Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 58 (2009) 443 – 448
Metabolism
Clinical and Experimental
www.metabolismjournal.com

Proinflammatory cytokines in response to insulin-induced hypoglycemic stress in healthy subjects

Laleh Razavi Nematollahi[a], Abbas Eghbal Kitabchi[a,*], Frankie B. Stentz[a], Jim Y. Wan[a], Bagher A. Larijani[b], Mohammad Mohajer Tehrani[b], Mohammad Hossein Gozashti[b], Kobra Omidfar[b], Eghbal Taheri[b]

[a]*The University of Tennessee Health Science Center, Memphis, TN 38163, USA*
[b]*Endocrinology and Metabolism Research Center, Tehran, Iran*

Received 21 July 2008; accepted 6 October 2008

Abstract

Hyperglycemic crises of diabetic ketoacidosis and nonketotic hyperglycemia are associated with elevation of counterregulatory hormones and proinflammatory cytokines, markers of lipid peroxidation, and oxidative stress. To investigate if other conditions besides hyperglycemia could evoke such a prompt increase in cytokine levels, lipid peroxidation, and oxidative stress markers, we induced hypoglycemic stress by standard insulin tolerance test and measured proinflammatory cytokines, markers of lipid peroxidation, reactive oxygen species (ROS), and counterregulatory hormones. Insulin tolerance test was performed in 13 healthy male subjects with no history of infection, cardiovascular risk factors, or abnormal glucose. At baseline and at 30, 45, 60, 120, and 240 minutes after insulin injection, the following parameters were measured: glucose, cortisol, corticotropin, epinephrine (EP), norepinephrine (NE), growth hormone, tumor necrosis factor (TNF)–α, interleukin (IL) 1β, IL-6, IL-8, free fatty acids, white blood cells, lipid peroxidation markers by thiobarbituric acid assay, and ROS by dichlorofluorescein method. The peak value of white blood cell count at 120 minutes was significantly associated with the peak values of NE at 30 minutes and cortisol at 60 minutes. By comparing the area under the curve of measured parameters, EP emerged as significant predictor of TNF-α ($P = .05$) and IL-8 ($P = .027$). Cortisol emerged as predictor of IL-1β significantly ($P = .05$). Corticotropin predicted area under the curve of IL-6 with borderline significance ($P = .06$). In the present study, insulin-induced hypoglycemia in nondiabetic male subjects is associated with increased proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8), markers of lipid peroxidation, ROS, and leukocytosis. Elevations of NE, EP, corticotropin, and cortisol in hypoglycaemia are associated with the elevation of the proinflammatory cytokines and leukocytosis.
Published by Elsevier Inc.

1. Introduction

We have documented that severe hyperglycemia of diabetic ketoacidosis (DKA) or nonketotic hyperglycemia provokes elevation of counterregulatory hormones, proinflammatory cytokines (tumor necrosis factor [TNF]–α, interleukin [IL]-6, IL-8, and IL-1β), markers of reactive oxygen species (ROS) measured by dichlorofluorescein (DCF) or lipid peroxidation measured as malondialdehyde (MDA), cardiac risk factors including C-reactive protein, and free fatty acids (FFA). These parameters, which are 2- to 3-fold higher than normal, return to normal values within 24 hours of insulin therapy and resolution of hyperglycemia and dehydration [1]. We had previously suggested that these prompt responses to elevation of cytokines and counterregulatory hormones may be due to either anti-inflammatory effects of insulin or, more likely, responses to stress of hyperglycemia. Because patients with diabetes on antidiabetic agents experience hyperglycemia and hypoglycemia, these excursions may provoke various responses in the body. We have chosen hypoglycemia in distinction to hyperglycemia to investigate if such a stress would also produce similar responses to that of hyperglycemia. Herein, we present the results of insulin-induced hypoglycemia in 13 nondiabetic male subjects.

2. Research design and methods

2.1. Participants

Healthy male subjects with no history of current infection, cardiovascular risk factors, metabolic syndrome, or abnor-

* Corresponding author. Division of Endocrinology, Department of Medicine, The University of Tennessee Health Science Center, Memphis, TN 38163, USA.
E-mail address: akitabchi@utmem.edu (A.E. Kitabchi).

0026-0495/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.metabol.2008.10.018

mal glucose metabolism were invited to participate in the study that was approved by the ethics committee of Endocrinology and Metabolism Research Center of Tehran University. On admission, after physical examination including measuring the vital signs, blood was drawn between 8:00 and 9:00 AM for the following tests: complete metabolic profile, cell blood counts with differential, hemoglobin A_{1c} (HbA_{1c}), thyroid function tests, oral glucose tolerance tests, and fasting lipid profile. Subjects with any abnormalities in the above-mentioned tests were excluded. Thirteen eligible healthy male subjects entered the study.

2.2. Procedure

We performed the standard insulin tolerance test (ITT) on the participants [2,3]. After a 30-minute rest in supine position, basal blood sample was obtained between 8:00 and 9:00 AM for measuring glucose, corticotropin (ACTH), cortisol, growth hormone (GH), epinephrine (EP), norepinephrine (NE), white blood cell count (WBC), proinflammatory cytokines (TNF-α, IL-6, IL-8, and IL-1β), and FFA. The ITT was performed by intravenous injection of 0.1 U of regular insulin (H-Insulin, Hoechst, Frankfurt, Germany) per kilogram of body weight. Vials of 50% glucose were available in case blood glucose dropped to values less than the critical level of 30 mg/dL or intolerable hypoglycemic symptoms occurred.

Blood glucose levels were measured every 5 to 10 minutes for the first 60 minutes and then every 30 to 60 minutes for the next 3 hours. Vital signs were monitored closely during the test. Venous blood samples for measurement of plasma cortisol, catecholamines (EP, NE), GH, ACTH, WBC, proinflammatory cytokines, DCF, MDA, leukocytes, and FFA were taken at 0, 30, 45, 60, 120, and 240 minutes after insulin injection. Adequate hypoglycemia was considered when the blood glucose became less than 39 mg/dL and/or when hypoglycemic symptoms including sweating and tachycardia were present [2,3].

2.3. Laboratory measurements

Levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8), C-reactive protein, GH, ACTH, and cortisol were measured in the plasma using a solid-phase, 2-site sequential chemiluminescent immunometric assay on an Immulite analyzer (Diagnostic Products, Los Angeles, CA). The coefficients of variation of the assays were less than 5%. The instrument calibrations for the assays were performed as recommended by the manufacturers and were within the specifications. For assessment of plasma NE and EP, blood was drawn into EDTA-coated Monovettes (Sarstedt, Numbrecht, Germany) and immediately centrifuged for 10 minutes at 2000*g*; the plasma was stored at −80°C until analysis. Plasma NE and EP were determined by high-pressure liquid chromatography (detection limit, 0.25 pg/mL; inter- and intraassay variance, <5%; Laboratory for Stress Monitoring, Göttingen, Germany). Lipid peroxidation markers (MDA) were measured by thiobarbituric acid assay, and ROS were determined by DCF method as described previously [1].

2.4. Statistical analysis

The means ± SD were calculated for all continuous variables. Data were analyzed using Stat View version 9.0.1 (SAS Institute, Cary, NC). All tests were 2-tailed, with the significance level set at P less than .05. Missing data were excluded listwise. Analysis of variance for repeated measures was computed to investigate whether changes in measured variables are significant across the various time points. The area under the curve (AUC) for cytokines and counterregulatory hormones was compared across time intervals by regression analysis to determine whether any excursion in counterregulatory hormones predicts the changes in cytokines [4].

3. Results

Table 1 demonstrates the baseline characteristics of the participants. During the ITT, no severe adverse events were detected; and infusion of 50% glucose was not required during the study, as hypoglycemia less than 30 mg/dL or intolerable hypoglycemic symptoms did not occur in any of participants. The mean nadir of hypoglycemia was 38.2 ± 4.3 mg/dL, which was recorded at 30 minutes. The levels of counterregulatory hormones (GH, ACTH, EP, and NE), WBC, proinflammatory cytokines, FFA, MDA, and DCF at baseline, peak, and 240 minutes after insulin injection are shown in Table 2.

Elevation of counterregulatory hormones in response to hypoglycemia was as expected [2,3], with the earliest peaks of EP and ACTH at 45 minutes after insulin injection. Peaks of GH and cortisol were detected at 60 minutes after insulin injection; and NE had an initial increase at 30 minutes after insulin injection, but its peak occurred at 240 minutes after insulin injection (Table 2).

Proinflammatory cytokines also increased in response to hypoglycemia. Tumor necrosis factor–α had a peak at 45 minutes after insulin injection, which plateaued at 240 minutes. Peak of IL-6 was detected at 120 minutes, which remained elevated at 240 minutes after insulin injection.

Table 1
Baseline characteristics of participants on admission

Parameters	
Subjects (n)	13 (male)
Age (y)	30 ± 4.8[a]
Blood pressure (mm Hg)	120 ± 0.4/76 ± 0.3[a]
BMI(kg/m^2)	23 ± 1.3[a]
Temperature (°F)	98.1 ± 1.2[a]
HbA_{1c}	5.6 ± 1.06[a]
White blood cells ×10^3 (/μL)	6.4 ± 1.1[a]

[a] Data are mean ± SD.

Table 2
Baseline values of measured counterregulatory hormones and cytokines, values at peak or nadir, and values at 240 minutes

Parameters	Baseline values ± SD	Values at peak or nadir ± SD	Time (min)	Values at 240 min ± SD
Glucose (mg/dL)	102.2 ± 10.2	38.23 ± 19.9 ($P < .001$)	30	99.1 ± 13.79
Cortisol (μg/dL)	10.12 ± 4.9	15.23 ± 5.8 ($P = .004$)	60	7.93 ± 4.17
GH (ng/mL)	0.1 ± 0.03	27.28 ± 17.6 ($P < .001$)	60	2.9 ± 6.3
ACTH (pg/mL)	7.9 ± 7.7	47.48 ± 41.7 ($P < .006$)	45	5.1 ± 3.05
NE (μg/mL)	488 ± 174.7	807.9 ± 282.9 ($P = .005$)	240	807.9 ± 282.9
EP (μg/mL)	70.1 ± 28.3	1198.9 ± 1030 ($P < .001$)	45	90.4 ± 31.1
WBC ×103/μL	6.4 ± 1.10	12.97 ± 2.9 ($P < .001$)	120	12.5 ± 2.8
TNF-α (μg/mL)	6.1 ± 2.8	8.1 ± 2.8 ($P < .001$)	45	6.9 ± 2.9
IL-6 (μg/mL)	2.18 ± 1.2	3.94 ± 1.2 ($P = .0009$)	120	3.6 ± 1.7
IL-8 (μg/mL)	8.43 ± 6.3	12.6 ± 7.7 ($P = .04$)	60	8.9 ± 5.3
IL-1β (μg/mL)	0.70 ± 1.04	1.12 ± 0.91 ($P = .9$)	240	1.12 ± 0.91
FFA (μmol/L)	0.5 ± 0.15	1.06 ± 0.27 ($P < .001$)	240	1.06 ± 0.27
MDA (μmol/L)	0.6 ± 0.1	2.7 ± 0.2 ($P < .001$)	45	1.1 ± 0.1
DCF (μmol/L)	1.9 ± 0.2	3.9 ± 0.2 ($P < .001$)	45	2.2 ± 0.1

Peak of IL-8 was detected at 60 minutes after insulin injection, with near to baseline values at 240 minutes. Peak of IL-1β was demonstrated at 240 minutes of insulin injection (Table 2).

White blood cell counts also increased in hypoglycaemia, with the peak at 120 minutes after insulin injection, which plateaued at 240 minutes. Peak of lipid peroxidation marker (MDA) and ROS (DCF) was detected at 45 minutes after insulin injection, with near to baseline values at 240 minutes.

As shown in Fig. 1, at 30 minutes after insulin injection, nadir of FFA was detected, which may be secondary to the antilipolytic effect of insulin. The subsequent incremental changes in FFA, parallel to the changes of NE, may be due to the lipolytic effects of NE. Elevation of GH in response to hypoglycemia, which demonstrated steep peak within 60 minutes of insulin injection, was also associated with lipolytic effect (Fig. 1).

We have measured AUCs of counterregulatory hormones and proinflammatory cytokines and compared these AUCs by regression analysis to detect the possible correlation between counterregulatory hormones and measured cytokines.

The AUC of EP correlated significantly with the AUC of TNF-α ($t = 2.14$, $P = .05$) and IL-8 ($t = 2.51$, $P = .027$) (Fig. 2). The AUC of cortisol correlated with the AUC of IL-1β ($t = 2.12$, $P = .05$) (Fig. 3).

The AUC of ACTH correlated with the AUC of IL-6 with borderline significance ($t = 2.01$, $P = .06$) (Fig. 4). By applying peak-to-peak analysis, the peak of WBC at 120 minutes was associated with the peaks of cortisol and NE ($P < .05$) (Fig. 5).

4. Discussion

In this study, we have demonstrated the responses of body to the stress of hypoglycemia; these include the well-known responses of counterregulatory hormones [2,3] and previously unknown elevation of proinflammatory cytokines, markers of lipid peroxidation and ROS, as well as leukocytosis.

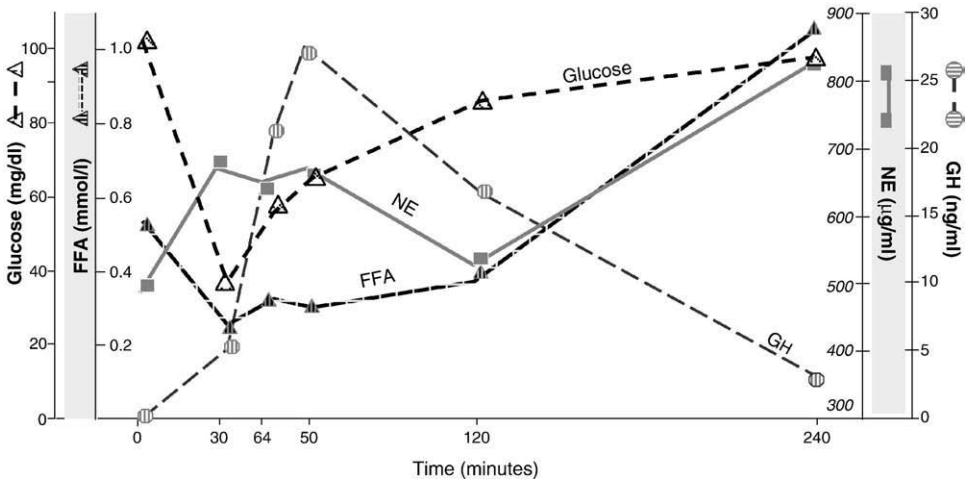

Fig. 1. Decremental excursions of FFA and glucose and incremental excursions of GH and NE in response to insulin-induced hypoglycemia.

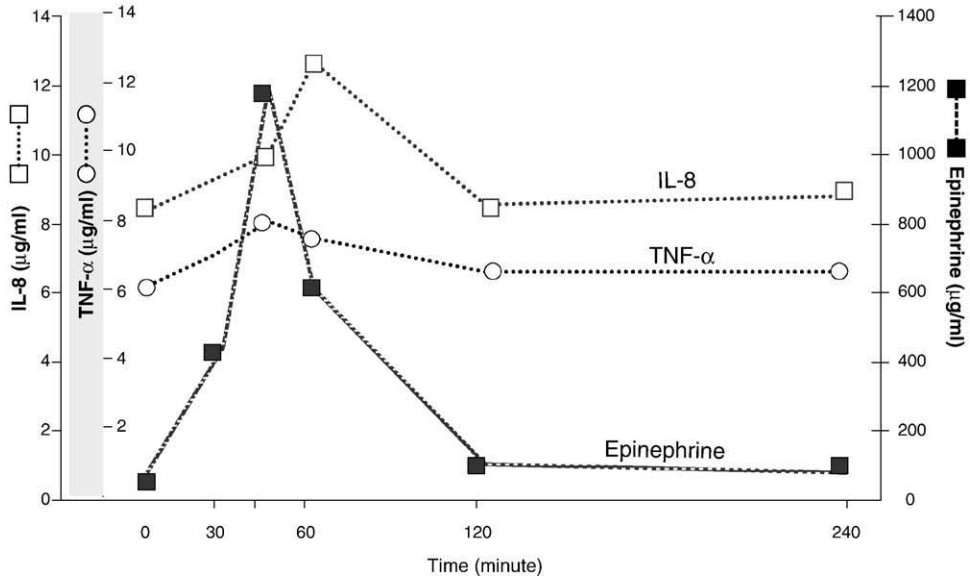

Fig. 2. The changes in glucose, ACTH, cortisol, and IL-6 in response to insulin-induced hypoglycemia before, during, and after insulin injection.

Based on the present findings, EP is a predictor of TNF-α and IL-8. Cortisol predicts the change of IL-1β. Corticotropin predicts IL-6 with borderline significance. Norepinephrine and cortisol are associated with peak value of leukocytes.

We have demonstrated that acute hypoglycemic event could provoke elevation of counterregulatory hormones and cytokines similar to the hyperglycemic crises reported by us previously [1]. However, quantitatively, hyperglycemia produces greater level of these measured hormones and cytokines than hypoglycemia. This could possibly be explained by the fact that duration of hyperglycemic stress was longer in DKA as shown by elevated HbA_{1c} and the presence of additional stressful conditions such as dehydration, hyperlipidemia, and acidosis in these patients [1,5]. Elevations of counterregulatory hormones are expected in hypoglycaemia [2,3] and are known to stimulate ketogenesis in DKA [6-9], but the actions of elevated proinflammatory cytokines in both hypo- and hyperglycemia have not been fully explained.

At the cellular level, we have shown that DKA induces in situ activation of T lymphocytes (T cells) with elaboration of proinflammatory cytokines, MDA, and DCF [9]. Similarly, incubation of T cells in the presence of high glucose [10] and palmitate [11] could also result in the elevation of proinflammatory cytokines, MDA, and DCF. However, the status of proinflammatory cytokines and the activation of T cell in hypoglycemic media have not been studied.

It has been recently established that elaboration of proinflammatory cytokines is not limited to one organ or tissue such as fat or liver but may include T cells and other hematologic cells in the fat tissue [12,13] as well as the presence of such cytokines in sympathoadrenal system

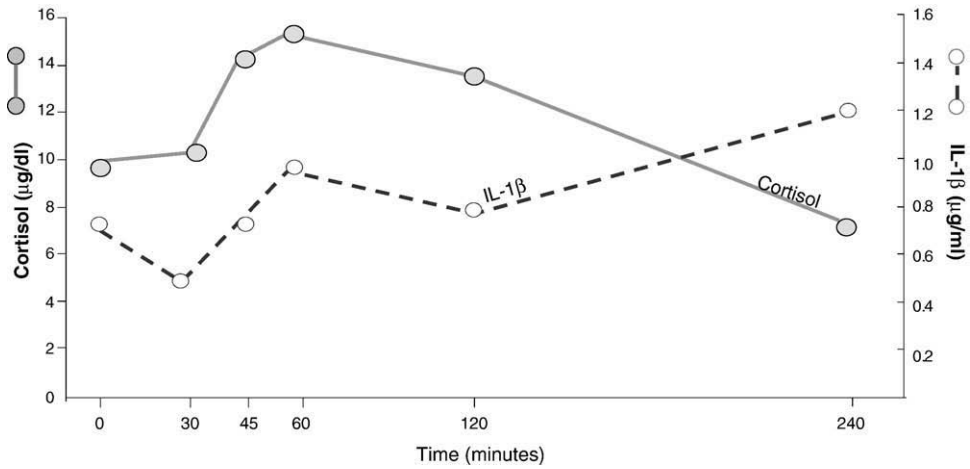

Fig. 3. Incremental changes in EP, IL-8, and TNF-α before, during, and after insulin-induced hypoglycemia.

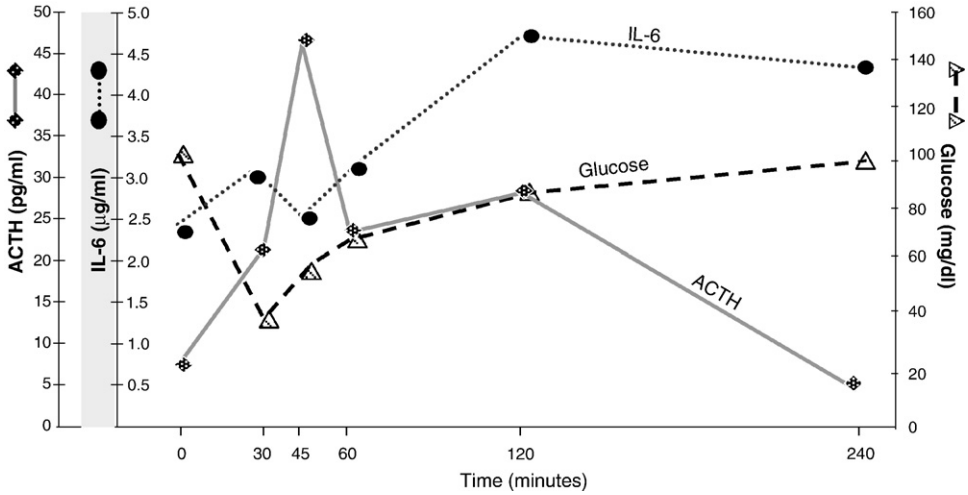

Fig. 4. Effect of insulin-induced hypoglycemia on excursion of cortisol, NEP, IL-1β, and leukocytes.

[14-18]. Furthermore, as an adaptive response, certain cytokines may act as either proinflammatory or anti-inflammatory mediators [19,20]. However, further studies are needed to elucidate the underlying mechanism of cytokines in response to different stressors such as glycemic excursions.

In conclusion, based on the present study and our previous in vivo and in vitro studies [1,9-11] as well as recent reports in the literature [21-25], we propose that elevation of proinflammatory cytokines may be an adaptive response to acute stress for maintaining glucose homeostasis in glycemic excursions. Additional studies are necessary to determine the origin and function of cytokines in other nonimmune systems including the hypothalamic-pituitary-adrenal and sympathoadrenal system in response to acute metabolic stresses in human.

4.1. Limitations of the present study

Our study has several limitations that must be noted.

1. Our investigation was limited to male subjects only. One reason for the selection of this sex, however, was to exclude the effects of various sex hormones during menstrual cycles.
2. The duration of our study was short and did not consist of prolonged hypoglycemia or step down glucose with the use of hypoglycemic clamp.
3. The study was not performed with control by the use of saline for 240 minutes, as intravenous ITT is a standard procedure and is used without control in clinical studies. The ITT tests were performed between 8:00 and 9:00 AM in all subjects, so diurnal variation was kept to a minimum.
4. This study was done in nondiabetic subjects. However, the purpose was to establish the changes in proinflammatory cytokines and correlate them with counterregulatory hormones, which we have previously shown to be increased in type 1 and type 2 diabetes mellitus patients with DKA [1].

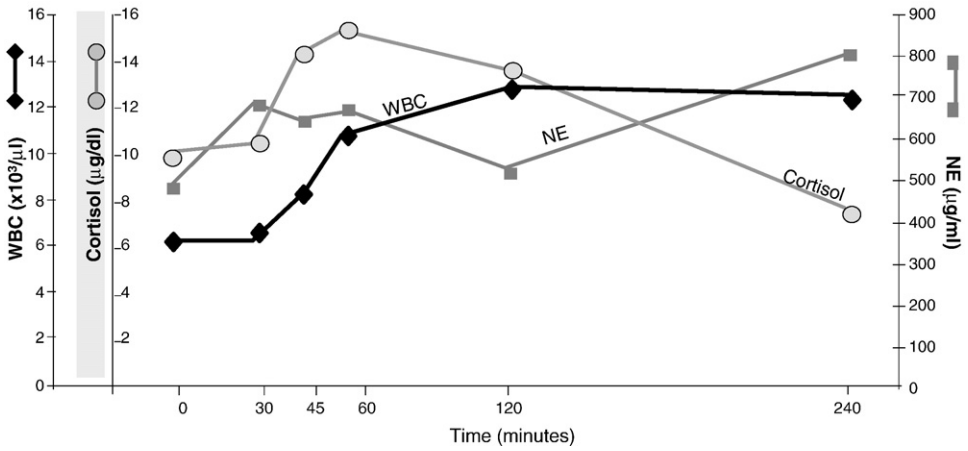

Fig. 5. Effect of insulin-induced hypoglycemia on excursion of WBC, cortisol, and NE.

Acknowledgment

We are grateful to our volunteers, whose participation made this study possible, and to Ms Brenda Scott for her secretarial assistance, Ms Cat Casey for her depiction of the results in the 5 figures, and Mr John Crisler for his assistance in laboratory assays.

None of the authors have any financial interests that might conflict with this study.

References

[1] Stentz F, Umpierrez GE, Cuervo R, Kitabchi AE. Proinflammatory cytokines, markers of cardiovascular risks, oxidative stress, and lipid peroxidation in patients with hyperglycemic crises. Diabetes 2004;53: 2079-86.

[2] Ertuck E, Jaffe CA, Barkan AL. Evaluation of the integrity of the hypothalamic-pituitary adrenal axis by insulin hypoglycemia test. J Clin Endocrinol Metab 1998;83:2350-4.

[3] Palmer KL, Kovac WJ. Addison's disease. In: Williams JA, Sholet SM, editors. Oxford textbook of endocrinology and diabetes. 2nd Ed. Oxford University Press; 2002. p. 837-44.

[4] Pruessner JC, Kirschbaum C, Meinlschmid G, Hellhammer DH. Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. Psychoneuroendocrinology 2003;28:916-31.

[5] Kitabchi AE, Umpierrez GE, Murphy MB, Barret EJ, Kriesberg RA, Malone JI, Wall BM. Management of hyperglycaemic crises in patients with diabetes. Diabetes Care 2001;24:241-69.

[6] Schade DS, Eaton RP. The regulation of plasma ketone body concentration by counter-regulatory hormones in man, effects of norepinephrine in diabetic man. Diabetes 1977;26:989-96.

[7] Schade DS, Eaton RP, Peake GT. The regulation of plasma ketone body concentration by counter-regulatory hormones in man, effects of growth hormone in diabetic man. Diabetes 1978;27:916-24.

[8] Schade DS, Eaton RP, Standefer J. Modulation of basal ketone body concentration by cortisol in diabetic man. JCEM 1978;47:519-28.

[9] Kitabchi AE, Stentz FB, Umpierrez GE. Diabetic ketoacidosis induces in vivo activation of human T-lymphocytes. Biochem Biophys Res Commun 2004;315:404-7.

[10] Stentz FB, Kitabchi AE. Hyperglycemia-induced activation of human T-lymphocytes with de novo emergence of insulin receptors and generation of reactive oxygen species. Biochem Biophys Res Commun 2005;335:491-5.

[11] Stentz FB, Kitabchi AE. Palmitic acid–induced activation of human T-lymphocytes and aortic endothelial cells with production of insulin receptors, reactive oxygen species, cytokines, and lipid peroxidation. Biochem Biophys Res Commun 2006;346:721-6.

[12] Fain JN, Bahouth SW, Madan AK. TNF-α release by the nonfat cells of human adipose tissue. Int J Obes Relat Metab Disord 2004;28: 616-22.

[13] Fain JN, Nesbit AS, Sudlow FF, Cheema P, Peeples JM, Madan AK, Tichansky DS. Release in vitro of adipsin, vascular cell adhesion molecule 1, angiotensin 1–converting enzyme, and soluble tumor necrosis factor receptor 2 by human omental adipose tissue as well as by the nonfat cells and adipocytes. Metabolism 2007;56:1583-90.

[14] Gonzalez-Hernandez JA, Ehrhart-Bornstein M, Spath-Schwalbe E, Scherbaum WA, Bornstein S. Human adrenal cells express tumor necrosis factor–alpha messenger ribonucleic acid: evidence for paracrine control of adrenal function. J Clin Endocrinol Metab 1996;81:807-13.

[15] Judd AM, Mac Leod RM. Adrenocorticotropin increases interleukin-6 release from rat adrenal zona glomerulosa cells. Endocrinology 1992;130:1245-54.

[16] De Simini MG, Sironi M, De Luigi A, Manfridi A, Mantovani A, Ghezzi P. Intracerebroventricular injection of interleukin 1 induces high circulating levels of interleukin 6. J Exp Med 1990;171: 1773-8.

[17] Bethin KE, Vogt SK, Muglia LJ. Interleukin 6 is an essential, corticotropin-releasing hormone-independent stimulator of the adrenal axis during immune system activation. Proc Natl Acad Sci 2000;97: 9317-22.

[18] Sarlis NJ, Stephanou A, Tephanou RA, Knight RA, Lightman SL, Chowdery HS. Effects of glucocorticoids and chronic inflammatory stress upon anterior pituitary interleukin-6 mRNA in the rat. Br J Rheumatol 1993;32:653-7.

[19] Glaser R, Kiecolt-Glaser JK. Stress-induced immune dysfunction: implications for health. Nat Rev Immunol 2005;5:243-51.

[20] Lemay LG, Vander AJ, Kluger MJ. The effects of psychological stress on plasma interleukin-6 activity in rats. Physiol Behav 1990;47: 957-61.

[21] Dagogo-Jack S. Hypoglycemia in type 1 diabetes mellitus, pathophysiology and prevention. Treat Endocrinol 2004;3:91-103.

[22] Shintani F, Nakai T, Kanba S, Kato R, Asai M. Role of interleukin-1 in stress responses. A putative neurotransmitter. Mol Neurobiol 1995;10: 47-71.

[23] Pitossi F, Del Rey A, Kabiersch A, Besedovsky H. Induction of cytokine transcripts in the CNS and pituitary following peripheral administration of endotoxin to mice. J Neurosci Res 1997;48:287-98.

[24] Stefana B, Ray DW, Meilmed S. Leukemia inhibitory factor induces differentiation of pituitary corticotroph function: an immuno-neuroendocrine phenotypic switch. Proc Natl Acad Sci 1996;93:12502-6.

[25] Elenkov IJ, Chrousos GP. Stress hormones, pro-inflammatory and anti-inflammatory cytokines, and auto-immunity. Ann NY Acad Sci 2002;966:290-303.