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The Deleterious Effects of Hyperglycemia on Platelet Function in Diabetic Patients With Acute Coronary Syndromes

Mediation by Superoxide Production, Resolution With Intensive Insulin Administration

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Objectives This study sought to assess the determinants of platelet nitric oxide (NO) responsiveness in diabetic patients admitted with acute coronary syndromes (ACS) and the short-term effects of aggressive glycemic control on these factors.

Background Hyperglycemia is an independent risk factor for mortality in both diabetic patients and nondiabetic patients with ACS. The mechanism(s) underlying this observation and potential benefit from its correction remain uncertain. Although a reduction in NO bioavailability has been proposed, this remains untested in the ACS setting.

Methods A total of 76 diabetic patients with ACS were studied. Putative correlations between admission blood sugar level (BSL), inhibition of platelet aggregation by the NO donor sodium nitroprusside (SNP), and superoxide (O_2^-) were assessed. Hyperglycemic patients ($n = 60$) were randomized to acute glycemic control with intravenous versus subcutaneous insulin, and changes in the aforementioned parameters were compared. Plasma levels of the endogenous inhibitor of NO synthase asymmetric dimethylarginine (ADMA) were also monitored.

Results There was an inverse correlation between admission BSL and both platelet SNP response ($p = 0.007$) and ADMA levels ($p = 0.045$), and a positive correlation with O_2^- generation ($p < 0.001$). Intravenous insulin infusion resulted in a greater reduction ($p < 0.001$) in BSL, differentially improved platelet responsiveness to SNP ($p = 0.049$), and decreased O_2^- ($p < 0.001$) and ADMA levels ($p = 0.049$).

Conclusions A component of platelet dysfunction in diabetic patients with ACS is impaired responsiveness to the antiaggregatory effects of NO, probably reflecting increased NO clearance by O_2^-. This phenomenon is reversed by acute aggressive glycemic control. These findings provide a further rationale for use of insulin therapy in acute myocardial infarction and suggest its extension to ACS patients. (J Am Coll Cardiol 2007;49:304–10) © 2007 by the American College of Cardiology Foundation

Mortality rates are significantly higher in diabetic patients who have an acute myocardial infarction (AMI) compared with nondiabetic patients (1,2), particularly in association with hyperglycemia (3,4). The mechanism(s) underlying these adverse outcomes remain uncertain.

One possible contributing factor is a reduction in nitric oxide (NO) bioavailability. In both animal (5) and human (6) diabetic studies, high glucose levels have been associated with a reduction in endothelium-dependent vasodilation. This may relate to an increase in oxidative stress and subsequent scavenging of NO (7,8) or an increase in concentration of the endogenous NO synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA) (9). These anomalies are reversed in part by insulin therapy.

Glucose levels in diabetic patients not only affect vascular reactivity, but also impact on platelet aggregability. Platelet-dependent thrombus formation is increased in diabetic patients with long-term poor glycemic control (10); conversely, decreases in platelet aggregability have been reported in association with improved diabetic control (11) and blood thrombogenicity (12). Moreover, an acute glucose load is also associated with an increase in platelet activation in type II diabetic patients (13), and a direct correlation exists between enhanced platelet activation and

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plasma glucose level after an AMI (14). The pro-aggregant effects of hyperglycemia are also in part reversed by insulin. The platelet–collagen interaction is inhibited by insulin (15), and in low doses these effects are mediated through incremental NO release (16); reversal of insulin's anti-aggregatory action occurs in the presence of NOS inhibition (17). Although NO is a physiologically important inhibitor of platelet aggregation, its anti-aggregatory effects are impaired in stable and unstable angina pectoris (18,19). The possible interactions of glucose with this pathway, however, remain uncertain.

Controversy continues to exist concerning the benefits of aggressive control of hyperglycemia in patients with acute coronary syndromes (ACS): even the results of studies in AMI (20,21) are less than completely conclusive regarding short-term beneficial effects. Furthermore, previously performed clinical studies in AMI shed little light on mechanism(s) of putative clinical benefit. The current study was therefore designed to examine the determinants of platelet responsiveness to NO in diabetic patients admitted with ACS and their possible modulation with short-term aggressive glycemic control. We tested the null hypotheses that: 1) admission blood sugar level (BSL), and 2) correction of hyperglycemia were independent of platelet NO responsiveness and its principal biochemical correlates (18), O_2^- generation and platelet cyclic guanosine-3′5′-monophosphate (cGMP) formation. We also examined the possible impact of both admission BSL and correction of hyperglycemia on plasma ADMA levels.

Methods

Seventy-six consecutive diabetic patients were enrolled in the study. Patients were eligible for the study if they were admitted to the coronary care unit with a diagnosis of ACS (either unstable angina pectoris or non–Q-wave AMI) with either known diabetes mellitus or an admission BSL >11.1 mmol/l. Criteria for exclusion were: 1) severe renal insufficiency (serum creatinine >0.30 mmol/l), 2) concomitant therapy with potent inhibitors of adenosine diphosphate (ADP)-induced platelet aggregation (clopidogrel and/or glycoprotein IIb/IIIa inhibitors) and/or therapy with perhexiline, which potentiates platelet NO responsiveness and lowers BSL (22).

Randomization regarding strategies for control of hyperglycemia was restricted to patients with admission BSL between 10 and 35 mmol/l. Patients randomized to aggressive control had intravenous (IV) Actrapid (Novo Nordisk Pharmaceuticals Pty Ltd., Copenhagen, Denmark) insulin infused according to the DIGAMI (Diabetes and Insulin-Glucose in Acute Myocardial Infarction) protocol (20) without incremental glucose supplementation for the first 12 h after randomization; control patients were treated via a subcutaneous (SC) protocol, whereas no insulin was administered if a glucose reading was registered between 0 and 10 mmol/l. With each subsequent 5-mmol/l increase in glucose, 4-U increments of insulin were administered. This was delivered thrice daily with meals, with a maximum of 12 U of insulin given at one time.

Abbreviations and Acronyms

ACS = acute coronary syndromes
ADMA = asymmetric dimethylarginine
AMI = acute myocardial infarction
BSL = blood sugar level
NO = nitric oxide
PRP = platelet-rich plasma
SC = subcutaneous

Other anti-ischemic therapy was initiated at the discretion of the treating cardiologist. The protocol was approved by the institutional Ethics of Human Research Committee of the local institution, and informed consent was obtained before study entry. Apart from monitoring of parameters specifically associated with the study, all patients underwent continuous electrocardiogram monitoring using a GE-Marquette (Milwaukee, Wisconsin) 12-lead system for the entire duration of the study to record episodes of significant ST-segment depression. The occurrence of AMI was defined on the basis of peak creatine kinase (CK) values of 3 times the upper limit of normal. The CK values were measured every 8 h for the first 24 h after admission and assayed via end-product absorption at 340 nm (Olympus auto-analyzer, Kobe, Japan).

Platelet studies. Blood sampling for platelet, O_2^-, and cGMP studies was performed at entry, and in randomized patients was repeated after 12 h. Blood obtained from the antecubital vein was transferred slowly to plastic screw-top tubes containing 1:10 volume of citric acid–sodium anticoagulant (2 parts of 0.1 mol/l citric acid to 3 parts 0.1 mol/l trisodium citrate, pH 5). For platelet-rich plasma (PRP) studies, blood was centrifuged at 250 *g* for 10 min at room temperature.

Platelet studies in whole blood (n = 76) and PRP (n = 36) were performed as previously described (18,19) using a dual-channel impedance lumi-aggregometer (model 560CA, Chrono-Log, Haverstown, Pennsylvania). Data were collected via a Chrono-Log model 810CA Aggro/link computer interface. To initiate aggregation experiments, ADP (1 μmol/l) was added at the end of the incubation period. Aggregation was monitored continually for 7 min and maximal responses recorded for electrical impedance, measured in ohms. Inhibition of ADP-induced aggregation by the NO donor sodium nitroprusside (SNP 10 μmol/l), added 1 min before ADP, was expressed as a percentage of response to ADP alone. The SNP was used in a previously validated concentration (18) as an index of platelet responsiveness to NO because unlike organic nitrates, it is a direct NO donor, and does not show cross-tolerance to nitroglycerin (23).

Chemiluminescence assay of O_2^-. Detection of O_2^- in whole blood was performed using a chemiluminescence technique (24), with lucigenin as a probe (n = 63). A low concentration of lucigenin (12.5 μmol/l) was used to minimize redox cycling. Chemiluminescence was monitored using a photoluminometer component of the dual channel

lumi-aggregometer (model 560CA, Chrono-Log, Haverstown, Pennsylvania). Data were collected via a Chrono-log model 810CA Aggro/link computer interface. After 1 min, the intensity of lucigenin-derived chemiluminescence (LDCL) was measured; results are expressed in millivolts.

Platelet cGMP assay. Intraplatelet cGMP was measured as previously described (25) from platelets harvested from PRP (n = 36). Briefly, PRP was diluted 2-fold by 0.9% saline and prewarmed at 37°C. If the final volume was >1 ml, subsequent concentrations of SNP were appropriately adjusted. One minute before platelet harvest, 10 μmol/l (or equivalent) of SNP or normal saline control was added. The PRP was then filtered through GF/C glass microfiber filters (Whatman P/L, Brentford, United Kingdom) held by plastic filter holders Swinnex-25 (Millipore Corp., Billerica, Massachusetts). Filters with absorbed platelets were rinsed with 0.9% saline and placed into 0.5 ml of 4 mmol/l ethylene diamine tetraacetic acid used to prevent cGMP decay. Tubes were then placed in a boiling water bath for 5 min. After this, samples were removed and centrifuged at 3,000 *g* for 10 min. The cGMP concentration in the supernatant was then assayed using a cGMP [^{125}I] radioimmunoassay kit (Amersham, Buckinghamshire, United Kingdom). Results were expressed as an SNP-mediated percentage increase of cGMP levels.

ADMA assay. The ADMA (N^G,N^G-dimethyl-L-arginine) was extracted from plasma by strong cation exchange, derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Fluor, Waters, Australia) to produce a highly stable derivative and analyzed by high-performance liquid chromatography (26). Recovery of ADMA from plasma was >92%, with intraassay and interassay variability of 2% and 6%, respectively.

Other parameters examined. C-reactive protein (CRP) levels (n = 61) were determined with a high-sensitivity CRP assay (Beckman Immage Immunochemistry System, Fullerton, California). The non-esterified fatty acid (NEFA) test kit (Wako Pure Chemical Industries Ltd., Osaka, Japan) used an in vitro enzymatic colorimetric method for the quantitation of NEFA or free fatty acids in serum (n = 61). Plasma glucose and creatine kinase values were derived via spectrophotometry. Glycated hemoglobin was measured by high-performance liquid chromatography (Variant Haemoglobin A_{1C} program, Bio-Rad Laboratories, Hercules, California), and the insulin levels (n = 50) were measured by an immunoassay kit (AxSym insulin, Abbott Laboratories, Abbott Park, Illinois) and expressed in μM/ml.

Statistical analysis and sample size calculation. Data were evaluated by the D'Agostino normality test for Gaussian/non-Gaussian distribution: non-Gaussian distributions were log transformed. Admission BSL values were correlated with platelet function and biochemistry using linear regression with 95% confidence intervals (Excel 2003, Microsoft, Seattle, Washington). We assessed the determinants of platelet NO response and blood O_2^- content by a stepwise multiple regression analysis (multivariate analysis).

For the intervention component of the study (intravenous versus subcutaneous insulin therapy in hyperglycemic patients), sample size calculation was based on preliminary data regarding SNP responsiveness in diabetic patients with BSL ≥ 10 mmol/l. On the assumption that in such patients baseline SNP responses were normally distributed and had values at presentation of approximately 35 ± 20%, a randomized study with 30 patients in each group would have approximately 76% power to detect a 20% (1 SD) relative change in SNP response at $p = 0.05$; power to detect 15% relative change was approximately 51%.

To assess the relative effects of IV versus SC insulin therapy, a 2-way analysis of variance with repeated measures was used. Statistical significance was assessed as $p < 0.05$ and is documented in relation to the interaction between time and therapy unless otherwise stated. All values are expressed as mean ± SEM, with non-parametric data expressed as median values.

Results

Characteristics of all 76 patients studied are shown in Table 1. Admission BSL was inversely correlated with platelet responsiveness to SNP in whole blood ($r = -0.31$, $p = 0.007$) (Fig. 1) but not in PRP. A significant correlation also existed between BSL and log LDCL ($r = 0.43$, $p < 0.001$) (Fig. 2), but not between BSL and SNP-mediated cGMP activity. The majority (n = 60) of patients had a BSL >10 mmol/l. The oral hypoglycemic agents used included sulfonylureas (n = 34), biguanides (n = 34), and thiazolidinediones (n = 1). Irrespective of prior therapy, all patients subsequently were treated with aspirin, heparin, organic nitrates (usually as intravenously infused nitroglycerin), and oral anti-ischemic agents (beta-adrenoceptor or non-dihydropyndine calcium antagonists).

Table 1 Admission Baseline Characteristics (n = 76)

Age (yrs)	66 ± 1.4
Gender (M:F)	49:27
Acute myocardial infarction (%)	29
Blood sugar level (mmol/l)	14.3 ± 0.6 (range 5.1–31.7)
Hemoglobin A_{1C}(%)	8.4 ± 0.2
Risk factors	
Hypertension (%)	54
Smoking (%)	17
Cholesterol (mmol/l)	4.6 ± 0.1
Diabetic medications (%)	
None	32
Oral hypoglycemic agents	54
Insulin	14
Concomitant medications on admission (%)	
Aspirin	50
Angiotensin-converting enzyme inhibitor	40
Statin	36

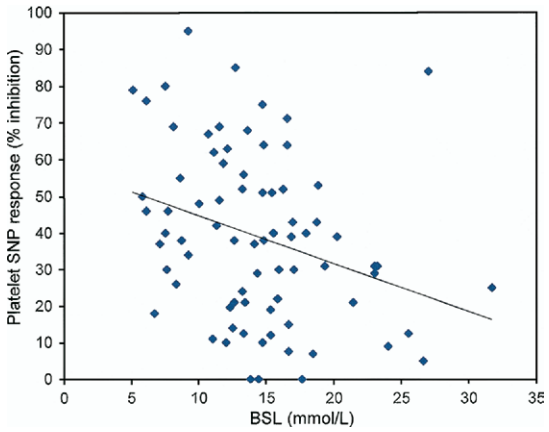

Figure 1 Relationship Between Platelet SNP Response and Admission BSL ($r = -0.31$, $p = 0.007$)

BSL = blood sugar level; SNP = sodium nitroprusside.

Admission BSL also was positively correlated with log CRP ($r = 0.28$, $p = 0.03$), log neutrophil count ($r = 0.36$, $p < 0.001$), and log peak CK ($r = 0.37$, $p < 0.001$). An inverse correlation existed between BSL and ADMA ($r = -0.27$, $p = 0.045$).

Parameters included in the stepwise multivariate analysis of determinants of SNP response and O_2^- content were: age, gender, BSL, statin therapy, insulin therapy, angiotensin-converting enzyme inhibitor therapy, and CK elevation. Admission BSL was a significant determinant of O_2^- generation ($p = 0.005$) and an inverse determinant of SNP response ($p = 0.02$), with increasing age also a significant inverse determinant ($p = 0.03$) of O_2^- generation.

The baseline characteristics of the patients randomized to 12 h of IV or SC insulin are shown in Table 2. The 2 groups were well matched regarding demographics and pharmacotherapy. As expected, IV insulin therapy was significantly more effective in lowering BSL than SC therapy: BSL decreased from 16.9 ± 0.9 mmol/l to 7.9 ± 0.6 mmol/l in the IV group and 15.4 ± 0.8 mmol/l to 10.5 ± 0.8 mmol/l in the SC group ($p < 0.001$). Interestingly, insulin levels decreased to a similar extent between the 2 groups ($p = 0.38$), from a median of 22.7 to 17 μmol/ml in the IV group and 23.5 to 15 μmol/ml in the SC group.

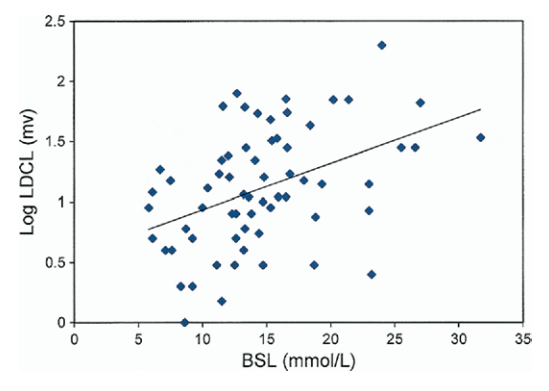

Figure 2 Relationship Between Log Lucigenin-Derived LDCL and Admission BSL ($r = 0.43$, $p < 0.001$)

BSL = blood sugar level; LDCL = lucigenin-derived chemiluminescence.

Table 2 Characteristics of Patients Randomized to Variable Strategies of Correction of Hyperglycemia: Intravenous versus Subcutaneous Insulin Therapy

	Intravenous (n = 30)	Subcutaneous (n = 30)
Age (yrs)	64 ± 14	68 ± 12
Gender (M:F)	20:10	17:13
Acute myocardial infarction (%)	38	32
Body mass index (kg/m^2)	27.6 ± 0.8	28.1 ± 0.9
Blood sugar level (mmol/l)	16.9 ± 0.9	15.4 ± 0.8
Hemoglobin A_{1c} (%)	9 ± 2	8.5 ± 1.6
Risk factors		
Hypertension (%)	60	50
Smoking (%)	20	23
Cholesterol (mmol/l)	4.8 ± 0.22	4.4 ± 0.19
Diabetic treatment (%)		
Diet	13	27
Oral hypoglycemic agents	66	50
Insulin	21	23
Other pharmacotherapy (%)		
Aspirin	47	47
Angiotensin-converting enzyme inhibitor	47	27
Statin	33	33

There were no significant differences between groups.

Whole-blood platelet SNP response significantly improved in the IV group compared with the SC group ($p = 0.049$) (Fig. 3A), whereas O_2^- generation was significantly reduced in the IV group compared with the SC group ($p < 0.001$) (Fig. 3B). These changes occurred in the absence of any significant fluctuation in cGMP generation, in aggregation responses to ADP, or any change in SNP response in PRP. Changes in plasma ADMA levels over the first 12 h of hypoglycemic therapy are shown in Figure 3C. There was a differential reduction in ADMA levels in the IV insulin group compared with the SC insulin group ($p = 0.049$).

Regarding other biochemical data, there was no significant differential effect of treatment on NEFA levels in patients randomized to receive IV insulin. The CRP values increased progressively with time ($p < 0.001$) without a differential effect according to treatment strategy (median CRP increases, 0.4 and 5.0 mg/l for IV insulin and SC insulin, respectively).

Discussion

The effects of clinical strategies including insulin administration on outcome in patients with acute myocardial ischemia are increasingly controversial. One-year

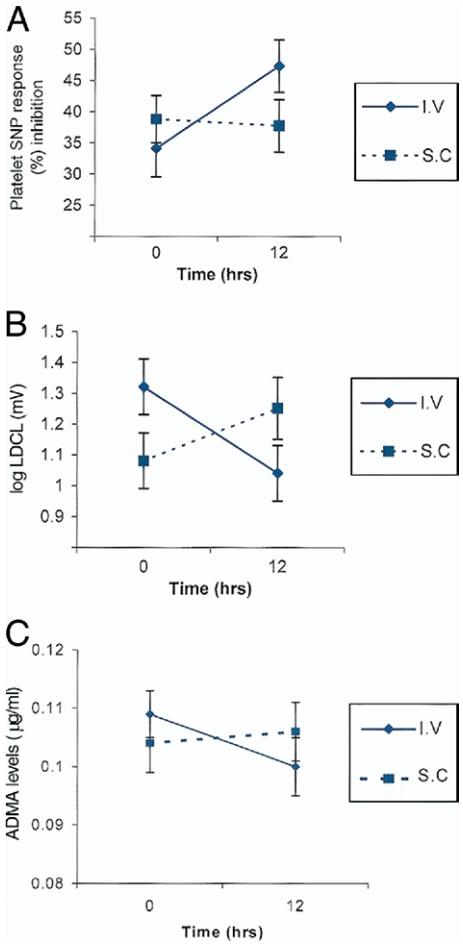

Figure 3 **Differential Effects of IV and SC Insulin Therapy on Changes Over 12 h**

(A) Platelet sodium nitroprusside (SNP) response ($p = 0.049$), **(B)** superoxide generation ($p < 0.001$), and **(C)** asymmetric dimethylarginine (ADMA) levels ($p = 0.049$). IV = intravenous; LDLC = lucigenin-derived chemiluminescence; SC = subcutaneous.

follow-up in the DIGAMI-1 study (20) showed that in patients with evolving AMI, aggressive glycemic control initiated with an IV insulin infusion significantly reduced mortality. Maintenance of normoglycemia using insulin infusion also has been shown to reduce mortality in intensive care patients (27), suggesting beneficial effects in circumstances of increased oxidative stress. On the other hand, the results of the DIGAMI-2 study provide no additional data to confirm these observations (21). Furthermore, the recently published results of the CREATE-ECLA (Clinical Trial of Reviparin and Metabolic Modulation in Acute Myocardial Infarction/Estudios Cardiologicas Latin American Study Group) (28) indicate that in patients with evolving ST-segment elevation, AMI treatment with a combination of glucose, insulin, and potassium does not alter short-term mortality rates. The aforementioned important studies therefore raise 2 critical additional issues. First, does the putative therapeutic impact of "metabolic" therapies such as insulin infusion primarily result from correction of hyperglycemia (and this will be greatest in patients with the poorest glycemic control), and second, what is the mechanism(s) underlying this effect?

This study is the first to evaluate NO responsiveness at the platelet level in diabetic patients presenting with ACS. These results provide a basis for previous observations of adverse outcomes associated with hyperglycemia, showing that admission BSL in this cohort is directly correlated with O_2^- generation and inversely correlated with platelet NO responsiveness. Moreover, rapid correction of hyperglycemia by IV insulin infusion over 12 h increases platelet responsiveness to NO while decreasing O_2^- generation.

Glucose-stimulated increases in activity of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase activity, the major source of O_2^- release, have been shown in both endothelial cells and neutrophils (29,30); the difference between the whole-blood results and those with PRP may be related to more intense O_2^- generation in whole blood from neutrophil-derived NAD(P)H oxidase.

At baseline, ADMA levels were inversely correlated with BSL. Consistent with this observation, Paiva et al. (31) found that ADMA levels in diabetic patients were inversely correlated with hemoglobin A_{1c}. The inverse correlation with BSL may relate to enhanced renal excretion. Glomerular filtration rate is increased in early diabetic kidney disease and inversely correlates with ADMA levels (31). Nevertheless, the finding that aggressive correction of hyperglycemia differentially and rapidly lowered ADMA levels is consistent with the current understanding of the mechanisms controlling ADMA kinetics: ADMA is metabolized by the enzyme dimethylarginine dimethylaminohydrolase, which is redox sensitive. In vitro studies have shown that dimethylarginine dimethylaminohydrolase activity is significantly impaired by in vitro high glucose levels, with subsequent increases in ADMA levels (9). Furthermore, ADMA levels correlated closely with LDCL. Available data suggest that a reduction in plasma ADMA concentration on the order of 10%, as observed in the current study, is likely to be associated with improved NO synthase activity (32) and, if sustained, with improved outcomes (33).

The study has a number of limitations. First, vascular and coronary endothelial function were not measured directly. Second, the study's findings that increased whole blood O_2^- correlated with platelet hyporesponsiveness to NO and that rapid correction of hyperglycemia both decreased O_2^- and restored platelet responsiveness to NO do not prove a cause-and-effect relationship. Indeed, the correlation between baseline BSL and platelet SNP response was only moderate, suggesting that other factors also modulate SNP responses. Furthermore, the results of this study are not necessarily relevant either to patients with ST-elevation

myocardial infarction (and thus to the majority of patients in DIGAMI-1 [20]) or to nondiabetic patients treated with glucose, insulin, and potassium regimens (27). Although we recently have shown that platelet hyporesponsiveness to NO is an independent predictor of incremental long-term cardiac morbidity and mortality (34), the current study did not address long-term outcomes, nor was it sized to detect differences in short-term event rates. It has also been shown recently (35) that high glucose concentrations decrease platelet NO production and diminish the anti-aggregatory effects of aspirin. Furthermore, the effects of insulin administration include activation of platelet NO synthase (36). It is possible that the observed effects of a high BSL might have been modulated to some extent by these processes, although SNP responsiveness is, in theory, independent of endogenous NO production.

The major findings of the current study also provide a physiological and biochemical rationale for urgent reversal of hyperglycemia in diabetic patients admitted with ACS. In doing so, the results suggest that the benefits of insulin therapy in ischemic diabetic patients may be largely independent of changes in myocardial metabolism; thus it would be expected that patients at risk for ongoing ischemia particularly would benefit from such therapy.

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