34) and thus validate the conditioning interpretation and to identify responder variables that enhance conditioning, we conducted the following post hoc analysis. Group 1

was divided into two subgroups on the basis of the maximum amount of blood glucose decrease after CS presentation. A median split resulted in five subjects (denoted as COND+) with a blood glucose decrease of >6 mg/dl on at least one time point after CS presentation and five subjects (COND-) with decreases of ≤ 6 mg/dl. The characteristics of COND+ and COND- subjects are summarized in Table 1. Because of the small sample size, descriptive data are given as medians.

COND+ and COND- subjects did not differ in hypoglycemia at the beginning of the acquisition phase (day 1), thus excluding preexperimental differences. However, COND+ subjects tended to react more intensively to the insulin injection on the last acquisition day (day 4; median = 40.5 mg/dl) than COND- subjects (median = 48.0 mg/dl; $U(n_1 = n_2 = 5) = 5$, $p = .075$). In accordance with conditioning predictions, subjects with a stronger CR (COND+) rated the CS as more intense (median = 6.0) than those with a weaker CR (COND-) (median = 3.0; $U = 4$, $p = .048$). COND+ subjects reported more neuroglycopenic symptoms ($p < .05$ in five of the descriptive comparisons) and had a lower noradrenaline level than COND- subjects

TABLE 1. Comparison of Conditionable (COND+) Versus Nonconditionable (COND-) Subjects of Group 1^a

Subgroups ^b	COND+ ^c (median)	COND- ^d (median)	<i>p</i> ^e	Features of COND+ Subjects
BG minimum				
Acquisition phase, day 1	40.0	43.0	.6905	
Acquisition phase, day 4	40.5	48.0	.075	Greater UR
Intensity of CS, test phase, day 5	6.0	3.0	.048	Greater CS intensity
Neuroglycopenic symptoms, test phase, day 5				
Symptom list, minutes -24, 24, 36, 48	1	0	<.05	Greater no. of symptoms
Symptom list, minute 72	2	0	<.05	Greater no. of symptoms
Noradrenaline (pg/ml), test phase, day 5				
Minute -24	177.0	312.0	.028	Less noradrenaline
Minute 72	176.0	318.0	.075	Less noradrenaline

^a Results of descriptively conducted intergroup comparisons (Mann-Whitney *U* test) and features of conditionable and nonconditionable subjects are also given.

^b Based on blood glucose (BG) decrease at day 5.

^c BG decrease of >6 mg/dl.

^d BG decrease of ≤6 mg/dl.

^e Mann-Whitney *U* test.

($U = 3$, $p = .028$) at baseline and a tendency for lower noradrenaline at the end of the test session (minute 72; $U = 5$, $p = .075$).

Insulin. Insulin injection resulted in the expected high insulin levels at the first postinjection measurement (minute 8, $p = .000$). Clear intergroup differences ($p < .001$) remained at minute 24 on all 4 days of the acquisition phase (Figure 4, *left*). Intergroup differences were no longer obvious at minutes 48 and 72. On days 3 and 4, comparisons revealed an even larger decrease for group 1 than for group 2 at minute 72 (day 3: $U = 23.5$, $p = .022$; day 4: $U = 26.5$, $p = .067$).

Insulin level at day 5 reached the same baseline value in group 1 (mean = 4.2 ± 0.4) and group 2 (mean = 4.2 ± 0.5). However, at the first postinjection measurement (minute 8), group 1 tended to differ from group 2, with group 1 showing a decrease (mean = -0.7 ± 0.3) compared with hardly any difference from baseline in group 2 (mean = 0.04 ± 0.3 ; $U = 30$, $p = .072$). Within group 1, an additional effect occurred: There was an increase of the baseline insulin level (minute -24) from day 1 (mean = 3.6 mU/liter) to day 5 (mean = 4.2 mU/liter) ($Z = 1.911$, $p = .028$, Lam-Longnecker test for paired data), whereas there was no change in group 2 ($Z = -0.216$, $p = .415$).

Counterregulatory hormones. Results for the counterregulatory hormones glucagon, adrenaline, and noradrenaline are summarized in Table 2, and cortisol data are shown in Figure 4.

Glucagon. Glucagon is the first responding counterregulatory hormone (25), with an early peak at minute 24 in group 1, resulting in strong group differences on days 1 to 4 (day 1: $p < .01$; days 2-4: $p < .001$). But at day 5 (test), groups did not differ ($U = 42$, $p = 1-0.290$).

Adrenaline. Intergroup differences in adrenaline during the acquisition phase peaked at minute 48 (day 1: $U = 10$, $p = .003$; day 2: $U = 11.5$, $p = .001$; day 3: $U = 14.5$, $p = .003$; day 4: $U = 17$, $p = .011$). At day 5, there were no effects in the difference-to-baseline scores.

Noradrenaline. Noradrenaline effects were weaker and fluctuating. At day 1, an early (minute 24) increase in group 1 ($U = 15.5$, $p = .012$) was found. At day 3, the peak was delayed (minute 48), and groups only tended to differ ($U = 32$, $p = .095$). No difference was found at days 2 and 4. Accordingly, no CR was detected at day 5. Baseline levels at day 5 were lower than at day 4 but then rose in both groups, mainly from minutes 48 to 72 ($p < .01$, Lam-Longnecker-test).

Cortisol. Our study was conducted in the morning (8:00-10:15 AM), when cortisol levels are decreasing (35). Thus, groups initially (minute 24) exhibited this circadian rhythm. But at minute 48, an opposite trend was observed; group 1 tended to have a lower decrease than group 2 ($U = 31$, $p = .083$) at day 1 that was more obvious at days 2 ($U = 26.5$, $p = .038$) and 3 ($U = 21$; $p = .014$), whereas p was only .062 at day 4 ($U = 29$). At day 5, groups again tended to differ at minute 48 ($U = 32.5$, $p = .095$). Baseline cortisol decreased from days 1 to 5 (group 1: $Z = -6.354$, $p = .000$; group 2: $Z = -2.437$, $p = .007$, Lam-Longnecker test for paired data).

Symptoms. Figure 5 summarizes the change scores of the subjective symptoms, classified as neuroglycopenic symptoms, autonomic symptoms, and total number of symptoms, on days 1 to 5. Baseline values did not differ between groups at any day.

Intergroup differences were related to the peak manifestation. With the exception of day 1, the difference-

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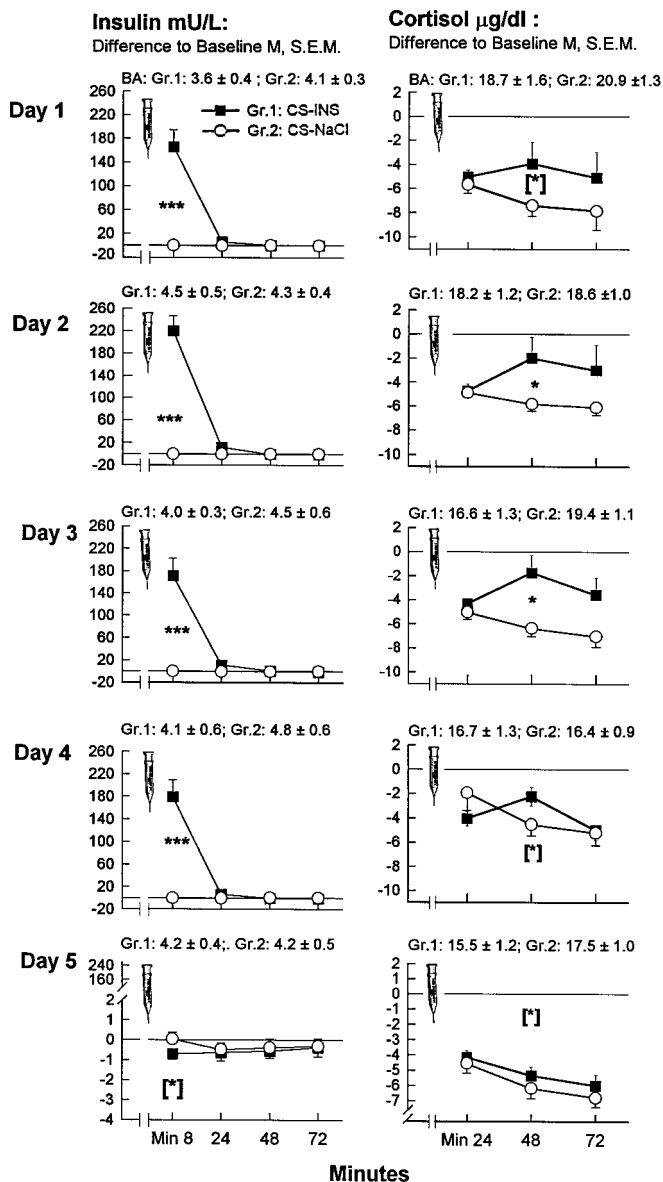


Fig. 4. Insulin and cortisol on days 1 to 5 in groups 1 and 2. *Left*, Insulin change (mU/liter). Difference from baseline value (minute -24). Mean (M) and SEM values are given. Baseline (BA) values are also shown. See break of y axis at day 5. *Right*, Cortisol change (µg/dl). Difference from baseline value (minute -24). Mean and SEM values are given. BA values are also shown. See break of y axis at day 5. Results of descriptively conducted Mann-Whitney U tests: [*] = $p < .10$; * = $p < .05$; *** = $p < .001$.

to-baseline score of neuroglycopenic symptoms (Figure 5, *left*) differed mostly at minute 36 after injection. Whereas the descriptively conducted comparisons revealed only minor effects on days 1 and 2 ($p < .10$), a higher neuroglycopenic score was clearly obvious at day 3 ($U = 11.5$, $p = .001$) but less obvious on day 4 ($U = 28$, $p = .053$); thus, an attenuation of the UR

(comparable to the cortisol response) occurred over the time course of the acquisition. At day 5, differences did not reach a p value of $< .05$ at any single time point. However, in the cumulative change of neuroglycopenic symptoms (sum of changes at minutes 12, 24, 36, 48, and 72), groups 1 and 2 tended to differ ($U = 32.5$, $p = .095$) because of an increase above baseline (mean = 0.3 ± 0.8) in group 1 and a decrease (mean = -0.9 ± 1.6) in group 2. From days 1 to 5, group 1, with the previous hypoglycemia experiences, showed a decrease ($Z = -1.872$, $p = .031$) in the baseline level of neuroglycopenic symptoms, whereas there was no change in group 2. Insulin-induced changes of symptoms during the acquisition phase became more obvious in the autonomic score (Figure 5, *middle*). A clear group difference culminated in minute 36 on all days of the acquisition (days 1, 2, and 4: $p < .001$; day 3: $p < .05$). During the test session, no difference was seen. The right column of Figure 5 shows the change in the total number of symptoms. Except on day 1, the intergroup difference culminated at minute 36 in the acquisition phase. At day 5 (test phase), group 1 exceeded the baseline level at all time points, whereas group 2 showed only minor changes. A first hint ($U = 28.5$, $p = .053$) for a difference between group 1 and 2 was detected at the end (minute 72) of the test session. Thus, the CR was temporarily delayed compared with the manifestation of the UR during acquisition.

DISCUSSION

A central aim of the present study has been to find out whether a conditioned decrease of blood glucose in healthy humans can be obtained when a placebo-controlled experimental design is used. So far, blood glucose change is the most commonly used indicator of conditioned responses after a CS-insulin experience. As expected, after the acquisition phase, which consisted of 4 days with a CS-insulin application (group 1) versus a CS-saline application (group 2), group 1 showed a significantly larger cumulative decrease of blood glucose after CS presentation than control group 2. A fine grain analysis of the time course covering the 13 equidistant measurement points after CS presentation revealed a lower blood glucose level in group 1 in 12 of 13 measurements with p values of $< .05$ in three samples (minutes 18, 36, and 48) and $< .10$ in four samples (minutes 0, 12, 42, and 66). Thus, the CR occurred earlier than the previous unconditioned hypoglycemia (minute 24). This corresponds to animal data (eg, Refs. 9 and 36). Our data revealed additional differences after the time point of the previous hypoglycemia. Because there was a smaller decrease of glucose in our study than has been reported

TABLE 2. Counterregulatory Hormones in Acquisition (Days 1–4) and Test (Day 5) Phases for Groups 1 and 2^a

	Glucagon (pg/ml)				Adrenaline (pg/ml)				Noradrenaline (pg/ml)			
	Group 1 ^b		Group 2 ^c		Group 1 ^b		Group 2 ^c		Group 1 ^b		Group 2 ^c	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Day 1												
Baseline, minute -24	308.8	±37.2	308.3	±35.2	33.7 ^d	±4.7	38.6 ^d	±2.6	247.4 ^d	±27.4	324.6 ^d	±16.3
Minute 24	+23.7 ^e	±8.6	-9.7 ^e	±4.6 ^h	+25.6 ^d	±16.7	-9.2 ^d	±3.1	+34.1 ^{de}	±36.2	-48.3 ^{de}	±14.2 ^g
Minute 48	+20.4	±11.8	-0.9	±7.7	+49.1 ^{de}	±13.2	-2.3 ^{de}	±4.2 ^h	+58.0 ^d	±27.1	-5.1 ^d	±27.9
Minute 72	+7.9	±6.3	-20.6	±5.1	+20.7 ^d	±9.8	-9.8 ^d	±4.7	+63.9 ^d	±30.0	+28.5 ^d	±30.1
Day 2												
Baseline, minute -24	296.3	±26.8	316.8	±42.0	40.6	±8.2	32.8	±5.6	344.5	±36.5	306.6	±28.5
Minute 24	+35.8 ^e	±8.0	+1.4 ^e	±4.9 ⁱ	+51.1	±25.8	+4.2	±12.3	+33.0	±21.2	-3.3	±28.0
Minute 48	+27.8	±9.3	+1.7	±6.3	+44.1 ^e	±14.8	-8.0 ^e	±4.6 ^h	+48.8	±20.5	+16.8	±23.1
Minute 72	+3.2	±7.0	-4.6	±6.9	+19.8	±8.8	+1.9	±7.3	+8.8	±21.3	+23.0	±26.8
Day 3												
Baseline, minute -24	286.1	±28.5	320.9	±45.1	32.2	±5.4	32.2	±8.7	315.4	±40.2	354.7	±45.5
Minute 24	+43.2 ^e	±9.6	-8.4 ^e	±6.6 ⁱ	+34.2	±12.4	-5.6	±6.3	+25.1	±12.1	+0.3	±34.5
Minute 48	+32.1	±8.3	-4.3	±7.4	+47.2 ^e	±14.5	-1.3 ^e	±7.8 ^h	+51.3 ^e	±17.1	+24.6 ^e	±15.0 ^f
Minute 72	+12.2	±6.2	-2.5	±6.7	+26.9	±6.9	+1.4	±13.5	+41.5	±15.0	+41.6	±21.9
Day 4												
Baseline, minute -24	308.1	±28.6	318.3	±44.6	46.3	±6.3	32.3	±3.9	269.1	±19.2	322.8	±15.7
Minute 24	+34.2 ^e	±6.7	-7.6 ^e	±5.5 ⁱ	+25.8	±9.4	+3.8	±6.9	+33.3	±19.1	+24.1	±26.2
Minute 48	+12.8	±7.7	+6.9	±6.6	+38.1 ^e	±9.7	+1.2 ^{de}	±3.9 ^g	+26.5	±16.7	+20.2 ^d	±30.7
Minute 72	-5.8	±9.3	+2.7	±3.5	+23.1	±7.6	+4.6	±4.0	+33.2	±13.9	+67.5	±27.8
Day 5												
Baseline, minute -24	304.0	±31.9	315.4	±40.3	40.2	±6.5	32.6	±5.8	237.3	±30.7	292.7	±36.6
Minute 24	-7.5	±3.8	-4.5	±4.5	+1.2	±6.5	+0.8	±4.5	+13.8	±23.3	+23.0	±25.8
Minute 48	-8.1	±4.4	-7.3	±4.9	+1.8	±8.1	+8.3	±7.1	+56.6	±38.7	+27.3	±16.5
Minute 72	-4.5	±6.1	-5.1	±6.9	+3.1	±4.5	-4.0	±3.6	+56.0	±18.8	+70.5	±16.8

^a Mean ± SEM of the baseline level (minute -24) and difference from baseline level for minutes 24, 48, and 72 are given. + = increase from baseline; - = decrease from baseline.

^b CS-insulin.

^c CS-saline.

^d *N* = 9 blood samples (see Catecholamines, day 1, groups 1 and 2, and day 4, group 2).

^e Time points of maximum intergroup differences (results of descriptively conducted Mann-Whitney *U* test).

^f *p* < .10.

^g *p* < .05.

^h *p* < .01.

ⁱ *p* < .001.

in animals, we speak of conditioned blood glucose decrease instead of conditioned hypoglycemia.

It is obvious that the conditioned decrease of blood glucose in the experimental group compared with the control group is not large, also with regard to the measurement error of the blood glucose assay. But several points support the relevance and consistency of our results. In accordance with the majority of results obtained in animal experiments (see Ref. 4 for review), a blood glucose decrease, not an increase, was found in all subjects of our previous CS-insulin group. The fine-grain analysis of the post-CS measurement points had revealed a systematically lower level for group 1 compared with group 2 in 12 of the 13 measurement points. This clearly differs from the random behavior in the four pre-CS measurement points. Thus,

while small in absolute terms, the decrease was present at almost every time point and thus differed from the pre-CS pattern. Thus, the results are more homogeneous than the response pattern obtained in a previous study with humans (23). Furthermore, the additional exploratory analyses (see below) are in accordance with the predictions.

To validate the conditioning interpretation, we conducted post hoc analyses, in which we again used blood glucose because it is the commonly used indicator of conditioning effects after the CS-insulin experience. On the basis of the amount of glucose reduction after CS presentation (ie, the amount of the CR), group 1 was divided into a subgroup with a higher (COND+) and a lower (COND-) conditioned blood glucose reduction. As expected from basic conditioning research

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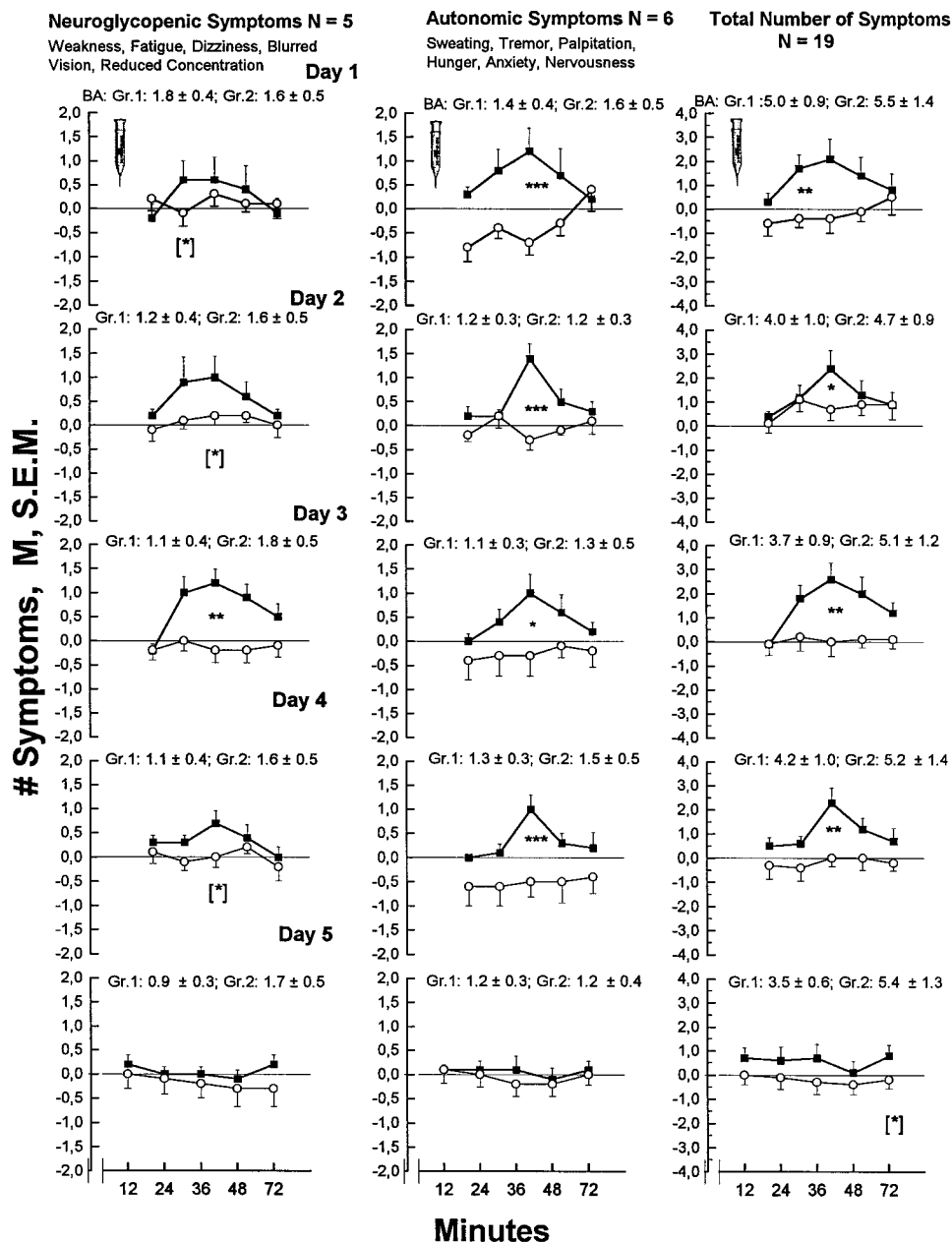


Fig. 5. Number of symptoms. Difference from baseline value (minute -24) on days 1 to 5 in groups 1 (■) and 2 (○). Mean (M) ± SEM baseline (BA) values are given. *Left*, Neuroglycopenic score. *Middle*, Autonomic score. *Right*, Total number of symptoms score. Mean ± SEM values are given. Results of descriptively conducted Mann-Whitney *U* tests: [*] = $p < .10$; * = $p < .05$; ** = $p < .01$; *** = $p < .001$.

(eg, Ref. 34), COND+ (ie, more conditionable) subjects showed features corresponding to parameters enhancing classical conditioning, such as a larger unconditioned blood glucose decrease (ie, a larger UR) at the end of the acquisition phase and they rated the CS as more intense. Furthermore, only COND+, but not COND-, subjects showed neuroglycopenic symptoms, whereas subgroups did not differ in the number of autonomic symptoms. Because neuroglycopenic symptoms

are indicative of CNS effects of hypoglycemia (eg, Refs. 30 and 31), and because classical conditioning always requires the CNS, subgroup differences in neuroglycopenic (not in autonomic) symptoms are in accordance with the assumed mediation of classically conditioned responses. Concerning hormones, a lower baseline noradrenaline level was found in the COND+ subgroup. Because central noradrenaline also has a blood glucose increasing effect (eg, Refs. 3 and 37), the lower noradren-

aline baseline corresponds with features that should facilitate conditioned blood glucose decrease. Although this is a post hoc analysis, it validates the conditioning interpretation of our data. Furthermore, it reveals endocrine and subjective responder variables that can be used as screening variables in insulin conditioning studies.

For insulin, group 1 compared with group 2 tended to decrease relative to baseline in minute 8 of the test session. This trend must be interpreted with caution, but it does not necessarily contradict the expected conditioned increase in insulin secretion (20). The first insulin sample was drawn only 8 minutes after injection. An increase might have occurred before that time point, as has been shown in animals (15). But our data also contain evidence of a conditioned insulin increase. In group 1, baseline insulin increased ($p < .05$) from day 1 (mean = 3.6 mU/liter) to day 5 (mean = 4.2 mU/liter). Although small in absolute level, this is a mean percentage increase of 27.8% in group 1 versus one of only 6.4% in group 2. This is the most obvious insulin effect in our study, which is the first to measure insulin after an acquisition phase with exogenous insulin. Such a baseline change might also indicate conditioning (2). We now suggest recording vagal activity continuously (eg, by measuring heart rate variability) (38–40) to examine whether the CS triggers a vagally mediated insulin secretion.

For glucagon, adrenaline, and noradrenaline, the descriptively conducted intergroup comparisons did not reveal conditioned effects in the difference-to-baseline values. However, there was a tendency for a conditioned cortisol response manifesting at the time point of the previous UR. This corresponds to findings of the only animal conditioning study with insulin in which corticosterone and blood glucose were both measured (19). Cortisol is also of interest for insulin conditioning because of its interaction with central insulin. Whereas insulin in the CNS leads to a reduction of food intake and body weight (4, 41, 42), glucocorticoids lead to increases in food intake (43, 44). Central insulin also interacts with the anorexic effects of peripheral cholecystokinin, now in an agonistic way (45). Thus, conditioning studies should also address the CNS effect of insulin, its role in food intake (46), and its interaction with neuropeptides.

Whereas group 1 and group 2 differed predominantly in autonomic symptoms during acquisition, neuroglycopenic symptoms were more sensitive to conditioning effects in the test session. Our results are in accordance with recent data (47) demonstrating that the expectation of receiving insulin led to a higher number of neuroglycopenic but not autonomic symptoms in comparison to subjects who were also injected with insulin but told they were receiving saline. Our

results complete this picture for a classical conditioning situation. Furthermore, in our experiment, neuroglycopenic symptoms turned out to be more sensitive to adaptation. From days 1 to 5, there was a tendency for a decrease of baseline neuroglycopenic, but not autonomic, symptoms within group 1, who had experienced antecedent hypoglycemia. Adaptation to antecedent hypoglycemia with blunted symptom perception has also been described in patients with insulin-dependent diabetes mellitus (eg, Ref. 48), in patients with insulin-producing tumors (eg, Ref. 25), and in healthy subjects experiencing reoccurring hypoglycemia (eg, Ref. 49). However, the results were never related to classical conditioning. It would be interesting to analyze the contribution of classical conditioning to this change in symptom perception. Attenuation of the UR with repeated insulin injections also occurred with cortisol, in which, similarly to the neuroglycopenic symptoms, a trend for a CR could be demonstrated in the test session. One interesting question arising from this observation is whether an endocrine or subjective parameter that is sensitive to experience during acquisition is more easily conditionable.

In addition to the basic research questions that still must be solved, especially for human subjects (eg, classical conditioning of insulin effects and moderator variables or analysis of the interaction between central insulin and peptides or hormones), in larger samples, future studies should also analyze the impact of classical conditioning on the diabetic patient.

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