Human Leukocyte/Endothelial Cell Interactions and Mitochondrial Dysfunction in Type 2 Diabetic Patients and Their Association With Silent Myocardial Ischemia

ANTONIO HERNANDEZ-MIJARES, MD, PHD[1,2,3,4]
MILAGROS ROCHA, PHD[1,3,4]
SUSANA ROVIRA-LLOPIS, PHD[1,3,4]
CELIA BAÑULS, PHD[1,3,4]
LORENA BELLOD, PHD[1,3,4]
CARMEN DE PABLO, PHD[5]
ANGELES ALVAREZ, PHD[5,6]
ILDEFONSO ROLDAN-TORRES, MD[7]
EVA SOLA-IZQUIERDO, MD, PHD[1]
VICTOR M. VICTOR, PHD[1,3,4,5,8]

OBJECTIVE—Diabetes is associated with oxidative stress and increased mortality, but a possible correlation between leukocyte-endothelium interactions, oxidative stress, and silent myocardial ischemia (SMI) is yet to be confirmed.

RESEARCH DESIGN AND METHODS—Mitochondrial dysfunction and interactions between leukocytes and human umbilical vein endothelial cells were evaluated in 200 type 2 diabetic patients (25 with SMI) and 60 body composition– and age-matched control subjects. A possible correlation between these parameters and the onset of SMI was explored, and anthropometric and metabolic parameters were also analyzed.

RESULTS—Waist, levels of triglycerides, proinflammatory cytokines (interleukin-6 and tumor necrosis factor-α), HbA_{1c}, high-sensitivity C-reactive protein (hs-CRP), glucose, and insulin, and homeostasis model assessment of insulin resistance were higher in diabetic patients than in control subjects. However, no statistical differences in hs-CRP and insulin levels were detected when the data were adjusted for waist. None of these parameters varied between SMI and non-SMI patients. Mitochondrial function was impaired and leukocyte-endothelium interactions were more frequent among diabetic patients, which was evident in the lower mitochondrial O_2 consumption, membrane potential, polymorphonuclear cell rolling velocity, and GSH/GSSG ratio, and in the higher mitochondrial reactive oxygen species production and rolling flux, adhesion, and vascular cell adhesion molecule-1 (VCAM-1) and E-selectin molecules observed in these subjects. Moreover, these differences correlated with SMI. Statistical differences were maintained after adjusting the data for BMI and waist, with the exception of VCAM-1 levels when adjusted for waist.

CONCLUSIONS—Oxidative stress, mitochondrial dysfunction, and endothelium-inducing leukocyte-endothelium interactions are features of type 2 diabetes and correlate with SMI.

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From the [1]Service of Endocrinology, University Hospital Doctor Peset, Valencia, Spain; the [2]Department of Medicine, University of Valencia, Valencia, Spain; the [3]Foundation for the Promotion of Healthcare and Biomedical Research in the Valencian Community (FISABIO), Valencia, Spain; the [4]INCLIVA Foundation, Valencia, Spain; the [5]Department of Pharmacology and CIBER CB06/04/0071 Research Group, CIBER Hepatic and Digestive Diseases, University of Valencia, Valencia, Spain; the [6]General Foundation of the University of Valencia, Valencia, Spain; the [7]Cardiology Service, University Hospital Doctor Peset, Valencia, Spain; and the [8]Department of Physiology, University of Valencia, Valencia, Spain.
Corresponding authors: Antonio Hernandez-Mijares, hernandez_antmij@gva.es, and Victor M. Victor, victor.victor@uv.es.
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A.H.-M. and M.R. contributed equally to this study.
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A diagnosis of diabetes worsens the prognosis of cardiovascular diseases (CVDs) to the extent that postinfarction mortality among these patients is double that of nondiabetic patients. In this sense, coronary artery disease is the major cause of morbidity and mortality in patients with diabetes, in which circumstances the prognosis is unfavorable (1), and silent myocardial ischemia (SMI) is thought to be a predictor of subclinical CVD (2).

Evidence points to the involvement of impaired mitochondrial energetics in cardiac dysfunction in obesity and diabetes (3,4). Multiple abnormalities associated with diabetes, such as hyperglycemia, hyperlipidemia, and insulin resistance, are thought to contribute to adverse outcomes in diabetes after myocardial ischemia (5).

The pathogenesis of mitochondrial dysfunction in obesity or diabetes-related heart disease is multifactorial and includes oxidative damage (4,6). In experimental models of obesity and diabetes, insulin resistance is commonly detected in tissues such as those in the heart (7).

Oxidative stress is implicated in the etiology of the insulin resistance associated with type 2 diabetes (8). Numerous studies of nonspecific antioxidant treatments provide indirect evidence of a link between oxidative stress and insulin resistance (9). Mitochondria are the main source of reactive oxygen species (ROS), which are important markers of mitochondrial function (10). Several studies have shown that the rate of mitochondrial H_2O_2 emission is significantly greater when basal respiration is supported by fatty acids (11), raising the possibility that mitochondrial H_2O_2 emission is a primary factor in the etiology of insulin resistance.

Peripheral polymorphonuclear leukocytes (PMNs) are one of the main inflammatory cell types. Once activated, PMNs release ROS, which contributes to oxidative stress and the inflammation and

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endothelial damage that follow. Oxidative stress occurs in the PMNs of insulin-resistant patients and is related to an impairment of mitochondrial function (8,12).

The onset of many CVDs is heralded by the movement and accumulation of leukocytes in the vessel wall. These processes are mediated by an interaction between the adhesion molecules expressed on white blood and/or endothelial cells. Different cellular adhesion molecules have been implicated in atherogenesis, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and selectins (13).

The current study was performed to throw light on the relationship between mitochondrial dysfunction, leukocyte activation, and the increase of leukocyte-endothelium interactions observed in type 2 diabetes and to explore a possible correlation between these factors and different clinical features, including SMI. We detected an increase in ROS production and PMN rolling flux, adhesion, and adhesion molecules, and a decrease in GSH levels, GSH/GSSG ratio, mitochondrial mass, membrane potential, and PMN rolling velocity, all of which are signs of an impairment of mitochondrial function and enhanced leukocyte-endothelium interaction. Our results endorse the hypothesis of an association between type 2 diabetes, mitochondrial dysfunction, and leukocyte-endothelium interaction. Additionally, they point to a correlation between these parameters and SMI, in the presence of which they were more affected.

RESEARCH DESIGN AND METHODS

Subjects

Our study population consisted of 200 patients (25 with SMI, 56.1 ± 2.0 years of age; those without SMI, 55.3 ± 1.7 years of age) consecutively attending the Department of Endocrinology's outpatient clinic of the University Hospital Dr. Peset and who were diagnosed with type 2 diabetes according to the criteria of the American Diabetes Association. Diagnosis was confirmed when a patient fulfilled one or more of the following criteria: *1*) levels of fasting serum glucose ≥7.0 mmol/L (126 mg/dL) or random serum glucose ≥11.1 mmol/L (200 mg/dL) on at least two occasions; 2) HbA_{1c} ≥6.5%; or *3*) antidiabetic medication. Patients taking allopurinol or any oral antioxidant supplements were excluded from the study.

Sixty volunteer control subjects (54.2 ± 1.8 years of age) were enrolled in the study and in any case they did not have the following criteria: *1*) levels of fasting serum glucose ≥7.0 mmol/L (126 mg/dL) or random serum glucose ≥11.1 mmol/L (200 mg/dL) on at least two occasions; 2) HbA_{1c} ≥6.5%; or *3*) antidiabetic medication. An additional inclusion criterion was the absence of any documented history of vascular disease (ischemic cardiopathy, peripheral arteriopathy, or cerebrovascular accident) and diabetes. Exclusion criteria were physical disability or alterations in the electrocardiogram (ECG) that made it impossible to perform an exercise stress test, or clinical signs of coronary artery, peripheral, or cerebrovascular disease. In accordance with the Declaration of Helsinki, all participants were informed of the purpose, risks, procedures, and possible benefits of the study and gave their expressed consent to take part. Approval was obtained from the hospital's ethics committee.

Study procedure

During their first visit, subjects provided their case history and underwent a physical examination that included measurement of BMI and waist circumference. Subjects were given an appointment at the Department of Endocrinology's functional testing unit for collection of blood for biochemical determinations. The appointment took place between 8:00 and 10:00 A.M. and patients were instructed to have fasted for at least 10 h. During the appointment, subjects were fitted with a Holter in order to monitor blood pressure over 24 h.

Evaluation of SMI

During the basal visit, a baseline ECG was performed and patients were monitored over the following 30 days. If a Q wave of previous myocardial necrosis was detected (i.e., <0.04 s with a depth greater than one-third the height of the QRS waves of the ECG in at least two anatomically consecutive leads), a Doppler echocardiography was performed. If this test also showed segmentary contractibility disorders, the subject was classified as positive for SMI. Otherwise, the subsequent step was 24-h Holter monitoring of the ECG, with any decrease in the ST segment ≥1 mV or any increase ≥1.5 mV and continuing for at least 1 min considered a positive result, even in the absence of symptoms of angina. If the result was negative, the patient was submitted to an exercise stress test consisting of the Bruce protocol and according to the recommendations of the American College of Cardiology (ACC) and American Heart Association (AHA) (14). When the result of this test was negative, SMI was ruled out. If inconclusive, a nuclear stress test was performed using the criteria for positivity established by the ACC, AHA, and American Society of Nuclear Cardiology (15). If the result of the Bruce protocol or nuclear stress was positive, the patient was offered a coronary angiograph in order to confirm the diagnosis.

Biochemical determinations

Blood samples were collected and centrifuged at 23,000*g* for 15 min at 4°C. Aliquots of serum were obtained to determine total cholesterol, glucose, and triglyceride (TG) levels using an enzymatic method and HDL levels using a Beckman LX20 analyzer (Beckman Corp., Brea, CA). The intraseries coefficient of variation (CV) was <3.5% for all these determinations. LDL content was calculated with the Friedewald formula. Insulin and lipoprotein(a) levels were determined by enzyme-linked immunosorbent assay (intratest CV <4%). Insulin resistance in patients not taking insulin was calculated using homeostasis model assessment of insulin resistance (HOMA-IR). High-sensitivity C-reactive protein (hs-CRP) levels were quantified by a latex-enhanced immunonephelometric assay (intra-assay CV of 8.7%).

Cells

PMNs were isolated from citrated blood samples and incubated with dextran (3%) for 45 min. The supernatant was obtained from the Ficoll-Hypaque gradient and centrifuged at 250*g* for 25 min, and the pellet was resuspended in lysis buffer after centrifugation at room temperature (100*g*, 5 min). PMNs were counted, washed in Hanks' balanced salt solution, and resuspended in complete RPMI medium.

Human umbilical vein endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were harvested from umbilical cords by means of collagenase treatment (16). Passage 1 from primary cultures was used in subsequent experiments. For adhesion studies, HUVECs were cultured on fibronectin (5 mg/mL)-coated, 25-mm plastic coverslips until confluent (~48 h).

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Measurements of O_2 consumption, membrane potential, and mitochondrial mass

PMNs were resuspended (5×10^6 cells/mL) in Hanks' balanced salt solution and placed in a gas-tight chamber. O_2 consumption was then measured with a Clark-type O_2 electrode (Rank Brothers, Bottisham, U.K.) (12). Sodium cyanide (10^{-3} mol/L) was used to confirm whether or not O_2 consumption was mainly mitochondrial (95–99%). Tetramethylrhodamine methyl ester (TMRM; 5×10^{-6} mol/L) fluorescent dye was used to assess $\Delta\Psi_m$. Mitochondrial mass was measured in cells treated with the fluorescent dye 10-*N*-nonyl acridine orange (NAO; 5×10^{-6} mol/L), which specifically binds to cardiolipin independently of $\Delta\Psi_m$ (17).

Measurement of ROS production

Three different methods were used to measure ROS. Total ROS production was evaluated by fluorimetry using a Fluoroskan plate reader (Thermo Labsystems, Thermo Scientific, Rockford, IL) after a 30-min incubation with the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; 5×10^{-6} mol/L), as described elsewhere (16). H_2O_2 was assessed with the Amplex Red[R] H_2O_2/Peroxidase Assay kit. H_2O_2 (0.5×10^{-4} mol/L) was used as a positive control. The fluorescent probe MitoSOX (5×10^{-6} mol/L, 30 min) was used to detect mitochondrial superoxide.

Glutathione content, adhesion assay, and levels of cytokines and adhesion molecules

The determination of glutathione content and the parallel plate flow chamber in vitro model have been described in detail previously (8,18,19). These techniques and the drugs and solutions used are described in the Supplementary Data.

Data analysis

Statistical analysis was carried out with R version 2.15.1 (The R Foundation for Statistical Computing, Vienna, Austria). Quantitative variables are expressed as mean and SD if normally distributed; if not, they are expressed as median and quartiles. The parametric data were compared with a one-way ANOVA, and nonparametric data were compared with a Kruskal-Wallis, followed in each case by a post hoc test (Student-Newman-Keuls or Dunn multiple comparison, respectively). Adjustment of variables was carried out using a univariate general linear model; the variable to be adjusted was the dependent variable and the group (control, SMI, or absence of SMI) the independent variables. Possible confounding variables (waist circumference or BMI) were defined as covariates. The χ^2 test was used to compare proportions among groups of subjects. Pearson correlation or Spearman correlation coefficients were used to measure the strength of the association between two variables for parametric and nonparametric data, respectively. All the tests used a confidence interval of 95% and differences were considered significant when $P < 0.05$.

RESULTS

Clinical and metabolic characteristics

The anthropometric characteristics of type 2 diabetic patients (with or without SMI) and control subjects are presented in Table 1, which shows a higher ($P < 0.05$) waist circumference among the former subjects. Fasting levels of serum TGs, HbA_{1c}, hs-CRP, glucose, insulin, and HOMA-IR were higher in type 2 diabetic patients ($P < 0.05$) (Table 1), whereas levels of HDL and LDL were lower ($P < 0.05$). No changes were found with respect to type of treatment between type 2 diabetic patients with or without SMI. Statistically significant differences remained after adjustment for BMI and waist, with the exception of those between hs-CRP and insulin levels after adjustment for waist alone.

Twenty-five patients presented SMI, of which 23 were diagnosed by exercise stress testing, 1 by Holter-ECG, and 1 due to a Q wave of previous myocardial necrosis. Nineteen patients agreed to undergo the coronary angiography test.

Mitochondrial O_2 consumption, membrane potential, and mitochondrial mass

An O_2-tight chamber was used to monitor the rate of O_2 consumption in PMNs from the blood of control subjects and type 2 diabetic patients with and without SMI. The O_2 requirement of the cells was mainly mitochondrial, since addition of sodium cyanide resulted in almost complete (95–99%) inhibition of O_2 consumption (not shown). The rate of O_2 consumption was lower in type 2 diabetic patients and more significant among those with SMI. Figure 1*A* represents the reduction in O_2 consumption in type 2 diabetic patients with SMI ($P < 0.001$) versus those without ($P < 0.01$). Both TMRM ($P < 0.01$ without SMI and $P < 0.001$ with SMI) and NAO ($P < 0.05$) fluorescence were diminished in diabetic patients ($P < 0.05$ without SMI and $P < 0.05$ with SMI), the former indicating a reduction in $\Delta\psi_m$ (Fig. 1*B*) and the latter a reduction in mitochondrial mass (Fig. 1*C*).

ROS production

DCFH-DA fluorescence was significantly higher among type 2 diabetes patients but was more evident in the group with SMI ($P < 0.01$ without SMI, and $P < 0.001$ in the group with SMI) (Fig. 1*D*). Diabetic patients presented higher levels of H_2O_2 ($P < 0.01$ without SMI and $P < 0.001$ with SMI) (Fig. 1*E*) and mitochondrial ROS production ($P < 0.05$ without SMI and $P < 0.001$ with SMI) (Fig. 1*F*). In general, the patients with SMI exhibited a higher level of ROS than those without SMI.

GSH levels

As shown in Fig. 2*A*, levels of GSH were significantly lower in diabetic patients ($P < 0.01$ without SMI and $P < 0.001$ with SMI). Figure 2*B* shows how the GSH/GSSG ratio was also lower among these patients ($P < 0.01$ without SMI and $P < 0.001$ with SMI). Type 2 diabetic patients with SMI exhibited lower levels of GSH and GSH/GSSG than those without SMI ($P < 0.001$), which is of great relevance with respect to oxidative stress.

Adhesion assay under flow conditions

Type 2 diabetes was related to lower rolling velocity of PMN ($P < 0.01$ without SMI and $P < 0.001$ with SMI) (Fig. 2*C*) and a significantly higher rolling flux (cells per minute; $P < 0.01$ without SMI and $P < 0.001$ with SMI) (Fig. 2*D*) and PMN adhesion (cells per square millimeter; $P < 0.01$ without SMI and $P < 0.001$ with SMI) (Fig. 2*E*). These results provide evidence that type 2 diabetes is characterized by a higher frequency of leukocyte-endothelium interactions that is even more pronounced among patients with SMI than those without SMI ($P < 0.05$) (Fig. 2*C–E*). After adding tumor necrosis factor-α (TNF-α; 10 ng/mL, 4 h) and PAF (1 μmol/L, 1 h) (positive controls) to HUVECs and leukocytes, respectively, an increase in rolling flux ($P < 0.001$) and adhesion ($P < 0.001$) and a decrease of rolling velocity ($P < 0.05$) were observed (Supplementary Fig. 1).

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Table 1—*Anthropometric, clinical, and metabolic characteristics of type 2 diabetic patients (with and without SMI) and control subjects*

	Control	non-SMI	SMI	*P* adjusted values
n	60	175	25	
Age (years)	54.2 ± 1.8	55.3 ± 1.7	56.1 ± 2.0	—
Duration of diabetes (years)		5.51 (2.03–12.02)	6.12 (2.01–11.53)	—
Mean SBP (mmHg)	125 ± 3	122 ± 2	122 ± 3	—
Mean DBP (mmHg)	74 ± 5	75 ± 3	75 ± 2	—
BMI (kg/m^2)	26.8 ± 1.4	30.3 ± 2.6	29.6 ± 2.9	—
Waist (cm)	97.6 ± 1.6[a]	107.5 ± 2.5[b]	106.9 ± 2.2[b]	—
Active smoker, %	15	19	20	—
Total cholesterol (mmol/L)	5.3 ± 0.4	5.1 ± 0.3	4.9 ± 0.2	$P > 0.05$* $P > 0.05$#
TG (mmol/L)	1.04 (0.86–1.41)[a]	2.05 (1.53–2.82)[b]	1.69 (1.34–2.41)[b]	$P < 0.05$* $P < 0.05$#
HDL (mmol/L)	1.20 ± 0.09[a]	1.05 ± 0.03[b]	0.96 ± 0.03[b]	$P < 0.05$* $P < 0.05$#
LDL (mmol/L)	3.57 ± 0.10[a]	3.06 ± 0.14[b]	3.03 ± 0.20[b]	$P < 0.05$* $P < 0.05$#
Lipoprotein(a) (μmol/L)	0.22 (0.11–0.62)	0.19 (0.12–0.69)	0.19 (0.11–0.68)	$P > 0.05$* $P > 0.05$#
HbA_{1c} (%)	3.9 ± 0.5[a]	7.5 ± 1.4[b]	7.4 ± 0.9[b]	$P < 0.05$* $P < 0.05$#
hs-CRP (mg/L)	1.62 (0.60–2.13)[a]	3.74 (1.52–4.91)[b]	3.83 (1.42–5.13)[b]	$P < 0.05$* $P > 0.05$#
Glucose (mmol/L)	5.4 (3.8–7)[a]	9.2 (7.1–13.9)[b]	8.3 (6.7–14.4)[b]	$P < 0.05$* $P < 0.05$#
Insulin (pmol/L)	60 (41–76)[a]	100 (63–137)[b]	98 (74–140)[b]	$P < 0.05$* $P > 0.05$#
HOMA-IR	3.1 (2.2–5.4)[a]	10.6 (6.6–11.4)[b]	12.3 (7.1–14.3)[b]	$P < 0.05$* $P < 0.05$#
		Treatment		
Treatment with metformin, %		61	54	—
Treatment with glitazone, %		15	21	—
Treatment with sulphonylurea, %		29	21	—
Treatment with statin, %		48	55	—
Treatment with fibrate, %		18	12	—

Data are expressed as mean ± SD for parametric data or as median (25th and 75th percentiles) for nonparametric data. Different superscript letters (a and b) indicate significant differences among the groups (a is different from b) ($P < 0.05$) when compared by means of one-way ANOVA or Kruskal-Wallis for parametric and nonparametric data, respectively, followed by a post hoc test or χ^2 test to compare proportions between groups. HOMA-IR = fasting insulin (mU/mL) × fasting glucose (mmol/L)/22.5. DBP, diastolic blood pressure; SBP, systolic blood pressure. **P* value after adjusting for BMI. #*P* value after adjusting for waist circumference.

Levels of cytokines and adhesion molecules

Type 2 diabetes was related to a significant increase in the levels of proinflammatory cytokines interleukin-6 (IL-6; $P < 0.05$ in both groups) and TNF-α ($P < 0.05$ without SMI and $P < 0.01$ with SMI) (Table 2) and higher levels of the adhesion molecules VCAM-1 and E-selectin ($P < 0.05$ without SMI and $P < 0.01$ with SMI). Type 2 diabetes was characterized by higher levels of proinflammatory cytokines and adhesion molecules, which were even higher in diabetic patients with SMI than those without SMI, with the exception of IL-6 ($P < 0.05$).

After adjusting for BMI and waist circumference, significant differences remained between the groups in relation to IL-6, TNF-α, and E-selectin but disappeared in the case of VCAM-1. No significant differences in VCAM-1 levels were observed between the groups.

Correlation studies

We studied possible correlations between leukocyte mitochondrial function, ROS (both total and mitochondrial), and leukocyte-endothelial interactions and type 2 diabetic patients with and without SMI (Supplementary Table 1).

Mitochondrial O_2 consumption was positively correlated with rolling velocity in SMI patients ($r = 0.954$, $P < 0.001$) and negatively correlated with PMN rolling in non-SMI patients ($r = -0.770$, $P < 0.05$) and with PMN adhesion in both groups ($r = -0.979$ and $P < 0.001$, $r = -0.873$ and $P < 0.05$, in non-SMI and SMI patients, respectively). Membrane potential was negatively correlated with PMN rolling in SMI patients ($r = -0.765$, $P < 0.05$) and with PMN adhesion in both groups ($r = -0.865$ and $P < 0.01$, $r = -0.929$ and $P < 0.01$, in non-SMI and SMI patients, respectively). ROS was positively correlated with PMN rolling ($r = 0.820$ and $P < 0.05$, $r = 0.777$ and $P < 0.05$ in non-SMI and SMI patients, respectively) and with PMN adhesion ($r = 0.965$ and $P < 0.001$, $r = 0.838$ and $P < 0.05$, in non-SMI and SMI patients, respectively). Interestingly, mitochondrial ROS production was negatively correlated with rolling velocity ($r = -0.814$,

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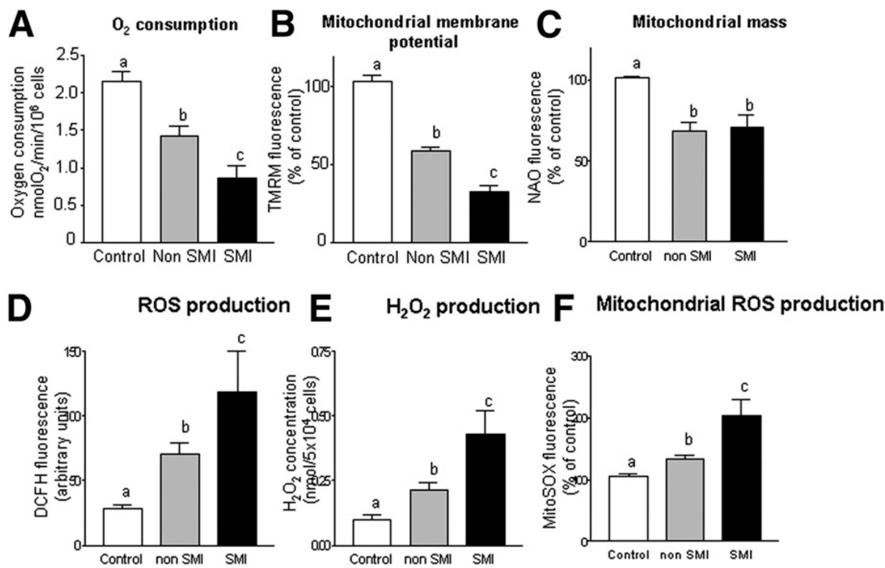

Figure 1—*Effects of type 2 diabetes on oxygen consumption and mitochondrial membrane potential and mass.* A: *O_2 consumption in control subjects* (n *= 30) and type 2 diabetic patients with* (n *= 25) or without SMI* (n *= 47) in a closed respiration chamber measured as nmol O_2/min/million cells.* B: *Mitochondrial membrane potential (TMRM fluorescence, % of control) in control subjects* (n *= 29) and type 2 diabetic patients with* (n *= 25) or without SMI* (n *= 68).* C: *Mitochondrial mass (NAO fluorescence, % of control) in control subjects* (n *= 60) and type 2 diabetic patients with* (n *= 25) or without SMI* (n *= 80). Effects of type 2 diabetes on ROS.* D: *Changes in the fluorescence of DCFH in control subjects* (n *= 37) and type 2 diabetic patients with* (n *= 25) or without SMI* (n *= 50).* E: *H_2O_2 production in control subjects* (n *= 28) and type 2 diabetic patients with* (n *= 25) or without SMI* (n *= 25).* F: *Mitochondrial ROS production (MitoSOX fluorescence, % of control) in control subjects* (n *= 25) and type 2 diabetic patients with* (n *= 25) or without SMI* (n *= 38). Data are expressed as mean + SEM. Different letters (a, b, and c) indicate significant differences among groups (a is different from b, a is different from c, and b is different from c)* (P < *0.05) when compared by means of one-way ANOVA followed by a post hoc test.*

$P < 0.05$) and positively correlated with PMN rolling ($r = 0.848$, $P < 0.05$) and with PMN adhesion ($r = 0.989$, $P < 0.001$) in SMI patients but not in non-SMI patients.

Regarding mitochondrial parameters, O_2 consumption was negatively correlated with ROS production in both groups ($r = -0.407$ and $P < 0.05$, $r = -0.647$ and $P < 0.01$, in non-SMI and SMI patients, respectively). Mitochondrial membrane potential was negatively correlated with mitochondrial ROS in SMI patients ($r = -0.468$, $P < 0.05$) but not in non-SMI patients.

CONCLUSIONS—We can report an increase in fasting levels of TG, HbA_{1c}, hs-CRP, glucose, insulin, and HOMA-IR in type 2 diabetic patients. Given that waist circumference and BMI were also higher in this group (but significantly only in the former case), we adjusted the data for these parameters. However, statistical differences remained, except between hs-CRP and insulin levels after adjustment for waist.

We observed a prevalence of SMI of 12.5%, which falls within the range of 10 to 20% attributed to the diabetic population (20). Furthermore, mitochondrial dysfunction (defined as a decrease in O_2 consumption and membrane potential and an increase in mitochondrial ROS production) in PMNs was identified as a characteristic of type 2 diabetes and was found to correlate with the presence of SMI. This effect was evident in the decrease in mitochondrial O_2 consumption, membrane potential, mitochondrial mass, GSH levels, and GSH/GSSG ratio and the increase in mitochondrial ROS production observed in these patients. Furthermore, we have observed how type 2 diabetes induces leukocyte-endothelium interactions by undermining PMN rolling velocity and increasing rolling flux and adhesion, which is accompanied by a simultaneous increase in proinflammatory cytokines IL-6 and TNF-α and adhesion molecules. These effects on the parameters under study were more evident in the SMI group, which may partially explain the increase of SMI and cardiovascular events during type 2 diabetes.

Our results provide evidence that the rate of mitochondrial dysfunction correlates with SMI in type 2 diabetes. It has been reported that leukocytes from patients with oxidative stress are in a proinflammatory state expressed by a heightened sensitivity to physiologic hyperglycemia and elevated plasma CRP (21).

It has been described that insulin signaling per se also regulates myocardial mitochondrial O_2 consumption and rates of ATP synthesis (22,23). The studies in question demonstrated a coordinated reduction in fatty acid enzymes and tricarboxilic acid that appeared to impair the delivery of reducing equivalents to the electron transport chain. Additionally, mitochondria from insulin receptor–deficient myocytes were found to be more susceptible to fatty acid–induced oxidative stress and mitochondrial uncoupling. These data highlight the important role of the insulin signaling pathway in modulating mitochondrial bioenergetics and integrity. Our results show that mitochondrial dysfunction may be related to the onset of SMI. Given that SMI seems to be a predictor of CVD driven by increased cardiovascular risk markers, it may be of relevance to monitor mitochondrial function as a marker of the appearance and prevalence of SMI.

In addition to mitochondrial dysfunction, insulin resistance in patients with type 2 diabetes is characterized by reduced maximal O_2 consumption (24). This parameter is positively correlated with insulin sensitivity and is considered to be a strong determinant of the insulin sensitivity index (25–27). In skeletal muscle of obese subjects and patients with type 2 diabetes, mitochondrial dysfunction appears to be caused by a lower number and decreased functional capacity of mitochondria (25,28).

Oxidative stress and ROS-mediated mitochondrial dysfunction are both major mechanisms of mitochondrial dysfunction. In the present work, we have demonstrated a higher production of mitochondrial ROS by leukocytes of type 2 diabetic patients in basal conditions, which is more pronounced among those with SMI.

ROS can be released from the endothelium and inflammatory cells, inducing oxidative damage (29). Indeed, excessive amounts of ROS are generally harmful to cells, as they can provoke lipid peroxidation and apoptosis. The antioxidant system of

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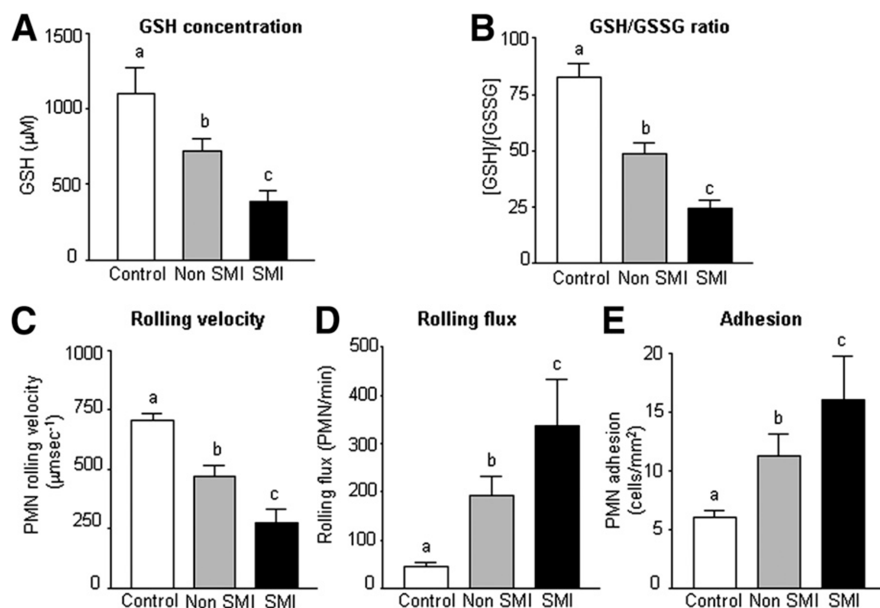

Figure 2—A: *The levels of GSH in control subjects* (n = *36) and type 2 diabetic patients with* (n = *25) or without SMI* (n = *36*). B: *The GSH/GSSG ratio in control subjects* (n = *37) and type 2 diabetic patients with* (n = *25) or without SMI* (n = *38*). *Effects of type 2 diabetes on* C: *PMN rolling velocity* (*μm sec^{-1}*) (n = *10 per group*), D: *rolling flux (PMN/min)* (n = *10 per group*), *and* E: *PMN adhesion (PMN/mm^2)* (n = *10 per group*). *Data are expressed as mean + SEM. Different letters (a, b, and c) indicate significant differences among the groups (a is different from b, a is different from c, and b is different from c)* (P < *0.05) when compared by means of one-way ANOVA followed by a post hoc test.*

organisms neutralizes the damaging effects of ROS and, together with GSH, plays a vital role in protecting cells from oxidative stress (30). In this way, an increase in the production of ROS, a decrease in GSH levels and the GSH/GSSG ratio, and a reduction in the mitochondrial membrane potential and mitochondrial mass, all of which are characteristics of diabetes, are likely to lead to dysfunction within the respiratory chain, which subsequently compromises the functioning of mitochondria as a source of energy. Mitochondria are known to be an important site for the generation of ROS, which are highly toxic to various sites of the mitochondrial respiratory chain. The inhibition of complex I is the most likely consequence of this toxicity.

Pathophysiological states such as atherosclerosis and hypertension are characterized by leukocyte recruitment to the arterial wall. To study this process, we have used an in vitro model in which human leukocytes flow over a monolayer of human endothelial cells with a shear stress similar to that observed in vivo (31). This reproduces the process that precedes inflammation in vivo (rolling and adhesion) and that is critical to homeostasis and vascular cell integrity. If these interactions are exacerbated, the vascular dysfunction and injury associated with many CVDs can occur (32). Our experimental system has been widely applied to visualize and analyze the multistep recruitment of leukocytes in these diseases, and allows the mechanisms of action implicated in this recruitment to be assessed (33,34). In the current study, we have observed how type 2 diabetes induces a significant increase in rolling flux and PMN adhesion and a decrease in the rolling velocity of PMN. These effects seem to directly correlate with SMI, as they were more pronounced in SMI patients in all cases. Thus, we can demonstrate that mitochondrial ROS production fully correlates with adhesion, rolling, and rolling velocity in type 2 diabetic patients with SMI. In relation to this, it is known that the causal link between elevated glucose and hyperglycemic damage is the increased production of ROS by mitochondria (35). Furthermore, increases in leukocyte-endothelium interactions have been related to oxidative stress in a model of insulin resistance (19). The present findings demonstrate a higher increase of ROS in diabetic patients with SMI. This is relevant, as ROS promotes a proinflammatory/prothrombogenic phenotype within the vasculature by different mechanisms (36), including the inactivation of nitric oxide, the activation of redox-sensitive transcription factors (e.g., nuclear factor-κB) that govern the expression of endothelial cell adhesion molecules, and the activation of enzymes (e.g., phospholipase A[2]) that produce leukocyte-stimulating inflammatory mediators.

Our data are in accordance with the evidence associating capillary occlusion by leukocytes with extravascular macrophage accumulation in animal models of diabetes (34). Additionally, an intravital microscopy study in which rats were made diabetic by streptozotocin injection has provided evidence that the diabetic state induces lower venular shear rates and an increased number of rolling leukocytes in mesenteric venules with respect to nondiabetic animals (37).

Table 2—*Cytokines and adhesion molecules in the serum of type 2 diabetic patients (with and without SMI) and control subjects*

	Control	non-SMI	SMI	*P* adjusted values
IL-6 (pg/mL)	1.8 (1.2–3.1)[a]	3.6 (2.0–4.2)[b]	3.9 (2.2–3.4)[b]	$P < 0.05$* $P < 0.05$#
TNF-α (pg/mL)	3.5 ± 0.3[a]	7.8 ± 0.4[b]	11.3 ± 0.5[b]	$P < 0.05$* $P < 0.05$#
VCAM-1 (ng/mL)	892 ± 38[a]	1,029 ± 61[b]	1,285 ± 102[b]	$P < 0.05$* $P > 0.05$#
ICAM-1 (ng/mL)	166 ± 69	153 ± 46	178 ± 25	$P > 0.05$* $P > 0.05$#
E-selectin (ng/mL)	23 ± 3[a]	49 ± 6[b]	68 ± 7[b]	$P < 0.05$* $P < 0.05$#

Data are expressed as mean ± SD for parametric data or as median (25th and 75th percentiles) for nonparametric data. Different superscript letters (a and b) indicate significant differences among the groups (a is different from b) ($P < 0.05$) when compared by means of one-way ANOVA or Kruskal-Wallis for parametric and nonparametric data, respectively, followed by a post hoc test. *P value after adjusting for age. #P value after adjusting for waist circumference.

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Endothelial activation can be assessed by measurement of the soluble adhesion molecules E-selectin, VCAM-1, and ICAM-1. In general, circulating levels of adhesion molecules are elevated in patients with type 2 diabetes (38) and are upregulated in healthy individuals in response to systemic inflammation (39). In the current study, we have observed an increase in adhesion molecules in accordance with a rise in the number of leukocyte-endothelium interactions and selectins that mediate the initial tethering and subsequent rolling of leukocytes. The elevated levels of adhesion molecules observed in the serum of type 2 diabetic patients are of particular interest given that they provide strong evidence of an ongoing inflammatory process in the endothelium that could, theoretically, lead to CVD. Furthermore, we have observed an increase in proinflammatory levels of the cytokines TNF-α and IL-6 in type 2 diabetic patients, and an enhanced release of TNF-α from leukocytes after activation by ROS-induced oxidative stress may inhibit insulin signaling and impair glucose uptake (40).

In conclusion, the current study provides evidence of oxidative stress, mitochondrial dysfunction, and endothelium-inducing leukocyte-endothelium interactions in type 2 diabetes. These alterations are more pronounced in patients with SMI, which suggests a correlation between the rate of mitochondrial dysfunction and leukocyte-endothelium interactions on the one hand and the prevalence/appearance of SMI in diabetes on the other. Future exploration of this oxidative stress may help to clarify the nature of the molecular mechanisms involved and the physiological significance of insulin resistance in type 2 diabetic patients with SMI. Such knowledge would no doubt help to develop strategies to reduce the risk of the onset of type 2 diabetes.

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A.H.-M. contributed to the discussion and wrote, reviewed, and edited the manuscript. M.R. researched data, contributed to the discussion, and reviewed and edited the manuscript. S.R.-L. researched data and contributed to the discussion. C.B. and L.B. researched data. C.d.P., I.R.-T., and E.S.-I. reviewed and edited the manuscript. A.A. contributed to the discussion and reviewed and edited the manuscript. V.M.V. wrote, reviewed, and edited the manuscript. A.H.-M. 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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