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Acute Hypoglycemia Decreases Myocardial Blood Flow Reserve in Patients With Type 1 Diabetes Mellitus and in Healthy Humans

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Background—Hypoglycemia is associated with increased cardiovascular mortality, but the reason for this association is poorly understood. We tested the hypothesis that the myocardial blood flow reserve (MBFR) is decreased during hypoglycemia using myocardial contrast echocardiography in patients with type 1 diabetes mellitus (DM) and in healthy control subjects.

Methods and Results—Twenty-eight volunteers with DM and 19 control subjects underwent hyperinsulinemic clamps with maintained sequential hyperinsulinemic euglycemia (plasma glucose, 90 mg/dL [5.0 mmol/L]) followed by hyperinsulinemic hypoglycemia (plasma glucose, 50 mg/dL [2.8 mmol/L]) for 60 minutes each. Low-power real-time myocardial contrast echocardiography was performed with flash impulse imaging using low-dose dipyridamole stress at baseline and during hyperinsulinemic euglycemia and hyperinsulinemic hypoglycemia. In control subjects, MBFR increased during hyperinsulinemic euglycemia by 0.57 U (22%) above baseline (B coefficient, 0.57; 95% confidence interval, 0.38 to 0.75; $P < 0.0001$) and decreased during hyperinsulinemic hypoglycemia by 0.36 U (14%) below baseline values (B coefficient, -0.36 ; 95% confidence interval, -0.50 to -0.23 ; $P < 0.0001$). Although MBFR was lower in patients with DM at baseline by 0.37 U (14%; B coefficient, -0.37 ; 95% confidence interval, -0.55 to -0.19 ; $P = 0.0002$) compared with control subjects at baseline, the subsequent changes in MBFR during hyperinsulinemic euglycemia and hyperinsulinemic hypoglycemia in DM patients were similar to that observed in control subjects. Finally, the presence of microvascular complications in the patients with DM was associated with a reduction in MBFR of 0.52 U (24%; B coefficient, -0.52 ; 95% confidence interval, -0.70 to -0.34 ; $P < 0.0001$).

Conclusions—Hypoglycemia decreases MBFR in both healthy humans and patients with DM. This finding may explain the association between hypoglycemia and increased cardiovascular mortality in susceptible individuals. (*Circulation*. 2011;124:1548-1556.)

Key Words: diabetes mellitus ■ echocardiography ■ hypoglycemia ■ insulin ■ regional blood flow

Several studies have shown that hypoglycemia is associated with an increase in cardiovascular mortality (CVM).¹⁻⁶ This association has been demonstrated in people with and without established coronary artery disease.¹⁻³ Importantly, patients with acute coronary syndromes appear to have worse short- and long-term outcomes if they experience hypoglycemia in the acute phase of their presentation.²⁻⁴ For example, in patients with diabetes mellitus (DM) and acute coronary syndromes, hypoglycemia within 48 hours of their admission was associated with a 2-fold increase in

all-cause mortality over a 2-year follow-up.² Similarly, Pinto et al³ showed that patients with ST-segment-elevation myocardial infarction and an admission blood glucose < 4.5 mmol/L had a 3-fold increased rate of adverse outcomes (defined as 30-day mortality and myocardial infarction). Furthermore, in the same study, patients with DM had an 18-fold increased risk of adverse cardiac outcomes. Subsequently, a more recent study showed that in patients after myocardial infarction, spontaneous hypoglycemia was associated with a 2-fold increase in in-hospital mortality.⁴

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Clinical Perspective on p 1556

Insulin, under euglycemic conditions, has important beneficial effects on the vascular tone by inducing nitric oxide-mediated vasodilatation.⁷ Studies on healthy humans and patients with DM show that insulin causes a marked increase in myocardial blood flow (MBF) and MBF reserve (MBFR) during euglycemia.^{8,9} In contrast, the mechanisms by which hypoglycemia adversely affects the cardiovascular system are unclear. Hypoglycemia has been associated with angina and, importantly, has been shown to increase the size of a myocardial infarct.^{10,11} Furthermore, low blood glucose also encourages a hypercoagulant state resulting from an increase in plasma concentrations of coagulation factors and by promoting platelet aggregation.^{12,13}

Myocardial contrast echocardiography (MCE) is an established technique used in the noninvasive quantification of MBFR with an accuracy similar to that of positron emission tomography and coronary Doppler flow wire measurements.^{14,15} MBFR is calculated as the ratio of peak MBF to resting MBF.¹⁴ In the absence of flow-limiting coronary artery disease, an MBFR <2.0 is indicative of underlying endothelial dysfunction.¹⁶ Furthermore, MBFR has been shown to be an independent predictor of CVM in diabetic and nondiabetic patients with normal stress echocardiograms and in patients after acute coronary syndromes.^{17–19}

We hypothesized that hypoglycemia would decrease the MBFR (measured by MCE) using a 1-step hyperinsulinemic clamp technique to induce hypoglycemia in patients with type 1 DM and in healthy control subjects.

Methods

Subjects

Twenty-eight subjects with type 1 DM (group DM) participated in the study after approval of the local research ethical committee. In addition, 19 healthy volunteers (group C) acted as control subjects. All volunteers underwent testing of MBF by MCE. Assessment of MBF was undertaken with an insulin clamp at 3 stages: at baseline, during hyperinsulinemic euglycemia (HE), and during hyperinsulinemic hypoglycemia (HH). During each stage, all volunteers underwent measurement of MBF during 2 states: at rest and after dipyridamole-induced stress.

None of the volunteers were active smokers or had a history of hypertension, coronary artery disease, or underlying lipid disorders. All volunteers had normal exercise stress echocardiograms. Within the DM group, 8 volunteers had evidence of microvascular complications (see the online-only Data Supplement). All volunteers provided written informed consent.

Hyperinsulinemic Clamps

Volunteers were admitted after an overnight fast. The overall study scheme is shown in Figure 1A and 1B. Written instructions were provided to avoid caffeine-containing products and alcohol for >12 hours. In the DM group, a standard sliding-scale insulin was begun to keep glucose levels close to 90 mg/dL (5.0 mmol/L). After a 30- to 40-minute rest period, baseline plasma glucose was determined, and the hyperinsulinemic clamp was begun.²⁰ Insulin was infused at $3 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 4 minutes, followed by $2 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for a further 3 minutes, after which the infusion rate was maintained at $1.5 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. Hyperinsulinemic euglycemia (90 mg/dL [5.0 mmol/L]) was maintained for 60 minutes after an initial 30-minute stabilization period. Glucose levels were subsequently reduced over a 30-minute period by decreasing the 20% (wt/vol) dextrose (Baxter Healthcare, Thetford, Norfolk, UK) infusion rate, and

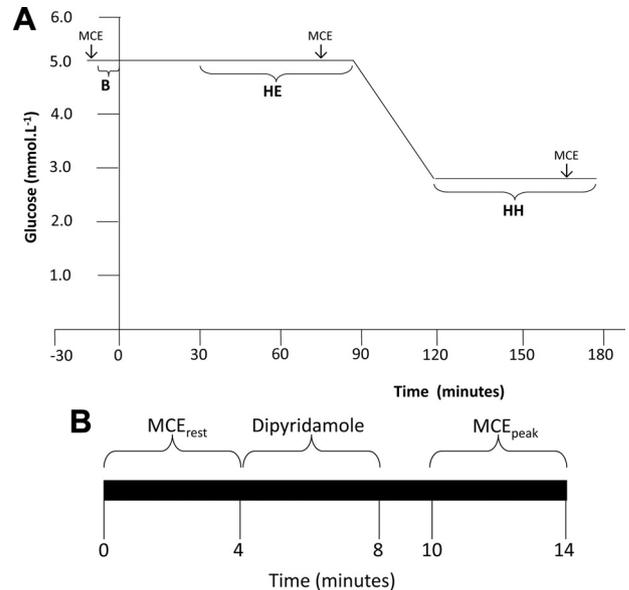


Figure 1. A, Study design showing glucose concentrations and timing of myocardial contrast echocardiography (MCE) at baseline (B), hyperinsulinemic euglycemia (HE), and hyperinsulinemic hypoglycemia (HH). B, Sequence of image acquisition during each MCE study at baseline and during HE and HH. MCE_{rest} indicates MCE at rest; MCE_{peak}, MCE after dipyridamole-induced stress.

symptomatic hypoglycemia (50 mg/dL [2.8 mmol/L]) was induced (see the online-only Data Supplement). The glucose concentrations were maintained for a further 60 minutes (HH), after which insulin infusion was terminated and normoglycemia was restored.

Myocardial Contrast Echocardiography

We performed MCE using a commercial ultrasound machine iE33 (Philips Medical Systems, Best, the Netherlands) and SonoVue (Bracco Research SA, Geneva, Switzerland) as the contrast agent as previously described.²¹ Real-time images were recorded within 3 to 4 minutes in the 3 apical views (apical 4-chamber, apical 2-chamber, and apical 3-chamber views) with low-power settings at a mechanical index of 0.1. The focus was set at the mitral valve level. SonoVue was initially started at 60 mL/h through the left anterograde cannula with the VueJect infusion syringe pump (BR-INF 100, Bracco Research, SA), which gently rotates and maintains the contrast agent in a suspension. Thereafter, the rate was set between 48 and 60 mL/h to maximize image quality with minimal attenuation. Once optimized, the machine settings were held constant throughout each participant study. Flash-impulse imaging at a high mechanical index (1.0) was performed to achieve complete myocardial bubble destruction, after which 10 end-systolic frames were recorded digitally in each apical view. After the resting images were acquired, dipyridamole was infused at 0.56 mg/kg over a 4-minute period. After an interval of 2 minutes, poststress images were recorded within 3 to 4 minutes. This entire sequence took 14 minutes (Figure 1B). The MCE studies were performed at baseline before insulin infusion and during HE and HH (Figure 1A). Continuous ECG monitoring was undertaken, and blood pressure was recorded before and after stress during each study.

Analytic Methods

Quantitative MCE analysis was performed offline with standard commercially available software, QLab version 7.0 (Q-Laboratory, Philips Medical Systems). Quantitative assessment of myocardial perfusion was performed for 10 consecutive end-systolic frames after microbubble destruction. A region of interest was placed over the entire thickness of the myocardium, and particular care was taken to exclude high-intensity epicardial and endocardial borders by manu-

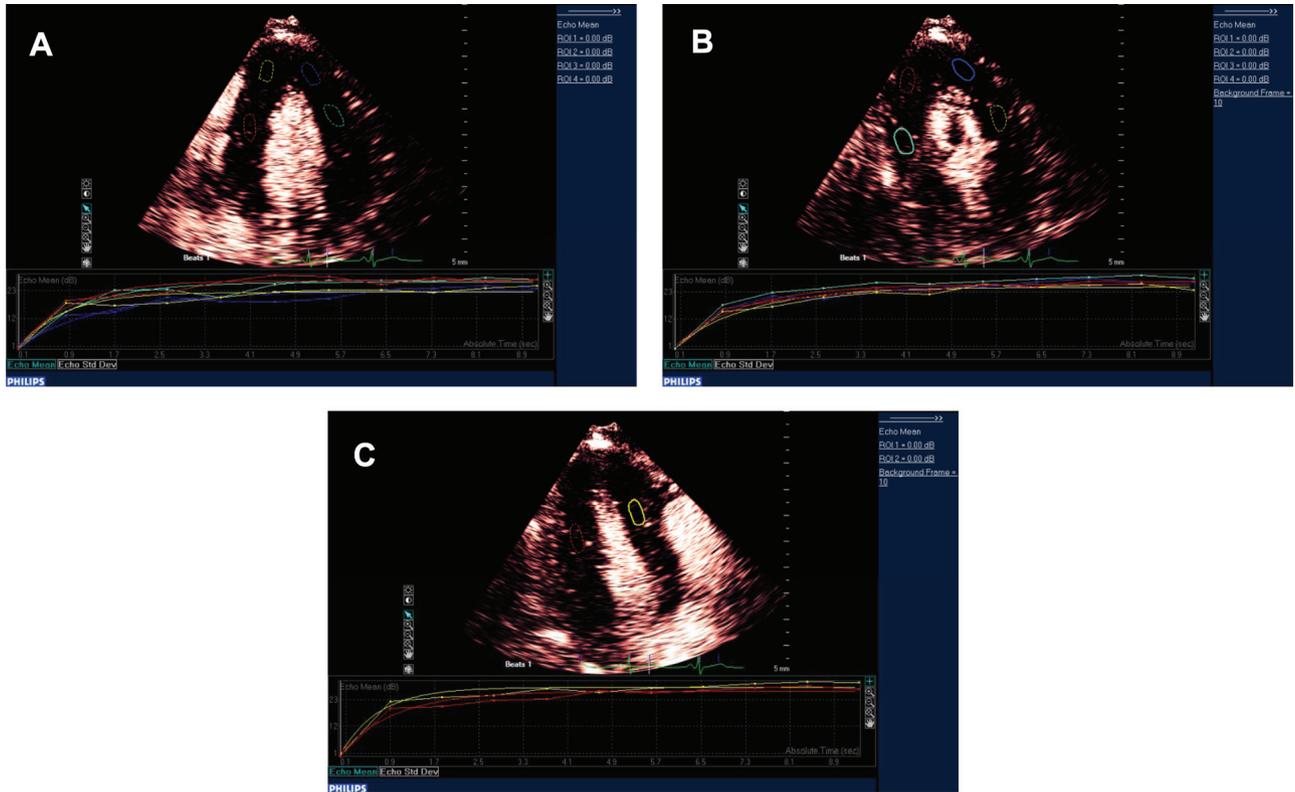


Figure 2. Model used for quantitative analysis of myocardial segments. **A**, Apical 4 chamber; **B**, apical 2 chamber; **C**, apical 3 chamber.

ally moving the region of interest between each frame (see Figure 2). Background-subtracted plots of peak myocardial contrast intensity (representing myocardial blood volume A , dB) versus pulsing intervals (representing time) were automatically constructed by QLab software to fit the monoexponential function conventional equation: $y = A(1 - e^{-\beta t})$.¹⁴ From these plots, the slope of the replenishment curve was determined (representing myocardial blood velocity β , dB/s). The product of A and β yielded resting MBF (dB²/s) and postdipyridamole MBF (peak MBF, dB²/s), respectively (Figure I in the online-only Data Supplement).¹⁴ We calculated MBFR by the ratio of peak MBF to resting MBF.¹⁴ Furthermore, MBFR was calculated by dividing the peak MBF by the resting MBF of the same segment at each of the 3 time frames (baseline, HE, and HH). The basal segments were not included in the analysis because of contrast attenuation.²² The remaining 10 mid and apical cardiac segments were analyzed as shown in Figure 2.²² A segment was not included in the analysis if there was artifact, inadequate microbubble destruction, attenuation, or a wide variation in contrast intensity to minimize errors. The average number of analyzable segments for baseline, HE, and HH was 6 for each. All studies were reanalyzed blindly for intraobserver variability, and for interobserver agreement, 100 myocardial segments were randomly analyzed by another observer (K.G.) who was blinded to the sequence of the studies. The intraobserver and interobserver variabilities were 7.7% and 8.2%, respectively.

Venous samples were taken at baseline and every 30 minutes thereafter (7 samples in total) for determination of plasma endothelin-1 (ET-1) and epinephrine levels, as well as serum high-sensitivity C-reactive protein and insulin levels. All assays were performed in duplicate by a single observer (S.Z.) who was blinded to the hemodynamic and MCE data. Plasma ET-1 levels were measured with a quantitative sandwich enzyme immunoassay (QuantiGlo ELISA, R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Intra-assay and interassay coefficients of variation were 3.4% and 8.9%, respectively, with a cross-reactivity of <0.02% for all human big ETs, 9% for ET-3, and 51% for ET-2. Plasma

high-sensitivity C-reactive protein was determined with a particle-enhanced immunoassay with an interassay coefficient of variation <10% (Roche Diagnostics, Burgess Hill, UK). Plasma epinephrine levels were measured after extraction and acetylation by competitive immunoassay (Labor Diagnostika Nord, Nordhorn, Germany). Both intra-assay and interassay coefficients of variation were <15%. Serum insulin concentrations were measured with electrochemiluminescence immunoassay (Roche Diagnostics, Burgess Hill, UK). The assay shows minimal cross-reactivity with proinsulin or recombinant insulin analogs, and the intra-assay and interassay coefficients of variation were <2% and <5%, respectively.

Statistical Analysis

All data are represented as mean \pm SD except ET-1 and high-sensitivity C-reactive protein values, which are presented as median (interquartile range).

For MBF, β , and A , the influence of measurement stage, stress state, and the presence of DM was assessed via mixed-effects regression modeling (to reflect the intraclass correlation resulting from repeated measurements made on each subject). For each of these 3 outcomes, a mixed-effects model was fitted in which the main effects of stage, stress state, and DM (together with all of their possible interactions) were assessed. Modeling was performed with the MIXED procedure in SAS software (version 9.2). Interpretation of these models is described in the online-only Data Supplement.

In addition to yielding regression parameter estimates, the models were used to estimate mean values for each combination of effects (via the LSMEANS option in the MIXED procedure) and to test for selected differences in these means. With 12 effects combinations (ie, 3 stages \times 2 stress states \times 2 diabetes states [present/absent]), the maximum number of possible between-group differences was 66. It was fully recognized that formal testing of between-group differences under these conditions was justified only when there was some a priori reason to anticipate the presence of an effect of interest and under the strict understanding that the primary purpose of such testing was the generation of hypotheses for future research rather

Table 1. Baseline Characteristics of Subjects

Variable	Group DM (n=28)	Group C (n=19)	P
Age, y	38.7±9	31.8±8	0.013
Men, n (%)	22 (79)	11 (58)	0.13
BMI, kg/m ²	25.7±3.5	24.9±2.6	0.39
SBP, mm Hg	125±13	115±10	0.007
DBP, mm Hg	77±7	73±7	0.05
Heart rate, bpm	78±14	79±16	0.71
Fasting glucose, mmol/L	10.3±3.9	4.9±0.3	<0.0001
Duration of diabetes mellitus, y	19.2±12		
Glycosylated hemoglobin HbA _{1c} , %	8.9±1.5		
Albumin/creatinine ratio, mg · mmol ⁻¹ · L ⁻¹	4.3±9.4		
VPT score	8.0±5.6		
TC, mmol/L*	4.8±0.9	4.3±0.7	0.03
LDL-C, mmol/L*	2.5±0.8	2.1±0.9	0.10
HDL-C, mmol/L*	1.8±0.7	1.5±0.3	0.10
TG, mmol/L†	1.2±0.7	1.3±1.2	0.81

DM, type 1 diabetes mellitus; C, control; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; VPT, vibration perception threshold; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; and TG, triglycerides. Data are presented as mean±SD.

*To convert mmol/L to mg/dL, multiply by 38.7.
 †To convert mmol/L to mg/dL, multiply by 88.6.

than the drawing of substantive inferences. For further detailed explanation, see the online-only Data Supplement.

For the main outcome (MBFR), the concept of stress state was not applicable. Consequently, this outcome was investigated via a further mixed-effects regression model in which MBFR was predicted by the main effects of measurement stage and of DM together with that of their interaction, by age, and by the subject’s systolic blood pressure.

Results

Subject Characteristics

The baseline characteristics of the 19 healthy volunteers (group C) and 28 volunteers with DM are summarized in Table 1.

Hemodynamic Data

Throughout the clamp, resting heart rate, resting systolic blood pressure, resting diastolic blood pressure, and resting

Table 3. Mixed-Effect Regression Model Showing the Effect of Stage (Baseline, Hyperinsulinemic Euglycemia, and Hyperinsulinemic Hypoglycemia), State (Resting and Dipyridamole-Induced Stress [After Stress]), and Diabetes Mellitus on Myocardial Blood Volume (A)

Variable	Estimate	95% CI	P
Measurement stage			
Baseline	19.3	18.4–20.2	<0.0001
Euglycemia	20.4	19.4–21.5	<0.0001
Hypoglycemia	21.7	20.7–22.7	<0.0001
State (poststress vs rest)	2.7	1.5–3.9	<0.0001
Interaction (stage with state)			
Euglycemia and poststress	0.7	–0.5–1.9	0.25
Hypoglycemia and poststress	–1.4	–2.9–0.1	0.068
Presence of diabetes mellitus (yes vs no)	0.7	–0.5–1.9	0.25
Interaction (stage with diabetes mellitus)			
Euglycemia and diabetes mellitus present	–0.9	–2.5–0.6	0.22
Hypoglycemia and diabetes mellitus present	–1.4	–2.9–0.1	0.075
Interaction (state with diabetes mellitus)			
Poststress and diabetes mellitus present	–0.4	–1.9–1.2	0.65
Interaction (stage with state with diabetes mellitus)			
Euglycemia, poststress, diabetes mellitus present	–0.5	–2.1–1.0	0.49
Hypoglycemia, poststress, diabetes mellitus present	1.9	–0.1–3.8	0.056

CI indicates confidence interval.

rate-pressure product were similar in groups C and DM (Table I in the online-only Data Supplement).

Myocardial Contrast Echocardiography–Derived Measurements

Myocardial Blood Volume

Mean myocardial blood volumes and 95% confidence intervals (95% CIs) at rest (A_r) and during dipyridamole-induced stress (A_d) are shown at baseline and during HE and HH in Table 2. Table 3 shows the mixed-effect regression modeling

Table 2. Myocardial Blood Volume (A) at Rest and After Dipyridamole-Induced Stress at Baseline and During Hyperinsulinemic Euglycemia and Hyperinsulinemic Hypoglycemia

Stage	State			
	DM		C	
	Rest (A _r), dB	Peak (A _d), dB	Rest (A _r), dB	Peak (A _d), dB
Baseline	20.0 (19.2–20.8)	22.4 (21.6–23.1)	19.3 (18.4–20.2)	22.0 (21.1–23.0)
HE	20.2 (19.3–21.1)	22.7 (21.9–23.5)	20.4 (19.4–21.5)	23.9 (22.9–24.8)
HH	21.0 (20.2–21.9)	23.9 (23.1–24.7)	21.7 (20.7–22.7)	23.0 (22.1–24.0)

Stage is baseline, hyperinsulinemic euglycemia (HE), or hyperinsulinemic hypoglycemia (HH). State is resting (resting blood volume=A_r) or during dipyridamole stress (peak=A_d). Values are means (95% confidence intervals).

Table 4. Myocardial Blood Velocity (β) at Rest and After Dipyridamole-Induced Stress at Baseline and During Hyperinsulinemic Euglycemia and Hyperinsulinemic Hypoglycemia

Stage	State			
	DM		C	
	Rest (β_r), dB/s	Peak (β_d), dB/s	Rest (β_r), dB/s	Peak (β_d), dB/s
Baseline	1.03 (0.96–1.09)	1.84 (1.70–1.98)	0.92 (0.84–1.00)	2.00 (1.83–2.17)
HE	1.11 (1.05–1.17)	2.37 (2.22–2.51)	0.96 (0.88–1.03)	2.41 (2.23–2.58)
HH	1.18 (1.12–1.24)	1.85 (1.74–1.97)	1.04 (0.96–1.11)	2.03 (1.89–2.17)

Stage is baseline, hyperinsulinemic euglycemia (HE), or hyperinsulinemic hypoglycemia (HH). State is resting (resting blood velocity= β_r) or during dipyridamole stress (peak= β_d). Values are means (95% confidence intervals).

testing the effect of stage, state, DM, and their interactions on A_r and A_d .

There was a significant increase in A_d in group C at baseline by 2.7 dB ($P<0.0001$), as shown in Table 3. In addition, there was marginal evidence that A_d was decreased during HH by 1.4 dB compared with baseline ($P=0.068$). Furthermore, the presence of DM did not affect either A_r or A_d at the baseline stage ($P=0.25$ and $P=0.65$, respectively). However, there was a suggestion that A_d was increased in group DM during HH compared with group C by 1.9 dB ($P=0.056$).

Myocardial Blood Velocity

Mean myocardial blood velocities and 95% CIs at rest (β_r) and during dipyridamole-induced stress (β_d) are shown at baseline and during HE and HH in Table 4. Table 5 shows the mixed effect regression modeling testing the effect of stage, state, DM and their interactions on β_r and β_d .

There was a significant increase in β_d in group C at baseline by 1.08 dB/s ($P<0.0001$), as shown in Table 5. During HE, β_d was further increased in group C compared with baseline values by 0.37 dB/s ($P<0.0001$). However, during HH, β_d declined and was not different from baseline stress values ($P=0.28$). In group DM, β_r was significantly elevated compared with group C at baseline by 0.11 dB/s ($P=0.035$). Importantly, at baseline, β_d was significantly decreased in group DM compared with group C by 0.27 dB/s ($P=0.005$).

In group DM, during HE and HH, a similar effect on β_d was observed compared with group C with no significant differences between the 2 groups at each stage.

Myocardial Blood Flow

Mean MBFs and 95% CIs at rest (resting MBF) and during dipyridamole-induced stress (peak MBF) are shown at baseline and during HE and HH in Table 6. Table 7 shows the mixed-effect regression modeling testing the effect of stage, state, DM, and their interactions on MBF.

In group C, peak MBF was significantly increased compared with resting MBF at baseline by 26.5 dB²/s ($P<0.0001$) as shown in Table 7. During HE, peak MBF was further increased in group C above baseline peak values by 11.6 dB²/s ($P<0.0001$). However, during HH, peak MBF declined and was not significantly different from baseline peak MBF values ($P=0.20$).

The resting MBF was significantly higher in group DM compared with group C at baseline by 2.6 dB²/s ($P=0.015$). There was no significant difference in the resting MBF values between the 2 groups at HE or HH. In group DM, peak MBF

was significantly decreased compared with group C at baseline by 6.0 dB²/s ($P=0.006$). In group DM, during HE and HH, a similar effect on peak MBF was observed compared with group C with no significant differences between the 2 groups at each stage.

Myocardial Blood Flow Reserve

We tested the effect of measurement stage, age, presence of DM, and systolic blood pressure on MBFR using regression modeling (Table 8 and Figure 3). In Table 8, the intercept of

Table 5. Mixed-Effect Regression Model Showing the Effect of Measurement Stage (at Baseline, During Hyperinsulinemic Euglycemia, and During Hyperinsulinemic Hypoglycemia), Presence of Diabetes Mellitus, and Stress State (Rest Versus After Dipyridamole-Induced Stress) on Myocardial Blood Velocity (β)

Variable	Estimate	95% CI	P
Measurement stage			
Baseline	0.92	0.84–1.00	<0.0001
Euglycemia	0.96	0.88–1.03	<0.0001
Hypoglycemia	1.04	0.96–1.11	<0.0001
State (after stress vs rest)	1.08	0.94–1.22	<0.0001
Interaction (stage with state)			
Euglycemia and poststress	0.37	0.25–0.49	<0.0001
Hypoglycemia and poststress	–0.08	–0.23–0.07	0.28
Presence of diabetes mellitus (yes vs no)	0.11	0.01–0.21	0.035
Interaction (stage with diabetes mellitus)			
Euglycemia and diabetes mellitus present	0.04	–0.05–0.14	0.36
Hypoglycemia and diabetes mellitus present	0.03	–0.05–0.12	0.46
Interaction (state with diabetes mellitus)			
Poststress and diabetes mellitus present	–0.27	–0.45–0.08	0.005
Interaction (stage with state with diabetes mellitus)			
Euglycemia, poststress, diabetes mellitus present	0.07	–0.08–0.23	0.35
Hypoglycemia, poststress, diabetes mellitus present	–0.05	–0.25–0.14	0.58

CI indicates confidence interval.

Table 6. Myocardial Blood Flow at Rest and After Dipyridamole-Induced Stress at Baseline and During Hyperinsulinemic Euglycemia and Hyperinsulinemic Hypoglycemia

Stages	State			
	DM		C	
	Rest MBF, dB ² /s	Peak MBF, dB ² /s	Rest MBF, dB ² /s	Peak MBF, dB ² /s
Baseline	20.4 (19.0–21.7)	40.9 (37.5–44.4)	17.7 (16.1–19.3)	44.2 (40.0–48.5)
HE	22.2 (20.6–23.8)	53.5 (49.3–57.6)	19.3 (17.3–21.2)	57.4 (52.4–62.4)
HH	24.5 (23.0–26.0)	44.4 (41.5–47.3)	22.2 (20.4–24.0)	46.7 (43.2–50.2)

Stage is baseline, hyperinsulinemic euglycemia (HE), or hyperinsulinemic hypoglycemia (HH). State is resting myocardial blood flow or during dipyridamole stress (myocardial blood flow MBF). Values are means (95% confidence intervals).

the mixed model, the B coefficient, was 3.16 (95% CI, 2.47 to 3.85) with baseline used as a reference point. In group C, MBFR increased during HE by 0.57 U (2.6±0.3 to 3.1±0.5; *P*<0.0001) (22%) above baseline and decreased during HH by 0.36 U (2.6±0.3 to 2.2±0.2; *P*<0.0001) (14%) below baseline values. Importantly, at baseline, MBFR was significantly lower in group DM compared with group C by 0.37 U (2.6±0.3 versus 2.1±0.3; *P*=0.0002). In group DM during HE (2.5±0.5), a similar effect on MBFR was observed compared with group C; however, there was a suggestion that

in group DM, there was a smaller decrease in MBFR during HH (1.9±0.4), compared with the decrease in MBFR observed in group C (*P*=0.05). Although there was a highly significant (*P*=0.003) and independent negative effect of age on MBFR, the B coefficient (−0.01) shows that the magnitude of this effect for each year of age was small. Finally, there was no independent effect of systolic blood pressure on MBFR.

Effect of Microvascular Complications on Myocardial Blood Flow Reserve

The mixed-model method was applied to explore whether the presence of microvascular complications in people with DM was predictive of a decreased MBFR. A mixed model was fitted (using data for subjects with DM only) in which MBFR was predicted by stage, the presence of microvascular complications, and a term representing the stage/complications interaction. This mixed model (Table II in the online-only Data Supplement) indicated that the presence of microvascular complications in people with DM was associated with a reduction in MBFR of 0.52 U (B coefficient, −0.52; 95% CI, −0.70 to −0.34; *P*<0.0001). There was no significant interaction of complications with the stage of measurement (ie, baseline, HE, or HH).

Table 7. Mixed-Effect Regression Model Showing the Effect of Measurement Stage (at Baseline, During Hyperinsulinemic Euglycemia, and During Hyperinsulinemic Hypoglycemia), Presence of Diabetes Mellitus, and Stress State (Rest Versus After Dipyridamole-Induced Stress) on Myocardial Blood Flow

Variable	Estimate	95% CI	<i>P</i>
Measurement stage			
Baseline	17.7	16.1–19.3	<0.0001
Euglycemia	19.3	17.3–21.2	<0.0001
Hypoglycemia	22.2	20.4–24.0	<0.0001
State (poststress vs rest)	26.5	23.3–29.7	<0.0001
Interaction (stage with state)			
Euglycemia and poststress	11.6	8.9–14.4	<0.0001
Hypoglycemia and poststress	−2.0	−5.1–1.1	0.20
Presence of diabetes mellitus (yes vs no)	2.6	0.5–4.7	0.015
Interaction (stage with diabetes mellitus)			
Euglycemia and diabetes mellitus present	0.3	−2.1–2.6	0.83
Hypoglycemia and diabetes mellitus present	−0.4	−2.4–1.6	0.71
Interaction (state with diabetes mellitus)			
Poststress and diabetes mellitus present	−6.0	−10.1–−1.8	0.006
Interaction (stage with state with diabetes mellitus)			
Euglycemia, poststress, diabetes mellitus present	−0.9	−4.4–2.6	0.60
Hypoglycemia, poststress, diabetes mellitus present	1.3	−2.7–5.3	0.51

CI indicates confidence interval.

Table 8. Effect of Measurement Stage, Age, Presence of Diabetes Mellitus, and Systolic Blood Pressure on Myocardial Blood Flow Reserve

Variable	B Coefficient	95% CI	<i>P</i>
Intercept	3.16	2.47–3.85	<0.0001
Measurement stage			
Euglycemia vs baseline	0.57	0.38–0.75	<0.0001
Hypoglycemia vs baseline	−0.36	−0.50–−0.23	<0.0001
Age (+1 y)	−0.01	−0.02–−0.00	0.003
Presence of diabetes mellitus	−0.37	−0.55–−0.19	0.0002
Interaction (stage with diabetes mellitus)			
Euglycemia and diabetes mellitus present	−0.14	−0.38–0.09	0.24
Hypoglycemia and diabetes mellitus present	0.17	0.00–0.35	0.05
Systolic blood pressure	−0.00	−0.01–0.00	0.56

CI indicates confidence interval.

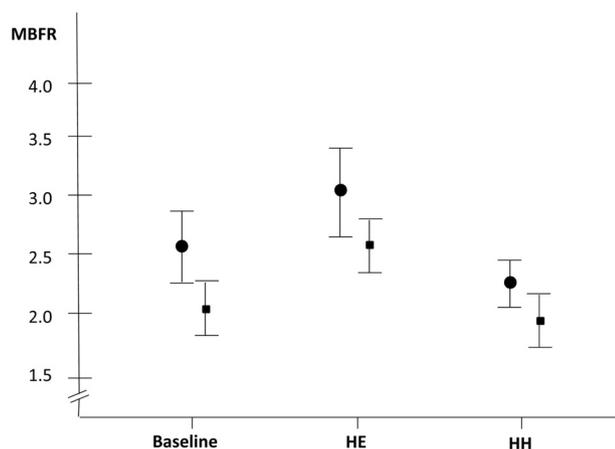


Figure 3. Myocardial blood flow reserve (MBFR) at baseline and during hyperinsulinemic euglycemia (HE) and hyperinsulinemic hypoglycemia (HH). ● Indicates healthy control subjects; ■, type 1 diabetes mellitus patients (means \pm SD).

Changes in Concentrations of Endothelin-1, High-Sensitivity C-Reactive Protein, Epinephrine, and Serum Insulin During the Hyperinsulinemic Clamp

To explore further the explanation for the decrease in MBFR during HH, we measured plasma ET-1 (as a potent vasoconstrictor) and serum high-sensitivity C-reactive protein (as a nonspecific marker of inflammation; Table III in the online-only Data Supplement). Plasma ET-1 concentrations at baseline were 0.19 pg/mL in control subjects and 1.44 pg/mL in the group with DM ($P < 0.0001$). In the group with DM, ET-1 remained markedly increased throughout the whole clamp, and there was a suggestion that ET-1 levels increased toward the end of the HH clamp in control subjects. Serum high-sensitivity C-reactive protein concentrations were not different between the 2 groups and did not change during the study. We also measured serum insulin levels (in view of the hyperinsulinemic clamp) and plasma epinephrine levels (to assess the counterregulatory response to hypoglycemia) in all individuals (Table III in the online-only Data Supplement). Serum insulin concentrations at baseline were 43 ± 23 pmol/L in control subjects and 208.3 ± 207 pmol/L in the group with DM. The plasma epinephrine levels were similar between the 2 groups at all stages.

Discussion

We have shown for the first time that insulin-induced hypoglycemia (HH) decreases the MBFR in both patients with type 1 DM and healthy subjects. We have demonstrated that in healthy controls during HE, insulin induced a marked increase in peak MBF and MBFR, whereas hypoglycemia led to a decline in peak MBF and a decrease in MBFR. Importantly, patients with type 1 DM behaved in a manner similar to the healthy control subjects (Figure 3) in the presence of HE and HH, although the presence of DM was associated with a more marked reduction in MBFR at baseline. The reduction in peak MBF during HH appeared to be due to a decrease in myocardial blood velocity rather than blood volume. We have also shown that the presence of microvascular complications is associated with a decrease in MBFR in patients with type 1 DM. Therefore, the overall effect of

hypoglycemia during HH is to suppress peak MBF, thereby mitigating the vasodilatory action of hyperinsulinemia that occurs during physiological glucose concentrations.

A significant amount of evidence has associated hypoglycemia with increased CVM.^{1–3,6,23,24} In a study including 40 069 patients, fasting hypoglycemia was independently associated with a 3-fold increased risk in CVM after a mean follow-up of 8 years.¹ Pinto et al³ observed that after ST-segment-elevation myocardial infarction, patients with a Thrombolysis in Myocardial Infarction risk score >4 and concomitant hypoglycemia had a >11 -fold increased risk of death within 30 days compared with those with normal glucose levels. Furthermore, another study including patients with established coronary artery disease showed that fasting hypoglycemia was associated with a 2-fold increase in all-cause mortality.²³ A subsequent study observed a 16% increase in the relative risk of CVM in the group receiving insulin therapy on admission to intensive care.⁶ Although this finding was unexplained, there was a 13-fold increased prevalence of severe hypoglycemia in the patients on insulin therapy compared with patients receiving conventional therapy. More recently, another study has demonstrated that fasting hypoglycemia was associated with a 33% increase in 3-year mortality rates in a cohort of 1854 elderly patients after an acute myocardial infarction.²⁴ This negative impact on survival was more pronounced in the subgroups with DM and those requiring coronary artery bypass grafting with a 2- and 3-fold increase in 3-year mortality rates, respectively. This evidence suggests that hypoglycemia is associated with short- and long-term adverse outcomes; however, the pathophysiological mechanisms are still ill defined and may vary.

Over the past few decades, several anecdotal case reports have associated hypoglycemia with episodes of angina and myocardial infarction.^{25–27} Although a direct causal link has not been established, animal studies have demonstrated that hypoglycemia can increase myocardial infarct size by $>40\%$.¹¹ Furthermore, in patients with DM and coexisting coronary artery disease, hypoglycemia was associated with a third of all episodes of angina and corresponding ischemic ECG changes.¹⁰

The endothelium is a highly biologically active single cell layer responsible for the release of several substances, the most important of which are nitric oxide and ET-1.^{28,29} A 21-amino acid peptide, ET-1 is the most potent vasoconstrictor but is identified in humans with a plasma half-life of 4 to 7 minutes.^{30,31} It induces its predominant vasoconstrictive effect by acting on receptors located on vascular smooth muscle cells and fibroblasts. This reduces nitric oxide bioavailability by either decreasing its production (caveolin-1-mediated inhibition of endothelial nitric oxide synthase activity) or increasing its degradation (via formation of oxygen radicals).³² One recent study demonstrated that direct infusion of ET-1 into the coronary sinus of 6 humans decreased the coronary blood flow in a dose-related manner by up to 25%.³³ In addition, ET-1 levels have been shown to be the strongest predictor of no reflow after primary angioplasty.³⁴ Several disease states have been shown to be associated with endothelial dysfunction (an imbalance between the bioavailability of nitric oxide and ET-1). Examples include atherosclerosis, pulmonary arterial hypertension, DM, and myocardial ischemia.^{29,34–36}

Acute hypoglycemia has also been shown to increase ET-1 concentrations.³⁷ Wright and coworkers³⁷ demonstrated that ET-1 levels in patients with type 1 DM rose by almost 70% above baseline values 1 hour after insulin-induced hypoglycemia. In our study, although baseline ET-1 levels were \approx 7-fold higher in the DM group compared with control subjects, the effect of HH versus HE on ET-1 is uncertain. We suggest that further work is needed specifically to elucidate the effects of more prolonged periods of hypoglycemia on ET-1 expression.

It is also plausible that other effects of hypoglycemia may have a deleterious impact on MBFR besides increases in ET-1. Hypoglycemia induces a hypercoagulant state in humans via increased platelet aggregation and changes in plasma concentrations of coagulation factors.^{12,13,38,39} For example, it has been shown that factor VIII was increased 2-fold after 30 minutes of hypoglycemia.¹³ Hypoglycemia may also be responsible for initiating an inflammatory response. In 1 study, hypoglycemia was associated with a 3-fold increase in the neutrophil count and an elevation in neutrophil elastase, a potent proteolytic enzyme.³⁹ Long-QT syndrome is well recognized as being associated with an increased risk of sudden cardiac death.⁴⁰ More worryingly, acute hypoglycemia has been demonstrated to produce prolongation of the corrected QT interval by up to 35% in patients with type 1 DM, with values reaching >550 milliseconds.^{41,42} Interestingly, this change seems to be attributed predominantly to a surge in catecholamine levels and is independent of electrolyte imbalance.⁴³ Finally, prolonged hypoglycemia can have a detrimental effect on cardiac metabolism because of the inability of the heart to use glucose, the preferred substrate instead of fatty acids (during acute myocardial ischemia), after exhaustion of myocardial glycogen reserves.⁴⁴

In light of our findings, it is plausible to suggest that hypoglycemia, by causing a decrease in MBFR, may increase the risk of CVM in susceptible individuals.

Limitations

Although dipyridamole was used 3 times in succession with our study protocol (Figure 1A and 1B), we consider that the repeated use of dipyridamole was unlikely to artifactually influence our results (see the online-only Data Supplement). We did not calculate absolute myocardial perfusion values because all settings and infusion parameters, once optimized at the start of each patient study, were kept constant for the rest of that individual procedure. We deliberately did not randomize the sequence of HE and HH because this allowed individuals to act as their own controls, permitting constant insulin levels, contrast infusion rates, and ultrasound machine settings.

Conclusions

This study has shown that insulin-induced hypoglycemia is associated with a decrease in MBFR in healthy control subjects as a result of a reduction in peak MBF and that patients with type 1 DM behave in a similar manner. In contrast, insulin infusion at normal plasma glucose concentrations is associated with an increase in MBFR caused by an increase in peak MBF. Exploratory analyses suggest that the presence of DM and microvascular complications are inde-

pendently associated with MBFR during HH. We speculate from our results that alterations in MBFR may explain the observed association between hypoglycemia and increased CVM in susceptible individuals.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Hypoglycemia is a common problem that occurs in almost 20% of patients receiving intensive insulin therapy in hospital. Several studies have recently shown that hypoglycemia is associated with an increase in cardiovascular mortality. This association has been demonstrated in people with and without established coronary artery disease. Importantly, patients with acute coronary syndromes appear to have worse short- and long-term outcomes if they experience hypoglycemia in the acute phase of their presentation. The pathophysiological mechanism responsible for this association is not known. This present study examined the effects of hypoglycemia on myocardial blood flow (MBF) reserve using myocardial contrast echocardiography in subjects with type 1 diabetes mellitus and healthy control subjects. With the use of a 1-step hyperinsulinemic clamp technique, insulin-induced hypoglycemia decreased the MBF reserve in both patients with type 1 diabetes mellitus and healthy subjects. Furthermore, during hyperinsulinemic euglycemia, insulin induced a marked increase in peak MBF and MBF reserve, whereas hypoglycemia led to an increase in resting MBF and a decrease in peak MBF, thereby decreasing the MBF reserve overall. This effect was observed in both healthy individuals and patients with type 1 diabetes mellitus. We speculate from our results that alterations in MBF reserve may provide an explanation for the observed association between hypoglycemia and increased cardiovascular mortality in susceptible individuals.

Supplemental Material

Methods

Subjects

Microvascular complications (retinopathy, neuropathy and nephropathy) were defined by the presence of pre- or proliferative diabetic retinopathy, on clinical examination and a vibration perception threshold score of >12 (measured on the Great Hallux using a Bio-thesiometer [Biomedical Instrument, Newbury, Ohio, USA]),¹ and an albumin/creatinine ratio of $>2.5 \text{ mg.mmol}^{-1}.\text{L}^{-1}$ for men and $>3.5 \text{ mg.mmol}^{-1}.\text{L}^{-1}$ for women.² Five patients with type 1 DM and microvascular complications who were taking an angiotensin converting enzyme inhibitor and a statin were instructed not to take their medications 48 hours prior to the study day to rule out any acute effects of medication on myocardial perfusion.³

Hyperinsulinemic Clamps

Two anterograde and one retrograde cannulae were sited after application of a local anaesthetic cream (Ametop gel 4.0% w/w, Smith and Nephew, UK) to minimise discomfort. The anterograde cannulae were inserted into the antecubital fossa on either side. The right anterograde cannula was used for insulin (Actrapid; Novo Nordisk, Copenhagen, Denmark) and 20% dextrose infusions. A retrograde cannula was inserted into the dorsum of the right hand and was kept patent with a slow infusion of 0.9% (w/v) saline to which 1000 units of heparin were added. This hand was placed in a heated box (55-60°C) to obtain arterialized samples.⁴ All studies were performed in a quiet and comfortable room (22-25°C) with the volunteers resting on a couch in a semi-reclined position. Arterialised glucose sampling was performed every 3-5 minutes and the 20% dextrose infusion was adjusted accordingly. Plasma glucose was determined using a glucose oxidase method (YSI 2300 STAT Plus, Yellow Springs, OH, USA).⁵

Volunteers were asked to report any symptoms that could be attributed to hypoglycemia which included general symptoms (dry mouth, headache, and weakness), autonomic symptoms (palpitations, trembling, tingling, sweating and feeling hungry) and neuroglycopenic symptoms (poor concentration, dizziness and blurred vision). All volunteers were provided with meals and observed for 1-hour at the end of which plasma glucose was rechecked before allowing them home.

Plasma glucose measurements

The inter-assay coefficient of variation (CV) was <2% while the calibration of the analyzer was checked at 30-minute intervals with a glucose standard (10 mmol.L⁻¹). Volunteers were not informed of their glucose levels during the study.

Potential additional limitations

Although dipyridamole was used three times in succession with our study protocol (Figure 1a and b), we consider that the repeated use of dipyridamole was unlikely to artefactually influence our results. Dipyridamole has a short half-life of 8-12 minutes and the time period between each dipyridamole infusion in our study was 76 minutes. Furthermore, dipyridamole-induced changes in left ventricular ejection fraction, end-systolic volume, heart rate and diastolic blood pressure have previously been shown to return to baseline after a 60-minute period using a much higher-dose (0.76 mg.kg⁻¹) protocol.⁶

Finally, the effects of administering dipyridamole three times in succession was tested in a healthy individual over the same time-course and there was no change in MBF, MBFR or other hemodynamic parameters. We did not calculate absolute myocardial perfusion values. This was because all settings and infusion parameters,

once optimised at the start of each patient study, were kept constant for the rest of that individual procedure. Furthermore, we achieved homogenous opacification of the left ventricular blood pool and the signal intensity received was consistently between 34-36 dB. Calculation of absolute myocardial blood flows to take into account regional blood flow variations that occur within individuals would have introduced an additional potential source of error.

Explanation of terms used in Tables 2b, 3b and 4b in the main manuscript

Tables 2b, 3b and 4b in the main text of the paper present parameter estimates from mixed effects regression models in which the outcome of interest (respectively: myocardial blood volume, myocardial blood velocity and myocardial blood flow) is predicted by the main effects of

- i. measurement stage (baseline, during hyperinsulinemic euglycemia and during hyperinsulinemic hypoglycemia)
- ii. stress state (at rest and post dipyridamole-induced stress)
- iii. diabetes status (controls vs. patients with diabetes)

and by all of their possible interactions: (i) with (ii); (i) with (iii); (ii) with (iii); and the single three-way interaction (i) with (ii) with (iii). Models were fitted using the MIXED procedure in SAS software version 9.2. Interpretation of the results presented in these Tables is now described.

MEASUREMENT STAGE is the estimated mean value of the outcome for control subjects, in the rest state, observed at each of the three stages (because models were fitted with the intercept suppressed). These values are identical to those given in the

'Rest' sub-column of the C (Controls) column in the corresponding table of means in the main text.

STATE is the estimated effect of the stress treatment on the outcome for control subjects, at the baseline stage.

INTERACTION (STAGE with STATE) estimates the extent to which the effect of the stress treatment in controls at, respectively, the euglycaemic and hypoglycemic stages varies relative to that observed at the baseline stage.

PRESENCE OF DIABETES estimates the difference in the outcome at the baseline stage, in the resting state, between control subjects and those with diabetes.

INTERACTION (STAGE WITH DIABETES) estimates the extent to which the effect of measurement stage (that is, the change in resting values of the outcome at the euglycemic and hypoglycemic stages relative to the value observed at baseline) differs between control subjects and those with diabetes.

INTERACTION (STATE WITH DIABETES) estimates the extent to which the effect of the stress treatment, at the baseline stage, differs between controls and those with diabetes.

INTERACTION (STAGE with STATE with DIABETES) estimates the additional influence on the outcome of the joint presence of all main and two-way interaction effects. This may be illustrated with reference to Table 2b, from which the predicted absolute value of myocardial blood volume under euglycemia, post-stress, in subjects with diabetes is given by:-

20.4 (main effect of euglycemic stage) +

2.7 (main effect of stress) +

0.7 (main effect of diabetes) +

$$\begin{aligned} &0.7 \text{ (euglycemia / stress interaction) +} \\ &-0.9 \text{ (euglycemia / diabetes interaction) +} \\ &-0.4 \text{ (stress / diabetes interaction) = 23.2} \end{aligned}$$

However, the three-way interaction term indicates that the estimated value of the outcome is 0.5 of a unit lower than that which would be predicted on the above basis (though the interaction is not statistically significant). That is, there is an additional effect arising from the joint presence of the euglycemia stage, the stress state and diabetes.

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Table 1.

Pulse rate, systolic blood pressure, diastolic blood pressure and rate pressure product at rest and after dipyridamole-induced stress during the hyperinsulinemic clamp.

Variable	Baseline		Hyperinsulinemic Euglycemia (HE)		Hyperinsulinemic Hypoglycemia (HH)	
	C	DM	C	DM	C	DM
Pulse _r b.min ⁻¹	63±9	68±9	66±8	71±14	75±11	78±15
Pulse _d b.min ⁻¹	90±11	92±14	93±14	91±15	97±10	94±14
SBP _r mmHg	121±14	123±16	124±13	121±15	124±13	120±14
SBP _d mmHg	125±15	127±18	125±11	120±14	121±11	124±14
DBP _r mmHg	79±12	73±12	78±9	71±12	69±12	65±9
DBP _d mmHg	72±9	72±10	71±9	63±11	68±6	62±7
RPP _r b.min ⁻¹ .mmHg	7739± 1533	8394± 1839	8163± 1448	8657± 2114	9454± 1692	9598± 2178
RPP _d b.min ⁻¹ .mmHg	11375± 2399	11705±2399	11533± 1839	10929±2534	11712± 1616	11596±2014

Pulse rate, systolic blood pressure, diastolic blood pressure and rate pressure product are presented as mean±SD.

C=Healthy controls, DM=group with diabetes mellitus, Pulse_r=resting pulse, Pulse_d=post-dipyridamole pulse, SBP_r=resting systolic blood pressure, SBP_d=post-dipyridamole systolic blood pressure, DBP_r=resting diastolic blood pressure, DBP_d=post-dipyridamole diastolic blood pressure, RPP_r= resting rate pressure product, RPP_d= post-dipyridamole rate pressure product.

Table 2:**Effect of measurement stage and presence of microvascular complications on myocardial blood flow reserve (MBFR) in subjects with diabetes.**

variable	B coefficient	95% CI	<i>p</i>
INTERCEPT	2.24	2.15 to 2.34	< 0.0001
MEASUREMENT STAGE:			
euglycaemia vs. baseline	0.47	0.30 to 0.65	< 0.0001
hypoglycaemia vs. baseline	-0.16	-0.30 to -0.02	0.023
PRESENCE OF COMPLICATIONS	-0.52	-0.70 to -0.34	< 0.0001
INTERACTION (STAGE with COMPLICATIONS):			
euglycaemia and complications present	-0.17	-0.50 to 0.16	0.30
hypoglycaemia and complications present	-0.11	-0.36 to 0.15	0.40

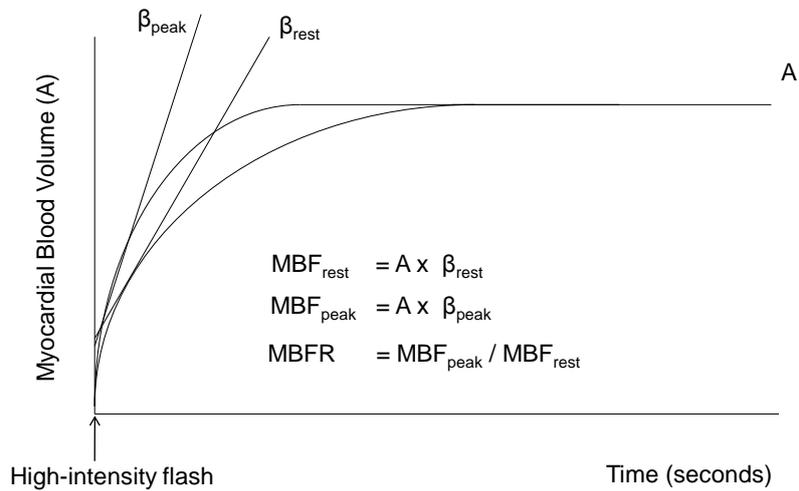
Table 3.**Endothelin-1, hs-CRP, epinephrine, and insulin concentrations during the hyperinsulinemic clamp.**

Variable	Baseline		Hyperinsulinemic Euglycemia				Hyperinsulinemic Hypoglycemia		
	Group	0 mins	30 mins	60 mins	90 mins	120 mins	150 mins	180 mins	
ET-1 pg.ml ⁻¹	C	0.19(1.0)	0.0(0.7)	0.0(0.8)	0.40(0.9)	0.2(0.7)	0.44(0.8)	0.52(0.8)	
	DM	1.44(0.5)	1.30(0.5)	1.32(0.7)	1.49(0.7)	1.44(0.8)	1.45(0.8)	1.49(0.7)	
hs-CRP mg.L ⁻¹	C	0.64(0.7)	0.60(0.6)	0.62(0.6)	0.61(0.6)	0.57(0.5)	0.61(0.5)	0.51(0.4)	
	DM	1.17(1.9)	0.82(1.9)	1.10(1.8)	0.93±1.9	1.10(1.8)	0.9(1.7)	0.82(1.8)	
Epinephrine pg.ml ⁻¹	C	76.3±77	77.7±79	91.6±108	96.3±56.7	106.7±84.4	347±199	405.7±310	
	DM	62.2±44.2	98.3±124.4	86.8±81.9	114.7±93.4	150.7±172	294.8±260.4	350.5±260.4	
Insulin pmol.L ⁻¹	C	43±23	741±180	728±222	763±160	643±167	683±133	714±160	
	DM	208.3±207	736.0±227.4	722.6±232.9	663.3±206.7	685.3±209.3	652.9±274.1	647.3±279.7	

Values are presented as median(interquartile range) for ET-1 and hs-CRP. Values are presented as mean±SD for epinephrine and insulin.

C=healthy controls, DM=group with diabetes mellitus, ET-1=endothelin-1, hs-CRP= high sensitivity CRP, Epinephrine=epinephrine.

Figure 1. Measurement of Myocardial Blood Flow Reserve using Flash Impulse Imaging.



A=myocardial blood volume, β_{rest} =myocardial blood velocity at rest, β_{peak} =myocardial blood velocity at peak, MBF_{rest} =myocardial blood flow at rest, MBF_{peak} =myocardial blood flow at peak, MBFR=myocardial blood flow reserve.