Repeated Hypoglycemia and Vascular Function

**Effects of Acute and Antecedent Hypoglycemia on Endothelial Function and Markers of Atherothrombotic Balance in Healthy Man**

Nino G. Joy, Donna B. Tate, Lisa M. Younk, and Stephen N. Davis*

Department of Medicine, University of Maryland School of Medicine, Baltimore, MD

*Please address all correspondence to:

Stephen N. Davis, MBBS, FRCP, FACP
Chairman, Department of Medicine
University of Maryland School of Medicine
22 S. Greene Street, Room N3W42
Baltimore, MD 21201
P: 410-328-2488
F: 410-328-8688
sdavis@medicine.umaryland.edu
ABSTRACT

The aim of this study was to determine the effects of single and repeated episodes of clamped hypoglycemia on fibrinolytic balance, pro-inflammatory biomarkers, pro-atherothrombotic mechanisms and endothelial function.

Twenty healthy individuals (12M/8F) were studied during separate 2 day randomized protocols. Day 1 consisted of either two 2hr hyperinsulinemic (812±50pmol/L) euglycemic (5±0.1mmol/L) or hypoglycemic (2.9±0.1mmol/L) clamps. Day 2 consisted of a single 2 hr hypoglycemic clamp. Two D Doppler ultrasound was used to determine brachial arterial endothelial function.

PAI-1, VCAM-1, ICAM-1, E-selectin, P-selectin, TAT (thrombin/anti-thrombin complex), TNF-α and IL-6 responses were increased (p<0.05) during single or repeated hypoglycemia as compared to euglycemia. Endogenous and exogenous NO mediated vasodilation were both impaired by repeated hypoglycemia. Neuroendocrine and autonomic nervous system (ANS) responses were also blunted by repeated hypoglycemia (p<0.05).

In summary, acute moderate hypoglycemia impairs fibrinolytic balance, increases pro-inflammatory responses, platelet activation and coagulation biomarkers and reduces NO mediated endothelial function in healthy individuals. Repeated episodes of hypoglycemia further impair vascular function by additionally reducing exogenously NO mediated endothelial function and increasing coagulation biomarkers. We conclude that despite reduced neuroendocrine and ANS responses, antecedent hypoglycemia results in greater endothelial dysfunction and an increased pro-atherothrombotic state as compared to a single acute episode of hypoglycemia.

Key Words: hypoglycemia, endothelial function, fibrinolytic balance, inflammation.
INTRODUCTION

Data are accumulating that hypoglycemia is associated with increased cardiovascular and cerebrovascular mortality (1, 2). Epidemiologic data as well as studies in ambulatory practice and intensive care settings, have demonstrated increased risk of serious cardiovascular adverse events following severe hypoglycemia (3-5). Several, recent large randomized controlled trials have demonstrated that both intensive and standard treated type 2 diabetic patients can suffer episodes of severe hypoglycemia (5-7). These episodes were associated with serious cardiovascular adverse events and in some trials, increased mortality (5, 8). Somewhat surprisingly, individuals with higher HBA\textsubscript{1c} in both standard and intensive treatment arms appeared to have worse cardiovascular outcomes after severe hypoglycemia (5-7). The possible mechanisms for these findings remain unexplained. Type 2 diabetes is a syndrome typically associated with increased cardiovascular risk (endothelial dysfunction, abnormal fibrinolytic balance, increased inflammation, premature atherothrombotic risks and vascular disease). It is unclear whether episodes of hypoglycemia may also contribute to the syndrome of premature and diffuse vascular disease (9,10). Recent work has started to investigate the effects of acute hypoglycemia on pro-inflammatory and pro-atherothrombotic mechanisms in both healthy and diabetic humans (9-12). Available data is consistent, demonstrating that acute insulin induced hypoglycemia can activate pro-inflammatory responses (12, 13).

However, lacking are data reporting the effects of repeated hypoglycemia on pro-inflammatory and pro-atherothrombotic markers. Similarly the effects of antecedent hypoglycemia on endothelial function are largely unknown. Therefore, in this present study, we have tested the hypothesis that acute or antecedent moderate hypoglycemia can 1) impair endothelial function 2)
activate pro-inflammatory and pro-atherothrombotic mechanisms and 3) reduce fibrinolytic balance in healthy individuals.

To control for the independent effects of insulin on pro-inflammatory responses and endothelial function (2), the hyperinsulinemic euglycemic and hypoglycemic clamp techniques were used to equate insulin levels in all studies. Using this approach the independent effects of acute and repeated hypoglycemia on in-vivo vascular physiology could be identified.

RESEARCH DESIGN AND METHODS

Study Participants:
Twenty adult volunteers (12M/8F), age (34±3yrs), BMI (27±1 kg/m^2), HBA\textsubscript{1C} (5±0.3%, 31±3.3 mmol/mol) participated in two randomized, single blind two day experiments. Twelve individuals participated in both protocols and an additional 8 individuals underwent a single two day visit resulting in n=16 for both two day studies. None of the subjects smoked, received anticoagulants, clopidogrel or statins. Subjects over age 40 were screened for silent ischemia with a standard treadmill stress test. Each subject had a normal blood count, plasma electrolytes, liver and renal function and no evidence of either impaired fasting glucose or overt diabetes mellitus. All gave written informed consent. Studies were approved by the Vanderbilt University Human Subjects Institutional Review Board.

Participants were instructed to avoid any alcohol and exercise and consume their usual weight maintaining diet for 3 days before each experiment. Patients were also asked not to use aspirin, NSAIDs or COX 2 inhibitors for three days prior to a study. Subjects were admitted to the general research clinical center (GCRC) the night prior to the study. After an overnight 10 hr fast, two intravenous cannulae were inserted under 1% lidocaine.
local anesthesia. One cannula was placed in a retrograde fashion into a vein on the back of the hand of the non-dominant arm. This hand was placed in a heated box (55-60 °C) during the study so that arterialized blood could be obtained (14). The other cannula was placed in the ipsilateral arm for infusions. Subjects were randomized to undergo the following two day protocols (Figure 1).

**Protocol 1**

**Day 1**

After placement of venous cannulae, a period of 120 min was allowed to elapse followed by a 120 min hyperinsulinemic-euglycemic experimental period. At time 120 min, a primed constant (9.0 pmol/kg/min) infusion of insulin (Human Regular Insulin, Eli Lilly, Indianapolis, IN) was started and continued until 240 min. During this period, plasma glucose was measured every 5 min and a 20% dextrose infusion was adjusted so that plasma glucose levels were held constant at 5.0±0.1mmol/L (15). Potassium chloride (20 mmol/L) was infused to reduce insulin-induced hypokalemia. At time 240 min the insulin infusion was turned off and a further 120 min was allowed to elapse before starting an identical 120 min afternoon euglycemic clamp. Following day 1 procedures participants received a standard meal and evening snack and underwent a 10 hr overnight fast.

**Day 2**

Day 2 consisted of an initial 120 min resting period. At time 120 min a primed constant (9.0 pmol/kg/min) infusion of regular insulin was started and continued until 240 min. The rate of fall of glucose was controlled (≈0.08mmol/min/) and the glucose nadir (2.9 mmol/L) was achieved using a modification of the glucose clamp technique (15, 16). Potassium chloride (20 mmol/L)
was infused during the clamp. At time 240 min insulin was turned off, plasma glucose was restored to euglycemia and participants were given a meal.

**Protocol 2**

**Day 1**

After placement of venous cannulae, a period of 120 min was allowed to elapse, followed by a 120 min hyperinsulinemic hypoglycemic experimental period similar to that described for day 2 protocol 1. After the morning clamp experiment a 120 min euglycemic period was established by infusing 20% dextrose. Following that a second 120 min hyperinsulinemic hypoglycemic clamp period similar to the morning study was performed. At completion of day 1 procedures, participants received a standard meal and an evening snack.

**Day 2**

Following a 10 hr overnight fast, day 2 morning procedures were identical to the hypoglycemic clamp studies performed during protocol 1, day 2.

**Analytical Methods**

The collection of blood samples have been described elsewhere (17). Plasma glucose concentrations were measured in triplicate using the glucose oxidase method with a glucose analyzer (Beckman, Fullerton, CA). Insulin, catecholamines, cortisol, and non-esterified fatty acids (NEFA) were drawn every 30 min. Insulin was measured as previously described with an interassay CV of 9% (18). Catecholamines were determined by HPLC with an interassay CV of 12% for epinephrine and 8% for norepinephrine (19). Cortisol was assayed using the Clinical
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Assays Gamma Coat radioimmunoassay (RIA) kit with an interassay CV of 6%. NEFA were measured using the WAKO kit with an interassay CV of 7%. (20)

During the experimental period blood for sVCAM-1, sICAM-1, E-selectin, P-selectin, IL-6, TNF-α, PAI-1, thrombin/anti-thrombin (TAT) complex was drawn every 60 min. Vascular adhesion molecules and adiponectin were assayed using LINCO Research Kits (St. Charles, Missouri) with an interassay CV of 8.5% (sVCAM-1), CV of 9.7% (sICAM-1), CV of 13.4%, (sE-selectin), CV of 9.02% (IL-6), CV of 9.98% (TNF-α), respectively (21). P-selectin was measured by Meso Scale Discovery assays (Gaithersburg, MD) with a CV of 9.9%. PAI-1 was determined by TintElize® PAI-1 Kit with interassay CV of 3.3%, and TAT Complex, CV of 7%, Assay Pro (St. Charles, MO).

Cardiovascular Measurements

Heart rate, systolic, diastolic, and mean arterial blood pressure were measured noninvasively by a Dinamap vitals monitor (Critikon, Tampa, FL) every 10 min throughout each 2-hr clamp study.

Endothelial Function

Measurements of endothelial function were conducted at baseline and during the final 30 minutes of each glucose clamp. Flow mediated dilation (FMD) of the dominant brachial artery was measured using 2D Doppler ultrasound during reactive hyperemia and exogenous nitroglycerin administration. Baseline images of the brachial artery were obtained during systole by scanning the artery in longitudinal section 5-10 cm above the antecubital fossa of the
dominant arm with the focal zone set to the depth of the mid vessel. Boundaries for diameter measurements were identified manually with electronic calipers. Reactive hyperemia was obtained by inflating the blood pressure cuff around the proximal forearm to a pressure of 50 mm Hg greater than the individual’s systolic blood pressure for 5 minutes (22). Brachial artery diameter measurements were taken at time points 30 seconds, 60 seconds, 90 seconds, 120 seconds and after cuff deflation. After a 15 minute rest period subjects received 0.4 mg sublingual nitroglycerin [as an exogenous nitric oxide (NO) donor]. Additional scans were performed as above with vessel diameter measurements obtained at 1, 2, 3 and 4 minutes (22).

Statistical Analysis

Data are expressed as mean ± SE and were analyzed using standard, parametric, one- and two-way analysis of variance (ANOVA) and with repeated measures where appropriate (Graph Pad Software, Inc., San Diego, CA). Tukey’s post hoc analysis was used to delineate statistical significance within each group. Data was also analyzed using paired and unpaired two-tailed t tests. In all cases p value of <0.05 was accepted as statistically significant.

RESULTS

Glucose and Insulin

Plasma glucose was maintained equivalently (5±0.1mmol/L) during the euglycemic clamps. During the hypoglycemic studies glucose reached steady state by 150 min and equivalent hypoglycemia was maintained (2.9±0.1mmol/L) during all hypoglycemic clamp procedures (Figure 1). Insulin levels (812±50 pmol/L) were similar during all clamp studies (Figure 1).
Neuroendocrine Counterregulatory Hormones

Epinephrine responses were significantly higher (p<0.001) during the final 30 min of day 1 hypoglycemia (3741±465pmol/L) and day 2 hypoglycemia following day 1 euglycemia (4251±568pmol/L) as compared to euglycemia (189±20pmol/L) or day 2 repeated hypoglycemia (2497±265pmol/L). Norepinephrine levels were also similar during all hypoglycemia protocols but significantly higher (p<0.001) as compared to euglycemia (1984±156 vs 1159±77pmol/L, respectively) (Table 2). Glucagon, cortisol and growth hormone levels were all reduced (p<0.05) during day 2 hypoglycemia following day 1 hypoglycemia (Table 2).

Intermediary Metabolism

NEFA baseline levels were similar at the start of glucose clamps. Plasma NEFA levels fell similarly (p<0.0001) during hypoglycemic and euglycemic clamps (Table 2).

Atherogenic Vascular Adhesion Molecules

Baseline values of ICAM-1, VCAM-1 and E –Selectin were similar (Table 1) and fell (p<0.05) during the hyperinsulinemic euglycemic clamps (Figures 2 and 3). End of clamp responses during all hypoglycemic protocols were similarly increased (p<0.05) as compared to euglycemia (Figures 2 and 3). Conceptually similar statistical differences were obtained when responses from the entire time course of the 2hr clamp studies (Figures 2 and 3) and incremental changes from baseline were evaluated.
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Platelet Activation and Fibrinolytic/Thrombotic Balance

Baseline values for PAI-1, TAT and tPA were similar for all protocols (Table 1). Basal day 2 hypoglycemia P-selectin values were lower (p<0.05) following day 1 hyperinsulinemia. PAI-1, P-selectin and TAT end of clamp responses were increased (p<0.05) during all hypoglycemic studies as compared to euglycemic clamp studies (Figures 2 and 3). Incremental responses from baseline for the entire time course during hypoglycemia for PAI-1, TAT and P-selectin were also increased as compared to euglycemia (Figure 3). Plasma tPA values remained similar to baseline during euglycemic and hypoglycemic studies.

Inflammatory Cytokines

Baseline values for IL-6 and TNF-α were similar at the start of all protocols of glucose clamp studies (Table 1). End of clamp responses were increased (p<0.05) during all hypoglycemic protocols as compared to euglycemia (Figures 2 and 3).

Cardiovascular parameters

Heart rate, systolic, diastolic and mean arterial blood pressure responses are shown in Table 3.

Endothelial Function

Endothelial function during endogenous NO stimulation was decreased (p<0.05) during day 1 and both day 2 hypoglycemia protocols as compared to day 1 euglycemia (Figure 4). Endogenous NO mediated endothelial function was also reduced (p<0.05) by repeated hypoglycemia as compared to day 2 hypoglycemia following day 1 euglycemia. There were also
reductions (p<0.05) in endothelial function during exogenous NO stimulation during repeated hypoglycemia as compared to day 1 euglycemia and day 1 hypoglycemia (Figure 4).

DISCUSSION

This study has investigated the effects of clamped hyperinsulinemic euglycemia and acute and repeated 2 hr episodes of moderate hypoglycemia (2.9 mmol/L) on endothelial function, fibrinolytic balance, pro-inflammatory and pro-atherothrombotic mechanisms in healthy man. Our results demonstrate that acute hypoglycemia reduces endogenous NO mediated endothelial vasodilation, activates inflammatory processes, impairs fibrinolytic balance and increases pro-atherothrombotic mechanisms. Secondly, repeated episodes of hypoglycemia can further impair vascular function by additionally reducing both endogenous and exogenous NO mediated endothelial function and increasing TAT complex formation.

Recent large randomized controlled trials investigating the effects of intensifying glucose control both in hospitals and out-patient settings have demonstrated an association with hypoglycemia and severe cardiovascular outcomes and even death (5, 8). The in-vivo vascular pathophysiologic mechanisms responsible for these findings are poorly understood. Additionally, the mechanistic role played by repeated hypoglycemia in these adverse events is largely unknown. In fact, it is argued that as counterregulatory responses during repeated hypoglycemia are blunted that antecedent hypoglycemia may have a reduced role in the pathophysiology of the adverse events in the above studies.

In this study, glucose clamp techniques were used to allow assessment of the effects of euglycemia and repeated hypoglycemia on in-vivo endothelial function, pro-atherothrombotic
and pro-inflammatory responses during equivalent insulinemia. This latter point is an important element of the study design, as hyperinsulinemia, per se, can acutely improve endothelial function and reduce pro-inflammatory responses (23, 24).

The results of the present study clearly demonstrate that despite reduced neuroendocrine and autonomic nervous system counterregulatory responses, repeated hypoglycemia produces a greater aggregate of deleterious in-vivo vascular biologic effects, compared to a single, similar hypoglycemic episode.

Relatively few studies have investigated the effects of acute hypoglycemia on pro-inflammatory and pro-atherothrombotic mechanisms (10, 12, 24, 25). In this present study we confirm and extend previous findings that 2 hr of acute moderate hypoglycemia can activate pro-inflammatory (ICAM-1, VCAM-1, E-selectin, TNF-α and IL-6) and pro-thrombotic (PAI-1, P-selectin, TAT complex) mechanisms. The increased TAT complexes (increased intravascular coagulation), elevated P-selectin (increased platelet aggregability) and increased PAI-1 relative to tPA (disordered fibrinolytic balance) responses demonstrate a broad activation of pro-hemostatic clotting mechanisms by hypoglycemia.

Supporting previous studies our results demonstrate that hyperinsulinemic euglycemia can acutely lower pro-inflammatory and pro-atherothrombotic responses (10, 12, 25). These data demonstrate that insulin per se can reduce inflammation acutely. However, despite the beneficial effects of insulin, our results demonstrate that two episodes of hyperinsulinemic euglycemia on day 1 had only limited effects on preventing the deleterious pro-inflammatory vascular action of next day hypoglycemia.

The time course of the increases in inflammatory biomarkers is worth noting. Adhesion molecules (ICAM-1, VCAM-1, E-selectin), pro-thrombotic markers (P-selectin, PAI-1, TAT
complex) and pro-inflammatory markers (TNF-α and IL-6) increased during the 2 hr hypoglycemic clamps. All of the above marker responses were elevated relative to the reductions observed during hyperinsulinemic euglycemic control studies (Figures 2 and 3). Whether extended periods or deeper levels of hypoglycemia would further increase pro-thrombotic and pro-inflammatory biomarkers is unknown.

Endothelial dysfunction is a key feature of macrovascular complications in both type 1 and type 2 diabetes. FMD is a well-validated assessment of impaired endothelial function (26). Using this technique, numerous studies have determined the association of impaired endothelial function with increased risk for atherosclerosis and coronary artery disease (26). However, there are few data describing the effects of hypoglycemia on the vascular endothelium (25, 27, 28) and none directly measuring endothelial function during repeated hypoglycemia.

Acute endothelial function was assessed by both exogenously and endogenously NO mediated vasodilatory mechanisms. Any episode of hypoglycemia significantly impaired endogenous NO mediated endothelial function. However, repeated hypoglycemia blunted both endogenous and non-endogenous NO mediated vasodilation and resulted in greater endothelial dysfunction compared to a single episode of hypoglycemia. This finding is notable for several reasons. Firstly, it appears that repeated hypoglycemia is the only physiologic stress that impairs both endogenous and exogenous NO mediated vasodilation (29). Secondly, this demonstrates that repeated acute episodes of hypoglycemia can result in greater endothelial dysfunction compared to a single episode of hypoglycemia. Thirdly, these present results indicate that repeated hypoglycemia can impair NO mediated vascular smooth muscle function (endogenous) as well as exogenously mediated inputs into vasculature (e.g., autonomic nervous system) (30).
Antecedent episodes of hypoglycemia typically blunt the majority of neuroendocrine and autonomic nervous system responses during subsequent hypoglycemia in non-diabetic, type 1 and type 2 DM individuals (31). Consistent with prior findings, day 2 epinephrine, cortisol, glucagon and growth hormone responses were also reduced in these present studies by day 1 antecedent hypoglycemia. Despite this, pro-inflammatory and particularly pro-atherothrombotic mediators were not reduced during day 2 of repeated hypoglycemia. In fact, responses of P-selectin (marker of platelet aggregation), PAI-1 (marker of reduced fibrinolytic balance) and TAT complexes (marker of thrombin activity) were either similarly or preferentially increased by repeated hypoglycemia. Thus our present findings demonstrate that three episodes of hypoglycemia have greater physiologic effects on endothelial function and pro-atherothrombotic markers compared to a single episode of comparable hypoglycemia.

The possible physiologic mechanisms responsible for the above findings deserve further discussion. Our present results strongly indicate that acute hyperinsulinemia per se does not have acute pro-inflammatory or deleterious effects on the vasculature (23, 24). The hyperinsulinemic euglycemic control studies improved acute vascular function and provided anti-inflammatory benefit. Rana et al. also have demonstrated that insulin induced hypoglycemia reduces myocardial blood flow, whereas hyperinsulinemic euglycemia has a beneficial effect on myocardial blood flow in healthy and type 1 DM individuals (32). However, it should be noted that the insulin levels achieved during the short duration of the present studies (euglycemic and hypoglycemic) are considerably higher than those chronically and typically observed in clinical practice.

Despite the beneficial acute anti-inflammatory effects of insulin, day 1 euglycemia appeared to have little effect on reducing endothelial dysfunction or pro-inflammatory and pro-atherogenic
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responses during next day hypoglycemia underscoring the powerful putative effects of repeated hypoglycemia on the vasculature. The only demonstrable beneficial effects of day 1 insulinemia (either following euglycemic or hypoglycemic studies) was that basal P-selectin levels were reduced at the start of day 2 hypoglycemia studies.

NEFA levels were similarly reduced during euglycemic and hypoglycemic clamp studies. Thus, it appears unlikely that NEFA levels could have been responsible for the pro-inflammatory and impaired endothelial function occurring during hypoglycemia (33).

This would indicate that either hypoglycemia per se or a consequence of hypoglycemia is the putative agent for vascular dysfunction and inflammation. Previous work has elegantly demonstrated that activation of $\alpha$ and/or $\beta$ receptors can reduce endogenously NO mediated smooth muscle vasodilation. The effects of catecholamines and direct sympathetic nervous system drive on pro-inflammatory, fibrinolytic balance and pro-atherothrombotic markers are complex, with reports of both activation and reduction of inflammatory responses (34-36). These effects may be tissue specific (e.g., platelet vs adipocyte) and may also depend upon the circulating neuroendocrine milieu (i.e., interactions with corticosteroids) (37). Thus the combination of the hypoglycemia driven increases in epinephrine, norepinephrine and/or direct sympathetic nervous system activity may have played a role in our findings (31).

In this study, antecedent hypoglycemia blunted corticosteroid responses during day 2 hypoglycemia. Thus it is possible that the reduced levels of corticosteroids may also have contributed to the relatively increased pro-inflammatory and endothelial dysfunction occurring during repeated hypoglycemia (38). Additionally, previous work by Wang et. al., has also demonstrated direct inflammatory and adverse effects of hypoglycemia per se on in-vitro vascular smooth muscle endothelial function (29). Lastly, we also can not exclude that increases
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in endothelin -1 may also have played a role in the pro-inflammatory responses and impaired endothelial function observed during acute and repeated hypoglycemia (25, 27).

In summary, this present study has demonstrated the complex, deleterious vascular biologic effects of acute and antecedent moderate hypoglycemia on endothelial function, fibrinolytic/thrombotic balance and pro-inflammatory mechanisms in healthy individuals. We conclude that in healthy individuals, acute moderate hypoglycemia can activate pro-inflammatory and pro-thrombotic mechanisms and secondly, antecedent hypoglycemia can result in greater pro-thrombotic responses and impaired endothelial function compared to a single episode of acute moderate hypoglycemia.

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N. J. performed studies, researched data and wrote the manuscript. D. T. and L. Y. helped perform studies, researched data, and reviewed and edited the manuscript. S. D. devised the study, reviewed and edited data and wrote the manuscript. All are affiliated with the University of Maryland, Baltimore.
Stephen Davis is the guarantor of this study and, as such, had full access to all the data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

There are no conflicts of interest to report.

REFERENCES


12. Gogitidze Joy N, Hedrington MS, Briscoe VJ, Tate DB, Ertl AC, Davis SN. Effects of acute hypoglycemia on inflammatory and pro-atherothrombotic biomarkers in individuals with type 1 diabetes and healthy individuals. Diabetes Care 2010; 33(7):1529-1535


20. Ho RJ. Radiochemical assay of long chain fatty acids using 63NI as tracer. Anal Biochem 1970; 26: 105-113


25. Ceriello A, Novials A, Ortega E, La Sala L, Pujadas G, Testa R, Bonfigli AR, Esposito K, Giugliano D. Evidence that hyperglycemia after recovery from hypoglycemia worsens...
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endothelial function and increases oxidative stress and inflammation in healthy control subjects and subjects with type 1 diabetes. Diabetes 2012; 61(11):2993-2997


### Pro-Inflammatory and Pro-Atherothrombotic Markers

<table>
<thead>
<tr>
<th></th>
<th>Day 1 euglycemia</th>
<th>Day 2 hypoglycemia following day 1</th>
<th>Day 1 hypoglycemia</th>
<th>Day 2 hypoglycemia following day 1</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Baseline</td>
<td>Baseline</td>
<td>Baseline</td>
</tr>
<tr>
<td>ICAM ng/ml</td>
<td>90±7</td>
<td>89±7</td>
<td>88±3</td>
<td>78±5</td>
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<tr>
<td>VCAM ng/ml</td>
<td>838±37</td>
<td>772±43</td>
<td>819±62</td>
<td>735±48</td>
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<tr>
<td>E-selectin ng/ml</td>
<td>22±2</td>
<td>18±2</td>
<td>21±2</td>
<td>16±2</td>
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<tr>
<td>P-selectin pg/mL</td>
<td>83±9</td>
<td>56±6*</td>
<td>63±13</td>
<td>45±9*</td>
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<tr>
<td>PAI-1 ng/ml</td>
<td>18±3</td>
<td>14±2</td>
<td>13±3</td>
<td>17±6</td>
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<td>TAT ng/ml</td>
<td>3.5±0.4</td>
<td>2.9±0.3</td>
<td>3.5±0.2</td>
<td>3.6±0.2</td>
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<tr>
<td>tPA ng/ml</td>
<td>6.1±1</td>
<td>5.9±1</td>
<td>5±1</td>
<td>4.2±1</td>
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*P<0.05- Significantly different from day 1 euglycemia baseline
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Table 2

**Neuroendocrine and Non-esterified Fatty Acids**
**Responses during**
**Euglycemic and Hypoglycemic Clamps**

<table>
<thead>
<tr>
<th>Units</th>
<th>Basal</th>
<th>Final</th>
<th>Basal</th>
<th>Final</th>
<th>Basal</th>
<th>Final</th>
<th>Basal</th>
<th>Final</th>
<th>Basal</th>
<th>Final</th>
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<tbody>
<tr>
<td>Epinephrine pmol/L</td>
<td>190±20</td>
<td>189±20</td>
<td>179±20</td>
<td>4251±568*†</td>
<td>184±40</td>
<td>3741±465*†</td>
<td>198±30</td>
<td>2497±265*†‡</td>
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<tr>
<td>Norepinephrine pmol/L</td>
<td>1083±80</td>
<td>1159±77</td>
<td>1128±124</td>
<td>1957±134*</td>
<td>1057±119</td>
<td>1966±149*</td>
<td>1161±124</td>
<td>2028±184*</td>
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<tr>
<td>Cortisol nmol/L</td>
<td>344±31</td>
<td>292±30</td>
<td>330±32</td>
<td>714±42*†</td>
<td>359±34</td>
<td>850±70*†</td>
<td>271±26</td>
<td>572±62*†‡</td>
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<td></td>
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<tr>
<td>Glucagon ng/L</td>
<td>65±5</td>
<td>43±3*</td>
<td>50±6</td>
<td>112±17*†</td>
<td>57±4</td>
<td>164±26*†</td>
<td>44±7</td>
<td>90±13*†‡</td>
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<tr>
<td>GH ng/mL</td>
<td>2.5±0.4</td>
<td>2.4±0.4</td>
<td>4±1.34</td>
<td>25±5*†</td>
<td>3.1±0.5</td>
<td>28±5*</td>
<td>2.2±0.2</td>
<td>20±3*†</td>
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<tr>
<td>NEFA mmol/L</td>
<td>379±48</td>
<td>103±40*</td>
<td>382±55</td>
<td>97±14*</td>
<td>288±32</td>
<td>110±19*</td>
<td>351±28</td>
<td>98±23*</td>
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*p<0.05 Significantly different from baseline
†p<0.05 Significantly different from day 1 euglycemia
‡p<0.05 Significantly different from day 1 hypoglycemia
Values are means ±SE
Table 3

**Cardiovascular Responses during Hyperinsulinemic Euglycemic and Hypoglycemic clamps**

<table>
<thead>
<tr>
<th>Basal</th>
<th>Final</th>
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<tr>
<td><strong>Systolic Blood Pressure mmHg</strong></td>
<td></td>
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<tr>
<td>Day 1 eugly</td>
<td>110±3</td>
</tr>
<tr>
<td>Day 2 hypo following day 1 eugly</td>
<td>109±3</td>
</tr>
<tr>
<td>Day 1 hypo</td>
<td>112±4</td>
</tr>
<tr>
<td>Day 2 hypo following day 1 hypo</td>
<td>113±4</td>
</tr>
<tr>
<td></td>
<td>110±3</td>
</tr>
<tr>
<td></td>
<td>116±4</td>
</tr>
<tr>
<td></td>
<td>121±7 †</td>
</tr>
<tr>
<td></td>
<td>123±5 †‡</td>
</tr>
<tr>
<td><strong>Diastolic Blood Pressure mmHg</strong></td>
<td></td>
</tr>
<tr>
<td>Day 1 eugly</td>
<td>63±2</td>
</tr>
<tr>
<td>Day 2 hypo following day 1 eugly</td>
<td>65±1</td>
</tr>
<tr>
<td>Day 1 hypo</td>
<td>65±2</td>
</tr>
<tr>
<td>Day 2 hypo following day 1 hypo</td>
<td>66±1</td>
</tr>
<tr>
<td></td>
<td>62±2</td>
</tr>
<tr>
<td></td>
<td>60±2 †</td>
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<tr>
<td></td>
<td>62±3</td>
</tr>
<tr>
<td></td>
<td>62±2 †</td>
</tr>
<tr>
<td><strong>Mean Arterial Pressure mmHg</strong></td>
<td></td>
</tr>
<tr>
<td>Day 1 eugly</td>
<td>79±2</td>
</tr>
<tr>
<td>Day 2 hypo following day 1 eugly</td>
<td>80±2</td>
</tr>
<tr>
<td>Day 1 hypo</td>
<td>81±3</td>
</tr>
<tr>
<td>Day 2 hypo following day 1 hypo</td>
<td>81±2</td>
</tr>
<tr>
<td></td>
<td>76±3</td>
</tr>
<tr>
<td></td>
<td>80±2</td>
</tr>
<tr>
<td></td>
<td>82±4</td>
</tr>
<tr>
<td></td>
<td>83±3</td>
</tr>
<tr>
<td><strong>Heart Rate beats/minute</strong></td>
<td></td>
</tr>
<tr>
<td>Day 1 eugly</td>
<td>64±3</td>
</tr>
<tr>
<td>Day 2 hypo following day 1 eugly</td>
<td>65±3</td>
</tr>
<tr>
<td>Day 1 hypo</td>
<td>66±3</td>
</tr>
<tr>
<td>Day 2 hypo following day 1 hypo</td>
<td>68±3</td>
</tr>
<tr>
<td></td>
<td>67±2</td>
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<tr>
<td></td>
<td>74±2 †‡</td>
</tr>
<tr>
<td></td>
<td>70±3</td>
</tr>
<tr>
<td></td>
<td>75±2 †‡</td>
</tr>
</tbody>
</table>

† p<0.05 compared to baseline  
‡ p<0.05 compared to final eugly  
*Values are means ±SE*  
**Eugly- Euglycemia**  
**Hypo-Hypoglycemia**
FIGURE LEGENDS

Figure 1
Experimental protocols, clamped glucose and insulin levels during hyperinsulinemic euglycemic and hypoglycemic clamps in healthy subjects. Values are mean ± SE.

Figure 2
Effects of hyperinsulinemic euglycemia (5.1 mmol/L) and hypoglycemia (2.9 mmol/L) in overnight fasted healthy subjects on vascular biologic markers. Response of VCAM-1, ICAM-1, E-selectin, IL-6, PAI-1, P-selectin and TAT are increased during all hypoglycemia protocols as compared to euglycemia. Values are mean ± SE.

Figure 3
Effects of hyperinsulinemic euglycemia (5.1 mmol/L) and hypoglycemia (2.9 mmol/L) in overnight fasted healthy subjects on vascular biologic markers. Response of VCAM-1, ICAM-1, E-selectin, IL-6, PAI-1, P-selectin and TAT are increased during all hypoglycemia protocols as compared to euglycemia. Values are mean ± SE.

Figure 4
Endogenously and exogenously (NO) flow mediated dilation during acute and repeated hyperinsulinemic/hypoglycemic protocols as compared to hyperinsulinemic/euglycemia in overnight fasted healthy individuals.
Endothelial Function Measurements - 0.4 mg Nitroglycerin + 2-dimensional images of the brachial artery and pulsed Doppler flow mediated dilation.

Arterialized blood was obtained via the heated hand vein technique.
* P<0.05 Significantly different from day 1 euglycemia
Figure: 3

Day1 Euglycemia
Day2 Hypoglycemia following euglycemia
Day1 Hypoglycemia
Day 2 Hypoglycemia following day 1 hypoglycemia

* P<0.05 Significantly different from day 1 euglycemia
† P<0.05 Significantly different from day 2 hypoglycemia following day 1 euglycemia
‡ P<0.05 Significantly different from day 2 hypoglycemia following day 1 hypoglycemia
Figure: 4

Diabetes

* p<0.01 significantly different from day 1 euglycemia
# p<0.01 significantly different to day 2 hypoglycemia following day 1 euglycemia
≠ p<0.04 significantly different to day 1 hypoglycemia

** p<0.01 Significantly different from baseline
§ p<0.001 Significantly different from day 1 euglycemia EC