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# Effects of Acute and Antecedent Hypoglycemia on Endothelial Function and Markers of Atherothrombotic Balance in Healthy Humans



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The aim of this study was to determine the effects of single and repeated episodes of clamped hypoglycemia on fibrinolytic balance, proinflammatory biomarkers, proatherothrombotic mechanisms, and endothelial function. Twenty healthy individuals (12 male and 8 female) were studied during separate 2-day randomized protocols. Day 1 consisted of either two 2-h hyperinsulinemic (812 ± 50 pmol/L)-euglycemic (5 ± 0.1 mmol/L) or hyperinsulinemic (812 ± 50 pmol/L)-hypoglycemic (2.9 ± 0.1 mmol/L) clamps. Day 2 consisted of a single 2-h hyperinsulinemic-hypoglycemic clamp. Two-dimensional Doppler ultrasound was used to determine brachial arterial endothelial function. Plasminogen activator inhibitor 1, vascular cell adhesion molecule-1, intracellular adhesion molecule-1, E-selectin, P-selectin, TAT (thrombin/antithrombin complex), tumor necrosis factor- $\alpha$ , and interleukin-6 responses were increased ( $P < 0.05$ ) during single or repeated hypoglycemia compared with euglycemia. Endogenous and exogenous nitric oxide (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 proinflammatory 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 proatherothrombotic state compared with a single acute episode of hypoglycemia.

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 after severe hypoglycemia (3–5). Several recent large randomized controlled trials have demonstrated that with both intensive and standard treatment of patients with type 2 diabetes, the 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<sub>1c</sub> 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 proinflammatory and proatherothrombotic mechanisms in both healthy and diabetic humans (9–12). Available data are

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See accompanying article, p. 2322.

consistent, demonstrating that acute insulin-induced hypoglycemia can activate proinflammatory responses (12,13).

However, lacking are data reporting the effects of repeated hypoglycemia on proinflammatory and proatherothrombotic 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 proinflammatory and proatherothrombotic mechanisms, and 3) reduce fibrinolytic balance in healthy individuals.

To control for the independent effects of insulin on proinflammatory responses and endothelial function (2), the hyperinsulinemic-euglycemic and hyperinsulinemic-hypoglycemic clamp techniques were used to equate insulin levels in all studies. With use of 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 (12 male and 8 female), age  $34 \pm 3$  years, BMI  $27 \pm 1$  kg/m<sup>2</sup>, and HbA<sub>1c</sub>  $5 \pm 0.3\%$  and  $31 \pm 3.3$  mmol/mol, participated in two randomized, single-blind 2-day experiments. Twelve individuals participated in both protocols, and an additional eight individuals underwent a single 2-day visit, resulting in  $n = 16$  for both 2-day studies. None of the subjects smoked or received anticoagulants, clopidogrel, or statins. Subjects over age 40 years were screened for silent ischemia with a standard treadmill stress test. Each subject had a normal blood count, plasma electrolytes, and liver and renal function and no evidence of either impaired fasting glucose or overt diabetes. 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, nonsteroidal anti-inflammatory drugs, or cyclooxygenase 2 inhibitors for 3 days prior to a study. Subjects were admitted to the general clinical research center the night prior to the study. After an overnight 10-h 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 nondominant arm. This hand was placed in a heated box ( $55\text{--}60^\circ\text{C}$ ) 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 2-day protocols (Fig. 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  $5.0 \pm 0.1$  mmol/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. After day 1 procedures, participants received a standard meal and evening snack and underwent a 10-h 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 ( $\sim 0.08$  mmol/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

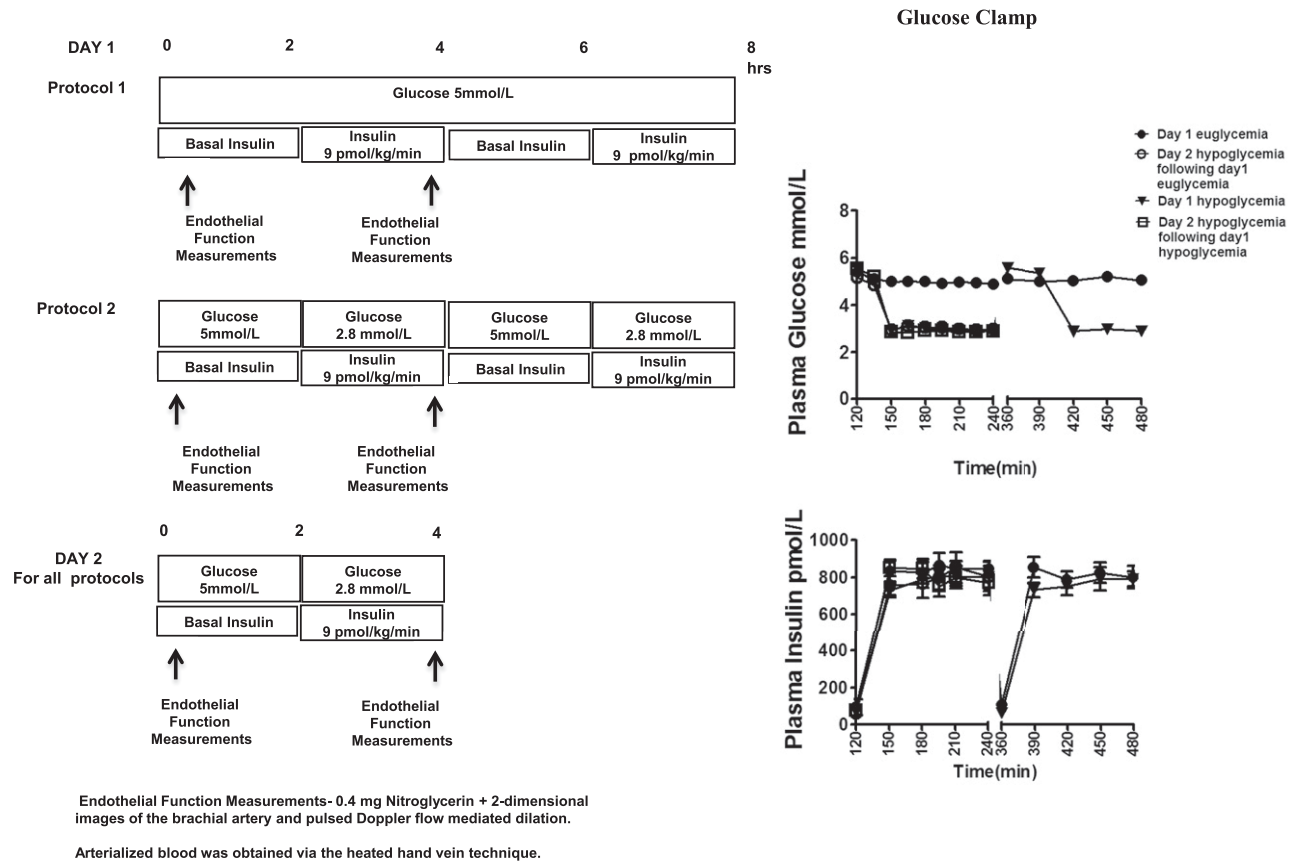
After placement of venous cannulae, a period of 120 min was allowed to elapse, followed by a 120-min hyperinsulinemic-hypoglycemic experimental period similar to that described for day 2 of protocol 1. After the morning clamp experiment, a 120-min euglycemic period was established by infusing 20% dextrose. After 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

After a 10-h overnight fast, day 2 morning procedures were identical to the hyperinsulinemic-hypoglycemic clamp studies performed during protocol 1, day 2.

### Analytical Methods

The collection of blood samples has previously been described (17). Plasma glucose concentrations were measured in triplicate using the glucose oxidase method with a glucose analyzer (Beckman, Fullerton, CA). Insulin, catecholamines, cortisol, and nonesterified fatty acids (NEFAs) were drawn every 30 min. Insulin was measured as previously described with an interassay coefficient of variation (CV) of 9% (18). Catecholamines were determined by high-performance liquid chromatography with an interassay CV of 12% for epinephrine and 8% for norepinephrine (19). Cortisol was assayed using the Clinical Assays Gammacoat radioimmunoassay kit with an interassay CV of 6%. NEFA were measured using the WAKO kit with an interassay CV of 7% (20).



**Figure 1**—Experimental protocols, clamped glucose, and insulin levels during hyperinsulinemic-euglycemic and hyperinsulinemic-hypoglycemic clamps in healthy subjects. Values are mean ± SE. Endothelial function measurements: 0.4 mg nitroglycerin plus two-dimensional images of the brachial artery and pulsed Doppler flow-mediated dilation. Arterialized blood was obtained via the heated hand vein technique. hrs, hours.

During the experimental period, blood for soluble vascular adhesion molecule (VCAM)-1, soluble intracellular adhesion molecule (ICAM)-1, E-selectin, P-selectin, interleukin (IL)-6, tumor necrosis factor (TNF)-α, plasminogen activator inhibitor (PAI)-1, and thrombin/antithrombin (TAT) complex was drawn every 60 min. VCAMs and adiponectin were assayed using LINCO Research kits (St. Charles, MO) with an interassay CV of 8.5% (soluble VCAM-1), CV of 9.7% (soluble ICAM-1), CV of 13.4%, (soluble E-selectin), CV of 9.02% (IL-6), and 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%, with Assay Pro (St. Charles, MO).

**Cardiovascular Measurements**

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

**Endothelial Function**

Measurements of endothelial function were conducted at baseline and during the final 30 min of each glucose

clamp. Flow-mediated dilation of the dominant brachial artery was measured using two-dimensional 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 mmHg greater than the individual’s systolic blood pressure for 5 min (22). Brachial artery diameter measurements were taken at time points 30 s, 60 s, 90 s, and 120 s and after cuff deflation. After a 15-min 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 min (22).

**Statistical Analysis**

Data are expressed as mean ± SE and were analyzed using standard, parametric, and one- and two-way ANOVA and with repeated measures where appropriate (GraphPad Software, Inc., San Diego, CA). Tukey post hoc analysis

**Table 1—Neuroendocrine responses and NEFA responses during euglycemic and hypoglycemic clamps**

	Day 1 euglycemia		Day 2 hypoglycemia after day 1 euglycemia		Day 1 hypoglycemia		Day 2 hypoglycemia after day 1 hypoglycemia	
	Basal	Final	Basal	Final	Basal	Final	Basal	Final
Epinephrine (pmol/L)	190 ± 20	189 ± 20	179 ± 20	4,251 ± 568*†	184 ± 40	3,741 ± 465*†	198 ± 30	2,497 ± 265*†‡
Norepinephrine (pmol/L)	1,083 ± 80	1,159 ± 77	1,128 ± 124	1,957 ± 134*	1,057 ± 119	1,966 ± 149*	1,161 ± 124	2,028 ± 184*
Cortisol (nmol/L)	344 ± 31	292 ± 30	330 ± 32	714 ± 42*†	359 ± 34	850 ± 70*†	271 ± 26	572 ± 62*†‡
Glucagon (ng/L)	65 ± 5	43 ± 3*	50 ± 6	112 ± 17*†	57 ± 4	164 ± 26*†	44 ± 7	90 ± 13*†‡
GH (ng/mL)	2.5 ± 0.4	2.4 ± 0.4	4 ± 1.34	25 ± 5*†	3.1 ± 0.5	28 ± 5*	2.2 ± 0.2	20 ± 3*†
NEFA (mmol/L)	379 ± 48	103 ± 40*	382 ± 55	97 ± 14*	288 ± 32	110 ± 19*	351 ± 28	98 ± 23*

Data are means ± SE. GH, growth hormone. \**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.

was used to delineate statistical significance within each group. Data were also analyzed using paired and unpaired two-tailed *t* tests. In all cases, a *P* value of <0.05 was accepted as statistically significant.

**RESULTS**

**Glucose and Insulin**

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

**Neuroendocrine Counterregulatory Hormones**

Epinephrine responses were significantly higher (*P* < 0.001) during the final 30 min of day 1 hypoglycemia (3,741 ± 465 pmol/L) and day 2 hypoglycemia after day 1 euglycemia (4,251 ± 568 pmol/L) compared with euglycemia (189 ± 20 pmol/L) or day 2 repeated

hypoglycemia (2,497 ± 265 pmol/L). Norepinephrine levels were also similar during all hypoglycemia protocols but significantly higher (*P* < 0.001) compared with euglycemia (1,984 ± 156 vs. 1,159 ± 77 pmol/L, respectively) (Table 1). Glucagon, cortisol, and growth hormone levels were all reduced (*P* < 0.05) during day 2 hypoglycemia after day 1 hypoglycemia (Table 1).

**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 1).

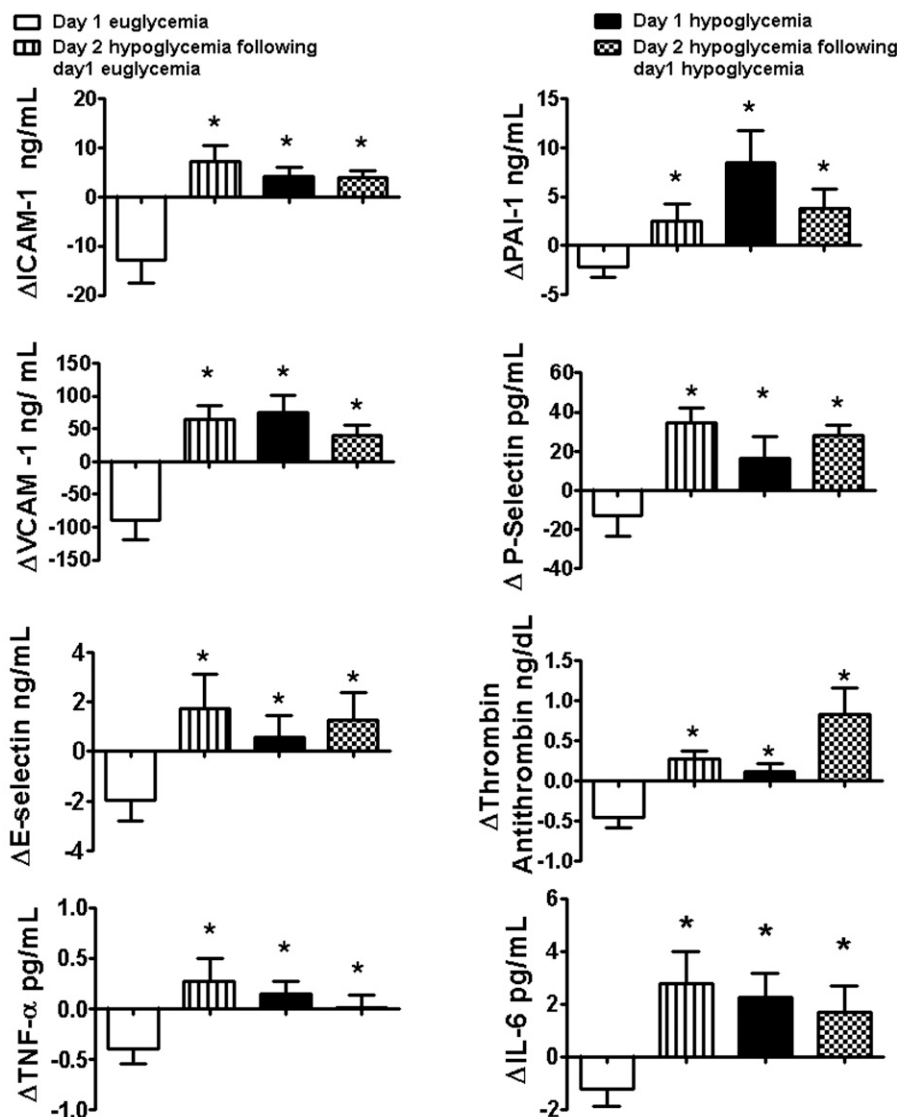
**Atherogenic VCAMs**

Baseline values of ICAM-1, VCAM-1, and E-selectin were similar (Table 2) and fell (*P* < 0.05) during the hyperinsulinemic-euglycemic clamps (Figs. 2 and 3). End of clamp responses during all hypoglycemic protocols were similarly increased (*P* < 0.05) compared with euglycemia (Figs. 2 and 3). Conceptually similar statistical differences

**Table 2—Proinflammatory and proatherothrombotic markers at baseline**

	Day 1 euglycemia	Day 2 hypoglycemia after day 1 euglycemia	Day 1 hypoglycemia	Day 2 hypoglycemia after day 1 hypoglycemia
ICAM (ng/mL)	90 ± 7	89 ± 7	88 ± 3	78 ± 5
VCAM (ng/mL)	838 ± 37	772 ± 43	819 ± 62	735 ± 48
E-selectin (ng/mL)	22 ± 2	18 ± 2	21 ± 2	16 ± 2
P-selectin (pg/mL)	83 ± 9	56 ± 6*	63 ± 13	45 ± 9*
PAI-1 (ng/mL)	18 ± 3	14 ± 2	13 ± 3	17 ± 6
TAT (ng/mL)	3.5 ± 0.4	2.9 ± 0.3	3.5 ± 0.2	3.6 ± 0.2
tPA (ng/mL)	6.1 ± 1	5.9 ± 1	5 ± 1	4.2 ± 1

Data are means ± SE. \**P* < 0.05, significantly different from day 1 euglycemia at baseline.



\* P<0.05 Significantly different from day 1 euglycemia

**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 is increased during all hypoglycemia protocols compared with euglycemia. Values are mean ± SE. \*P < 0.05, significantly different from day 1 euglycemia.

were obtained when responses from the entire time course of the 2-h clamp studies (Figs. 2 and 3) and incremental changes from baseline were evaluated.

**Platelet Activation and Fibrinolytic/Thrombotic Balance**

Baseline values for PAI-1, TAT, and tissue plasminogen activator (tPA) were similar for all protocols (Table 2). Basal day 2 hypoglycemia P-selectin values were lower (P < 0.05) after day 1 hyperinsulinemia. PAI-1, P-selectin, and TAT end-of-clamp responses were increased (P < 0.05) during all hypoglycemic studies compared with euglycemic clamp studies (Figs. 2 and 3). Incremental responses from baseline for the entire time course during hypoglycemia for PAI-1, TAT, and P-selectin were also increased compared with

euglycemia (Fig. 3). Plasma tPA values remained similar to baseline during euglycemic and hypoglycemic studies.

**Inflammatory Cytokines**

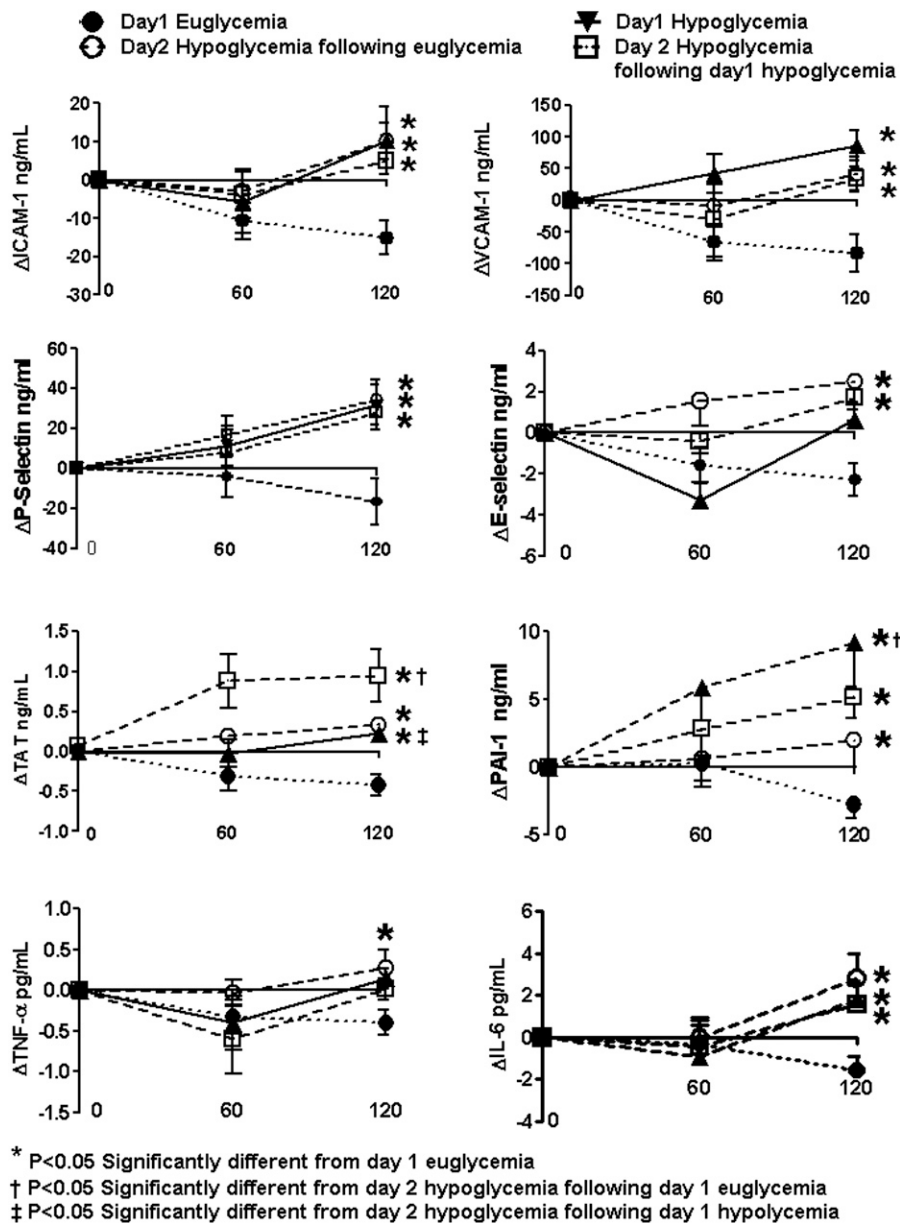
Baseline values for IL-6 and TNF-α were similar at the start of all protocols of glucose clamp studies (Table 2). End of clamp responses were increased (P < 0.05) during all hypoglycemic protocols compared with euglycemia (Figs. 2 and 3).

**Cardiovascular Parameters**

Heart rate and 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



**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 is increased during all hypoglycemia protocols compared with euglycemia. Values are mean  $\pm$  SE. \* $P < 0.05$ , significantly different from day 1 euglycemia. † $P < 0.05$ , significantly different from day 2 hypoglycemia after day 1 euglycemia. ‡ $P < 0.05$ , significantly different from day 2 hypoglycemia after day 1 hypoglycemia.

hypoglycemia protocols compared with day 1 euglycemia (Fig. 4). Endogenous NO-mediated endothelial function was also reduced ( $P < 0.05$ ) by repeated hypoglycemia compared with day 2 hypoglycemia after day 1 euglycemia. There were also reductions ( $P < 0.05$ ) in endothelial function during exogenous NO stimulation during repeated hypoglycemia compared with day 1 euglycemia and day 1 hypoglycemia (Fig. 4).

## DISCUSSION

This study has investigated the effects of clamped hyperinsulinemic euglycemia and acute and repeated 2-h episodes

of moderate hyperinsulinemic hypoglycemia (2.9 mmol/L) on endothelial function, fibrinolytic balance, and proinflammatory and proatherothrombotic mechanisms in healthy man. Our results demonstrate that acute hypoglycemia reduces endogenous NO-mediated endothelial vasodilation, activates inflammatory processes, impairs fibrinolytic balance, and increases proatherothrombotic 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.

**Table 3—Cardiovascular responses during hyperinsulinemic-euglycemic and hyperinsulinemic-hypoglycemic clamps**

	Basal	Final
Systolic blood pressure (mmHg)		
Day 1 eugly	110 ± 3	110 ± 3
Day 2 hypo after day 1 eugly	109 ± 3	116 ± 4
Day 1 hypo	112 ± 4	121 ± 7†
Day 2 hypo after day 1 hypo	113 ± 4	123 ± 5†‡
Diastolic blood pressure (mmHg)		
Day 1 eugly	63 ± 2	62 ± 2
Day 2 hypo after day 1 eugly	65 ± 1	60 ± 2†
Day 1 hypo	65 ± 2	62 ± 3
Day 2 hypo after day 1 hypo	66 ± 1	62 ± 2†
Mean arterial pressure (mmHg)		
Day 1 eugly	79 ± 2	76 ± 3
Day 2 hypo after day 1 eugly	80 ± 2	80 ± 2
Day 1 hypo	81 ± 3	82 ± 4
Day 2 hypo after day 1 hypo	81 ± 2	83 ± 3
Heart rate (bpm)		
Day 1 eugly	64 ± 3	67 ± 2
Day 2 hypo after day 1 eugly	65 ± 3	74 ± 2†‡
Day 1 hypo	66 ± 3	70 ± 3
Day 2 hypo after day 1 hypo	68 ± 3	75 ± 2†‡

Data are means ± SE. eugly, euglycemia; hypo, hypoglycemia.  
†*P* < 0.05 compared with baseline. ‡*P* < 0.05 compared with final euglycemia.

Recent large randomized controlled trials investigating the effects of intensifying glucose control both in hospitals and in outpatient 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 and proatherothrombotic and proinflammatory 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 proinflammatory responses (23,24).

The results of the current 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 with a single similar hypoglycemic episode.

Relatively few studies have investigated the effects of acute hypoglycemia on proinflammatory and proatherothrombotic mechanisms (10,12,24,25). In this present study, we confirm and extend previous findings that 2 h

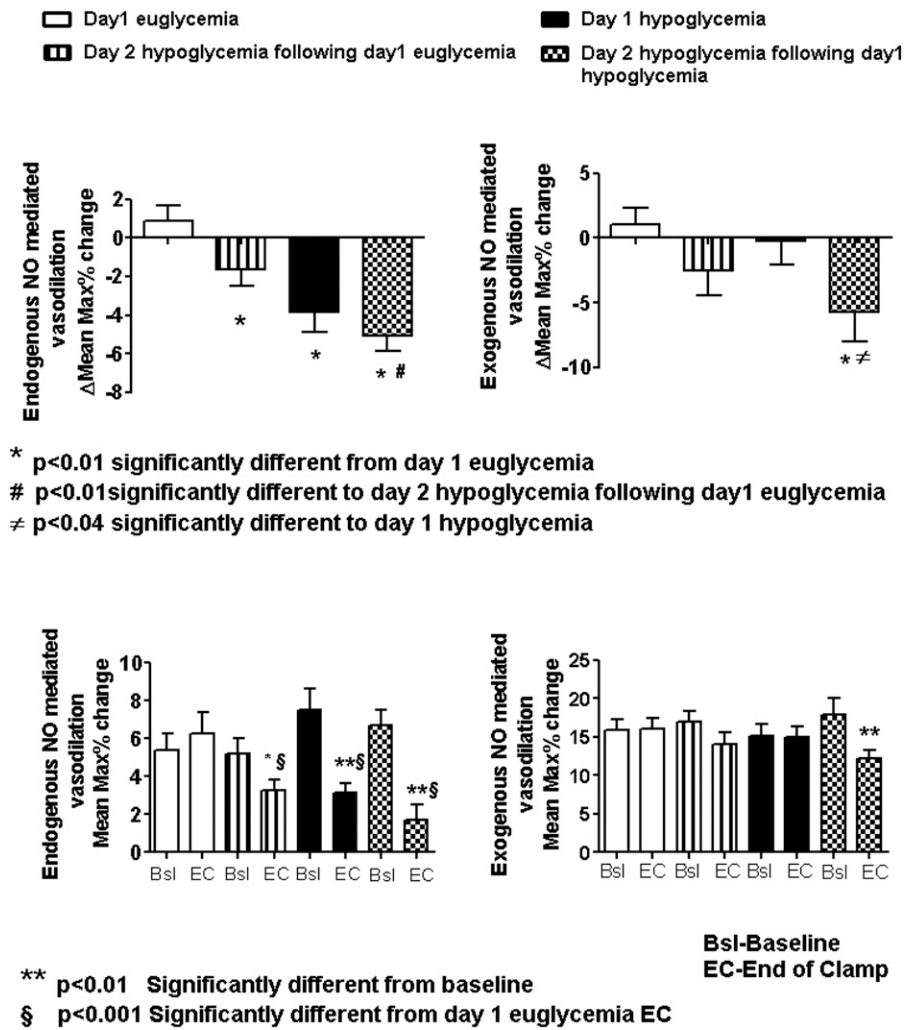
of acute moderate hypoglycemia can activate proinflammatory (ICAM-1, VCAM-1, E-selectin, TNF- $\alpha$ , and IL-6) and prothrombotic (PAI-1, P-selectin, and TAT complex) mechanisms. The increased TAT complexes (increased intravascular coagulation), elevated P-selectin (increased platelet aggregability), and increased PAI-1 relative to tPA (disordered fibrinolytic balance) responses demonstrate a broad activation of prohemostatic clotting mechanisms by hypoglycemia.

Supporting previous studies, our results demonstrate that hyperinsulinemic euglycemia can acutely lower proinflammatory and proatherothrombotic 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 proinflammatory vascular action of next day hyperinsulinemic hypoglycemia.

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

Endothelial dysfunction is a key feature of macrovascular complications in both type 1 and type 2 diabetes. Flow-mediated dilation 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 nonendogenous NO-mediated vasodilation and resulted in greater endothelial dysfunction compared with 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 with a single episode of hypoglycemia. Thirdly, these present results indicate that repeated hypoglycemia can impair NO-mediated vascular smooth



**Figure 4**—Endogenously and exogenously (NO) flow-mediated dilation during acute and repeated hyperinsulinemic/hypoglycemic protocols compared with hyperinsulinemic euglycemia in overnight-fasted healthy individuals. Bsl, baseline; EC, end of clamp; Max, maximum. \**P* < 0.01, significantly different from day 1 euglycemia. #*P* < 0.01, significantly different from day 2 hypoglycemia after day 1 euglycemia. ≠*P* < 0.04, significantly different from day 1 hypoglycemia. \*\**P* < 0.01, significantly different from baseline. §*P* < 0.001, significantly different from day 1 euglycemic end of clamp.

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 nondiabetic individuals and individuals with type 1 or type 2 diabetes (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, proinflammatory and particularly proatherothrombotic 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 proatherothrombotic markers compared with 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 proinflammatory 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. (32) also have demonstrated that insulin-induced hypoglycemia reduces myocardial blood flow, whereas hyperinsulinemic euglycemia has a beneficial effect on myocardial blood flow in healthy individuals and individuals with type 1 diabetes. 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 proinflammatory and proatherogenic 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 (after either euglycemic or hypoglycemic studies) were 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 proinflammatory 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  $\alpha$ - or  $\beta$ -receptors can reduce endogenously NO-mediated smooth muscle vasodilation. The effects of catecholamines and direct sympathetic nervous system drive on proinflammatory, fibrinolytic balance, and proatherothrombotic 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 on the circulating neuroendocrine milieu (i.e., interactions with corticosteroids) (37). Thus, the combination of the hypoglycemia-driven increases in epinephrine, norepinephrine, 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 proinflammatory and endothelial dysfunction occurring during repeated hypoglycemia (38). Additionally, previous work by Wang et al. (29) has also demonstrated direct inflammatory and adverse effects of hypoglycemia per se on in vitro vascular smooth muscle endothelial function. Lastly, we also cannot exclude that increases in endothelin-1 may also have played a role in the proinflammatory 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 proinflammatory mechanisms in healthy individuals. We conclude that in healthy individuals, acute moderate hypoglycemia can activate proinflammatory and prothrombotic mechanisms and secondly, antecedent hypoglycemia can result in

greater prothrombotic responses and impaired endothelial function compared with a single episode of acute moderate hypoglycemia.

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**Author Contributions.** N.G.J. performed studies, researched data, and wrote the manuscript. D.B.T. and L.M.Y. helped perform studies, researched data, and reviewed and edited the manuscript. S.N.D. devised the study, reviewed and edited data, and wrote the manuscript. S.N.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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