# Reactive oxygen species production is increased in the peripheral blood monocytes of obese patients

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## Abstract

Infiltrating macrophages play an important role in the production of inflammatory mediators by the adipose tissue of obese subjects. To reach the adipose tissue, peripheral monocytes are recruited by locally produced chemoattractants. However, little is known about the activation of monocytes in the peripheral blood of obese subjects. The objective of this study was to determine reactive oxygen species and endoplasmic reticulum stress as early markers of monocytic commitment with an inflammatory phenotype in the peripheral blood of nondiabetic obese patients. Patients were recruited from an academic general hospital; controls were voluntary students. Seven lean controls and 6 nondiabetic obese patients were included in the study. Monocytes were prepared from peripheral blood. Immunoblot, flow cytometry, and polymerase chain reaction were used to determine reactive oxygen species and endoplasmic reticulum stress. Increased reactive oxygen species and activation of endoplasmic reticulum stress were detected in the monocytes from obese patients. Reducing endoplasmic reticulum stress with a chemical chaperone reversed monocytic activation, as determined by the reduction of reactive oxygen species production. Thus, monocytes from nondiabetic obese patients are already committed with an inflammatory phenotype in peripheral blood; and reducing endoplasmic reticulum stress negatively modulates their activation.

## 1. Introduction

The adipose tissue of obese subjects secretes a number of inflammatory mediators such as tumor necrosis factor–α (TNF-α), interleukin-6, resistin, and type 1 plasminogen activator inhibitor [1-3]. Some of these proteins play an important role in the common association of obesity with diabetes because they can activate intracellular mechanisms involved in the induction of insulin resistance [4].

Although a number of studies have shown that adipocytes have an autonomous capacity for producing inflammatory mediators [5-7], recent evidence has suggested a need for macrophagic infiltration of the adipose tissue to achieve full induction of the inflammatory response [8,9]. The infiltrating macrophages are derived from peripheral blood monocytes, which are recruited by different chemoattractants produced in the adipose tissue [10,11]. When entering the hypertrophic adipose tissue, macrophages express several inflammatory markers that denote their activation [9]; however, little is known about the commitment of peripheral blood monocytes of obese patients with an inflammatory phenotype.

In at least 2 studies, markers of activation of peripheral monocytes were detected in obese patients [12,13]; however, data also exist to suggest the converse [14]. In these studies, the phenotypic characterization of monocytes relied predominantly on the determination of cytokines and CD11b expression [12-14].

An earlier event in the activation of phagocytic cells is the production of reactive oxygen species (ROS) [15]. According to a recent study, a connection between ROS

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production and activation of endoplasmic reticulum stress (ERS) appears as a very premature event during the activation of macrophages by lipopolysaccharide [16]. Therefore, we decided to evaluate the activation of peripheral monocytes from nondiabetic obese patients by determining ROS production and the potential role for ERS in this phenomenon. Reactive oxygen species production was evaluated by fluorometry and by determining the levels of superoxide dismutase (SOD) and catalase (CAT), whereas ERS was evaluated by measuring the ER proteins PERK and eIF2α, which lead to the control of antioxidant gene expression, and by the chaperone GRP78 (BiP), which control, through the IRE pathway, the splicing of XBP-1 and the regulation of expression of a number of genes involved in the unfolded protein response.

## 2. Methods

### 2.1. Reagents and antibodies

Reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, HEPES, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween 20, and glycerol were from Bio-Rad (Richmond, CA). Anti–superoxide dismutase 1 (sc-6525, goat polyclonal), F4/80 (sc-71085, rabbit polyclonal), GRP78/BiP (sc-13968, rabbit polyclonal), anti-pPERK (Thr981) (sc-7383, rabbit polyclonal), and anti-peIF2α (Ser52) (sc-12412, mouse polyclonal) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). 5-Phenylbutiric acid (PBA, P21005), thapsigargin (T-9033), and anti-CAT antibody (C0979, mouse monoclonal) were from Sigma (St Louis, MO). RPMI 1640 and bovine fetal serum were from Cultilab (Campinas, SP, Brazil). Chemicals for real-time polymerase chain reaction (PCR) were from Invitrogen (Carlsbad, CA) and Applied Biosystems (Foster City, CA).

### 2.2. Subjects

Six nondiabetic obese patients (3 men, 3 women) were randomly selected from the obesity clinic of the Clinics Hospital, University of Campinas. Seven lean controls (3 men, 4 women) were selected among students of the university. All subjects were informed about the study, and a written informed consent was obtained. Exclusion criteria were impaired glucose tolerance; hypertension; hypercholesterolemia; and the use of corticosteroids, sulfonylureas, and thiazolidinediones. The study was approved by the university's Ethics Committee (protocol no. C04-0193). A complete clinical evaluation was performed on each subject. Blood samples were collected by venous puncture to perform laboratory tests and to obtain monocytes. Blood biochemical parameters were determined using kits from Roche Diagnostics (Sao Paulo, Brazil). Insulin was determined by radioimmunoassay (Linco, St Charles, MO). Homeostasis

model assessment of insulin resistance (HOMA-IR) was calculated as previously described [17] using the following formula: HOMA-IR = fasting insulin (in microunits per milliliter) × plasma glucose (in millimoles per liter)/22.5. Patients were considered insulin resistant when HOMA-IR was greater than 2.71, according to a predefined threshold for the Brazilian population [18].

### 2.3. Cell culture and peripheral blood mononuclear cell purification

Blood samples were collected in heparin and overlaid onto a Ficoll-Paque PLUS layer (Amersham-Biosciences AB, Uppsala, Sweden), with density adjusted to 1.076 g/mL, and centrifuged at 1300$g$ at room temperature for 20 minutes. The interface layer containing mononuclear cells was recovered by Pasteur pipette, washed twice in phosphate-buffered saline, and centrifuged at 800$g$ for 10 minutes. Cells were counted in a Neubauer chamber, and cell viability was determined by the trypan blue exclusion method. Cells were only used when viability was greater than 98%. Monocytes were isolated from remaining mononuclear cells by allowing them to adhere to tissue culture plastic (1.5-cm$^2$ plate wells) for 2 hours at an initial density of $10^6$ cells per milliliter in RPMI 1640 in a humidified atmosphere (5% $CO_2$ at 37°C). Nonadherent cells were removed by washing, and adherent cells were analyzed based on staining for CD14 using flow cytometry. This method allowed specific determination of monocytes. Adherent cells were only used for assays when positive staining to CD14 was greater than 90%. These cells were cultured for an additional 12 hours without or with TNF-α 10 ng/mL, thapsigargin 5 μmol/L, or PBA 100 μmol/L, as described in figure legends.

### 2.4. Determination of SOD and CAT expressions

The expressions of the antioxidant enzymes SOD and CAT were determined by 2 methods. Firstly, total protein extracts of peripheral blood monocytes were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes, and blotted with specific antibodies. Secondly, total protein extracts of peripheral blood monocytes were used in immunoprecipitation assays using the anti-GRP78/BiP antibody. The immunocomplexes obtained were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes, and blotted with specific antibodies against SOD and CAT. Details of the immunoprecipitation and immunoblotting methods are described below.

### 2.5. Immunoprecipitation and immunoblotting

For evaluation of protein expression, monocytes were centrifuged for 10 minutes at 800 rpm in a 70-Ti rotor (Beckman-Coulter, Fullerton, CA) at 28°C; and pellets were immediately homogenized in solubilization buffer at 4°C (1% Triton X-100, 100 mmol/L Tris-HCl [pH 7.4], 100

mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium orthovanadate, 2.0 mmol/L phenylmethylsulfonyl fluoride, and 0.1 mg aprotinin/mL). Insoluble material was removed by centrifugation for 40 minutes at 11 000 rpm in a 70-Ti rotor (Beckman) at 4°C. The protein concentration of the supernatants was determined by the Bradford dye method. Aliquots of the resulting supernatants containing 0.5 mg of total protein were used for immunoprecipitation with specific antibodies at 4°C overnight, followed by SDS/PAGE, transfer to nitrocellulose membranes, and blotting. The dilution of the GRP78/BiP antibodies used for immunoprecipitation was 1:200 (1.0 $\mu$g/mL). In direct immunoblot experiments, 50 $\mu$g of protein extracts was separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with antibodies. The dilutions of the antibodies used in the immunoblots were 1:100 (2.0 $\mu$g/mL) for pPERK and peIF2$\alpha$, 1:1000 (0.2 $\mu$g/mL) for CAT and F4/80, and 1:500 (0.4 $\mu$g/mL) for SOD-1. Specific bands were detected by chemiluminescence, and visualization was performed by exposure of the membranes to RX films (Kodak, Rochester, NY).

### 2.6. XBP-1 messenger RNA expression and splicing

The XBP-1 messenger RNA (mRNA) expression was examined by reverse transcription PCR. The RNA was isolated using the TRIZOL method and extracted with chloroform after tissue homogenization (GIBCO BRL, Gaithersburg, MD). The RNA was then recovered from the aqueous phase by precipitation with isopropyl alcohol and suspended in diethylpyrocarbonate (DEPC)-treated water. Complementary DNA (cDNA) was synthesized from 10 $\mu$g of total RNA using 1 $\mu$L of reverse transcriptase (MMLV, GIBCO BRL). The cDNA samples were stored at −20°C until use. The nucleotide sequence of the primers for XBP-1 has been previously reported [19], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the internal control for the PCR. Forty cycles of PCR amplification for XBP-1 and 33 for GAPDH were chosen after pilot experiments to define amplification conditions. The PCRs were performed in a final volume of 25 $\mu$L containing 2.5 $\mu$L cDNA, 2.5 $\mu$L 10 Taq buffer, 0.75 $\mu$L MgCl$_2$ (1.5 mmol/L), 0.5 $\mu$L dNTPs (0.2 mmol/L), 1.5 $\mu$L (0.5 $\mu$mol/L), 15.5 $\mu$L H$_2$O Milli Q (Millipore, Billerica, MA), and 0.25 $\mu$L Taq DNA polymerase (1.25 U). The amplification cycle was performed with denaturation for 1 minute at 94°C, annealing for 45 seconds at 65°C, and extension for 1.5 minutes at 72°C. The PCR products (10 $\mu$L) were previously normalized to provide equivalent amounts of the GAPDH control and were PstI digested and separated on 1.5% agarose gel containing 10% ethidium bromide. Gels were visualized under UV light, and images were captured (Chemimager 5500; Alpha Innotech, San Leandro, CA). The band sizes for the XBP-1 spliced and nonspliced forms were 1036 and 1158 base pairs, respectively. Band densitometry was determined to assess the expression of each isoform relative to GAPDH, and the results are presented as a ratio of spliced to unspliced forms.

### 2.7. Flow cytometry

The samples were analyzed in a FACSCalibur flow cytometer equipped with an argon laser and CellQuest software (Becton Dickinson, San Jose, CA). Ten thousand events were acquired from each sample. The monocyte

Table 1
Clinical and metabolic characteristics of the subjects

| | | Lean | | | | Obese | | | P |
|---|---|---|---|---|---|---|---|---|---|
| | n | Median | Minimum | Maximum | n | Median | Minimum | Maximum | |
| Age (y) | 7 | 28 (mean, 29.2) | 24 | 32 | 6 | 28.5 (mean, 29.6) | 27 | 34 | NS |
| BMI (kg/m$^2$) | 7 | 21.2 | 16.9 | 24.7 | 6 | 43.1 | 32.2 | 56.2 | <.05 |
| Waist (cm) | 7 | 71 | 56 | 85 | 6 | 118 | 90 | 137 | <.05 |
| SBP (mm Hg) | 7 | 107.8 | 90 | 130 | 6 | 124 | 100 | 140 | NS |
| DBP (mm Hg) | 7 | 75.7 | 60 | 90 | 6 | 90.8 | 70 | 100 | <.05 |
| T-chol (mg/dL) | 7 | 172 | 121 | 208 | 6 | 169.3 | 134 | 199 | NS |
| LDL-chol (mg/dL) | 7 | 86.6 | 55 | 143 | 6 | 96 | 70 | 121 | NS |
| HDL-chol (mg/dL) | 7 | 69 | 45 | 108 | 6 | 48 | 31 | 96 | NS |
| Tg (mg/dL) | 7 | 82.4 | 54 | 121 | 6 | 123 | 51 | 230 | NS |
| Glucose (mg/dL) | 7 | 76.1 | 70 | 84 | 6 | 87 | 76 | 96 | <.05 |
| Insulin (ng/dL) | 7 | 6.4 | 3.8 | 11.9 | 6 | 20.1 | 5.4 | 34.2 | <.05 |
| HOMA | 7 | 1.21 | 0.67 | 2.28 | 6 | 4.30 | 1.10 | 7.50 | <.05 |
| ALT (U/L) | 7 | 19.8 | 9 | 32 | 6 | 26.3 | 17 | 45 | NS |
| AST (U/L) | 7 | 18.7 | 15 | 24 | 6 | 21.5 | 18 | 29 | NS |
| Leukocyte (/mL) | 7 | 6,962 | 4,991 | 10,311 | 6 | 7,713 | 6,081 | 10,189 | NS |
| CRP (mg/L) | 7 | 0.22 | 0.08 | 0.42 | 6 | 0.95 | 0.1 | 1.6 | <.05 |

BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T-chol, total cholesterol; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; Tg, triglycerides; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C reactive protein; NS, not significant.

populations were identified by their light-scattering characteristics, enclosed in electronic gates, and analyzed for the intensity of the fluorescent probe signal. For pPERK and peIF2$\alpha$ determination, cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% saponin. Primary antibodies were used in a final concentration of 2.5 $\mu$g/mL; and the secondary, fluorescein-5-isothiocyanate (FITC)-conjugated antibody was used in a final dilution of 1:100.

### 2.8. Measurement of cytosolic free $Ca^{2+}$ concentrations

Changes in cytosolic $Ca^{2+}$ concentrations ($[Ca^{2+}]_{cyt}$) were monitored with the Fluo-3,AM (5 $\mu$mol/L) (Sigma, St. Louis, MO) green fluorescent probe, a single-wavelength indicator, by flow cytometry. Monocytes ($10^6$ cells per milliliter) in RPMI 1640 medium were loaded with 5 $\mu$mol/L Fluo-3,AM containing 1 $\mu$mol/L pluronic acid F-127 and 30 $\mu$g/mL bovine serum albumin in a humidified $CO_2$ incubator (5% $CO_2$) at 37°C for 40 minutes. Nonhydrolyzed Fluo-3, AM was removed by washing the cells with medium just before fluorescence acquisition. Calibration was performed at the end of each experiment. $[Ca^{2+}]_{cyt}$ was calculated considering Kd of the $Ca^{2+}$-Fluo-3.

### 2.9. Measurement of intracellular ROS levels

Intracellular ROS generation was assessed using 1 $\mu$mol/L $H_2DCF$-DA. Monocytes ($10^6$ cells per milliliter) were preincubated in RPMI 1640 medium containing 3 $\mu$mol/L dihydroethidium (DHE) at 37°C in a humidified $CO_2$ incubator (5% $CO_2$) for 40 minutes.

### 2.10. Statistical analysis

For blots, PCR, ROS production, and cytometry, the experiments were always performed in duplicate, with results displayed as mean ± SD; significance was assessed by analysis of variance and a post hoc Tukey test. For clinical and biochemical variables, the results are presented as median (minimum-maximum). Comparisons were performed by Mann-Whitney nonparametric test. Level of significance was set at $P$ less than .05 using SAS software (Statistical Analysis System 6.12; SAS Institute, 1989-1996, Cary, NC).
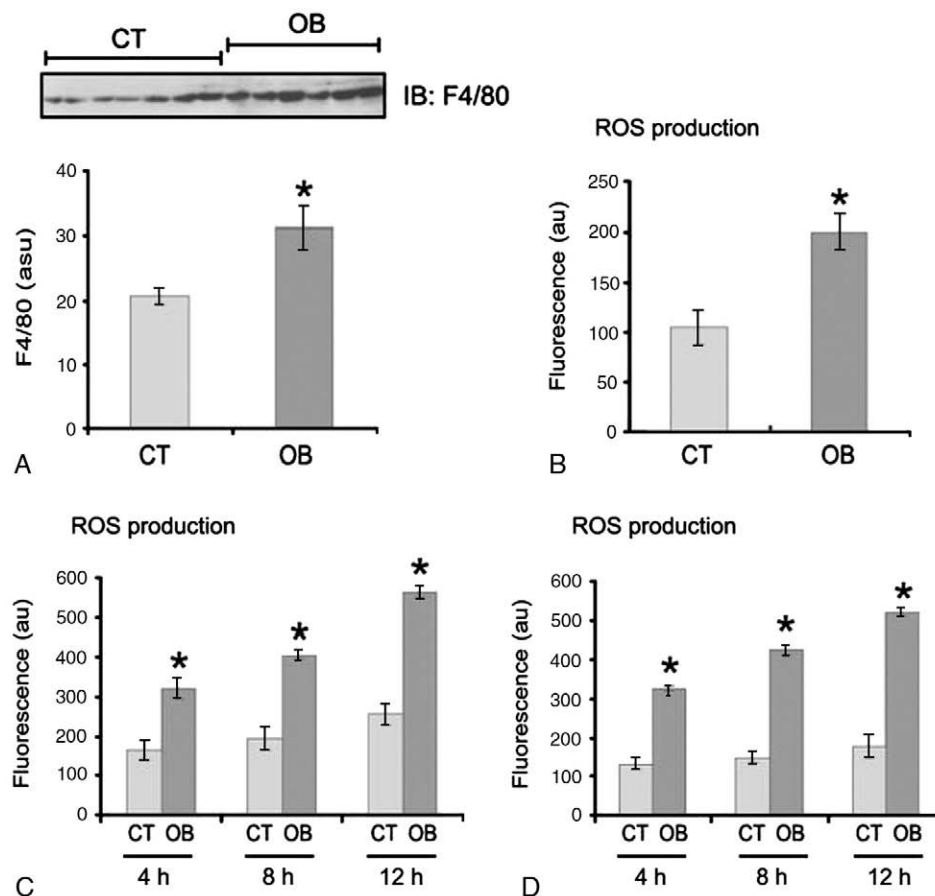


Fig. 1. A, F4/80 expression was determined by immunoblot (IB) analysis of total protein extracts of monocytes obtained from the peripheral blood of lean (CT) and obese (OB) subjects; results are expressed as arbitrary scanning units (asu). B-D, Reactive oxygen species were determined by the $H_2DCF$-DA method in freshly isolated monocytes (B) from CT and OB or in monocytes treated with TNF-$\alpha$ (C) or thapsigargin (D) for 4 to 12 hours. In all experiments, n = 7 for CT and n = 6 for OB; *$P$ < .05 vs CT (in A and B) or vs respective time CT (in C and D).

# 3. Results

## 3.1. Clinical and metabolic characterization of subjects

As presented in Table 1, obese patients had significantly higher body mass index and waist circumference, increased diastolic but not systolic blood pressure, and increased blood glucose and insulin levels, which reflected an increased HOMA index and increased C-reactive protein levels. The remaining parameters evaluated, including leukocyte and monocyte counts in peripheral blood, were similar between control and obese subjects.

## 3.2. Increased basal and stimulated ROS in peripheral blood monocytes from obese patients

F4/80, a member of the epidermal growth factor–transmembrane 7 family of proteins, is expressed in distinct populations of macrophages [20]; and its presence increases in migrating monocytes [21]. Here, we show that peripheral blood monocytes from obese patients express significantly higher levels of F4/80 (Fig. 1A), suggesting that increased numbers of peripheral blood monocytes from these patients are actively migrating. Because active monocytes are known
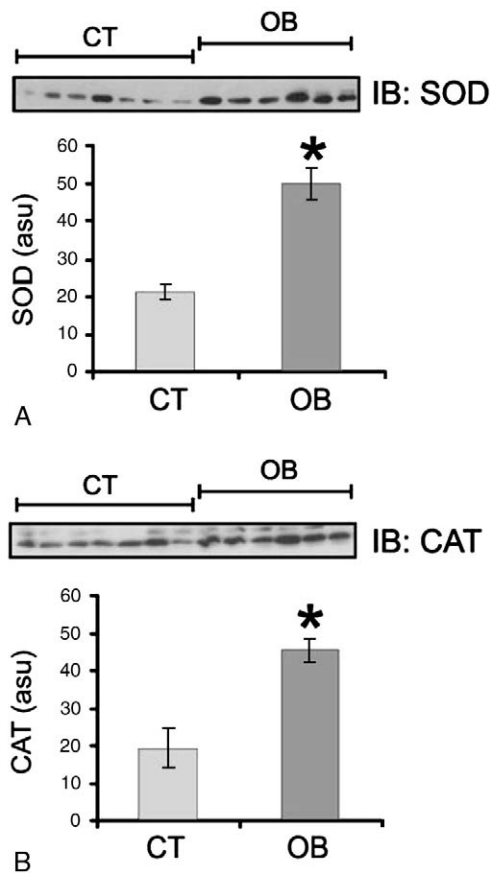


Fig. 2. Superoxide dismutase-1 (A) and CAT (B) expressions were evaluated by IB analysis of total protein extracts of monocytes obtained from the peripheral blood of CT and OB; results are expressed as asu. In all experiments, n = 7 for CT and n = 6 for OB; *P < .05 vs CT.
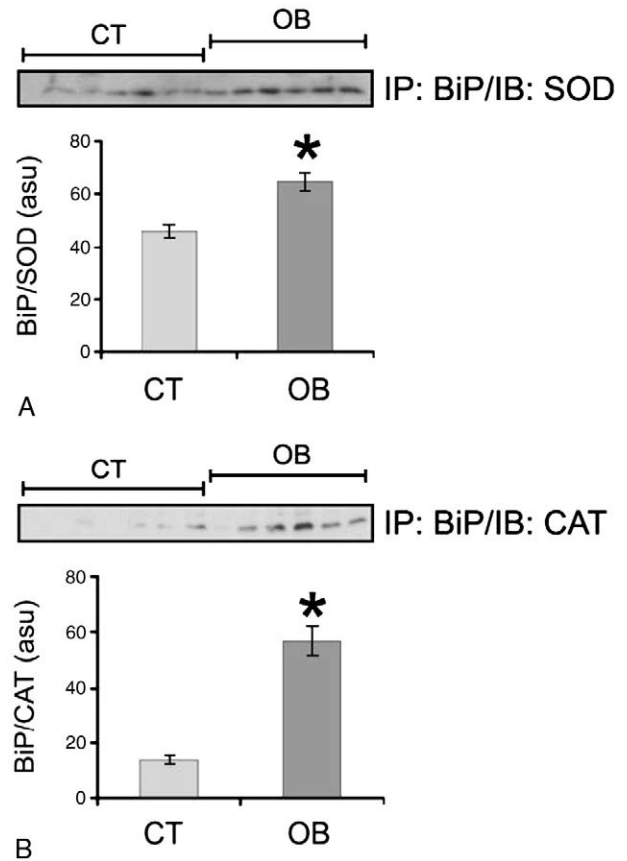


Fig. 3. The associations of GRP78/BiP with SOD (A) or CAT (B) were determined by immunoprecipitation using anti-GRP78/BiP antibody and IB with anti-SOD or anti-CAT antibodies, respectively; results are expressed as asu. In all experiments, n = 7 for CT and n = 6 for OB; *P < .05 vs CT. IP indicates immunoprecipitation.

to produce ROS, we determined basal and stimulated ROS production in isolated monocytes. As shown in Fig. 1B, monocytes from obese subjects produce significantly higher amount of ROS; and this is further stimulated by a pretreatment with TNF-α (Fig. 1C) or by the inhibitor of $Ca^{2+}$-ATPase, thapsigargin (Fig. 1D). Finally, in Fig. 2 (A and B), we provide further evidence for peripheral blood monocyte activation by showing that monocytes from obese patients express significantly higher levels of the antioxidant enzymes SOD and CAT.

## 3.3. Increased association of SOD and CAT with the endoplasmic reticulum chaperone GRP78/BiP in monocytes from obese patients

To evaluate whether increased SOD and CAT expression may result in an increased association of these proteins with the endoplasmic reticulum chaperone GRP78/BiP, total protein extracts from peripheral blood monocytes were used in immunoprecipitation assays using anti-GRP78/BiP antibody. The immunocomplexes obtained were resolved by SDS-PAGE and blotted with anti-SOD or anti-CAT antibodies. As depicted in Fig. 3 (A and B), both SOD and

CAT were significantly more associated with the chaperone GRP78/BiP in the monocytes from obese subjects.

### 3.4. Increased expression of markers of ERS in monocytes from obese patients

Induction of ERS was evaluated in peripheral blood monocytes by immunoblot analysis of Ser52-eIF2α and
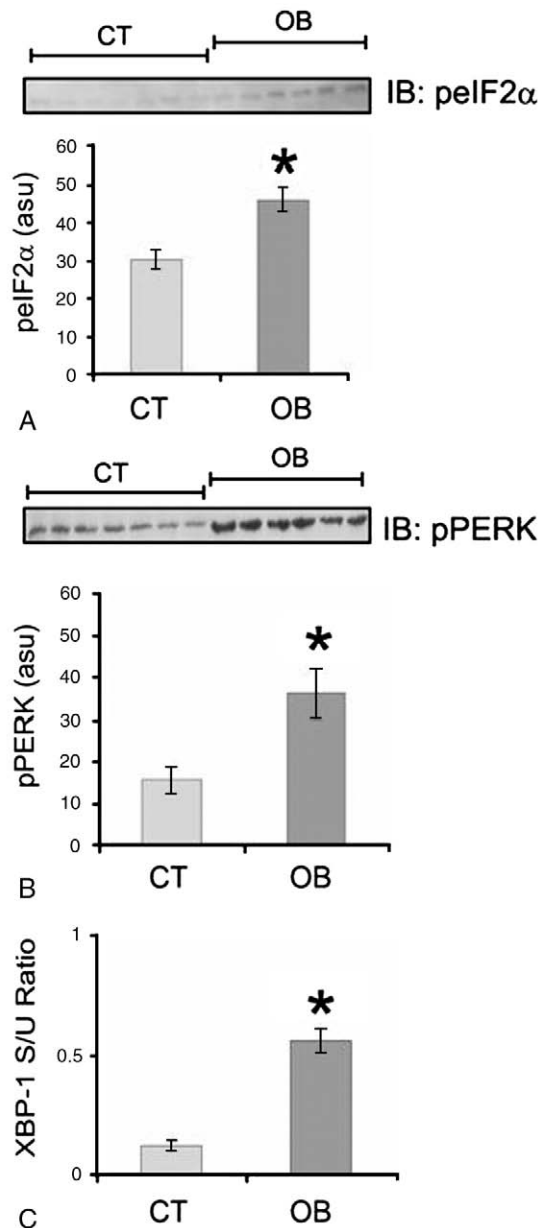


Fig. 4. Phosphor-eIF2α (A) and phosphor-PERK (B) expressions were evaluated by IB analysis of total protein extracts of monocytes obtained from the peripheral blood of CT and OB subjects; results are expressed as asu. The ratio of spliced to unspliced XBP-1 (C) was evaluated in PCR-amplified, PstI-digested cDNA samples prepared from total RNA obtained from the monocytes of peripheral blood of CT and OB subjects. In all experiments, n = 7 for CT and n = 6 for OB; *P < .05 vs CT. S/U indicates ratio of spliced to unspliced.

Thr981-PERK phosphorylation. As depicted in Fig. 4 (A and B), both markers were significantly increased in the monocytes from obese subjects. In addition, the ratio of spliced to unspliced XBP-1, an additional indicator of ERS, was significantly increased in the monocytes from obese patients. To explore the mechanisms involved in the induction of ERS in monocytes from obese patients, peripheral blood monocytes were treated with thapsigargin or TNF-α; and Ser52-eIF2α and Thr981-PERK phosphorylation was measured by immunoblot. As depicted in Fig. 5 (A and B), in monocytes from control and obese subjects, thapsigargin increased both Ser52-eIF2α and Thr981-PERK phosphorylation; however, TNF-α exerted no effect on monocytes from obese patients and increased only Thr981-PERK phosphorylation in monocytes from control subjects. This finding was associated with an increased cytosolic free $Ca^{2+}$ concentration in monocytes from both lean and obese subjects treated with thapsigargin, but only in monocytes from lean subjects when treated with TNF-α (Fig. 5C).

### 3.5. Reduction of ERS with a chemical chaperone reduces ROS production by peripheral blood monocytes

The treatment of isolated monocytes with the chaperone PBA resulted in a significant reduction of Ser52-eIF2α and Thr981-PERK phosphorylation in cells from both control and obese subjects, as determined by immunoblotting (Fig. 6A and B). Similar results were obtained by flow cytometry analysis (not shown). This effect was accompanied by significant reduction in ROS production in obese but not control subjects (Fig. 6C and D).

## 4. Discussion

The cytokines/adipokines produced by the hypertrophic adipose tissue of obese subjects play a central role in the development of insulin resistance that frequently accompanies obesity [4]. These inflammatory mediators activate intracellular mechanisms such as the serine kinases JNK, IKK, and PKC [22-24]; the expression of SOCS3 [25,26]; the expression of tyrosine phosphatase PTP1B [27,28]; and the nitrosation of important components of the insulin signaling pathway [29], all of which contribute to hampering insulin action in peripheral tissues.

According to recent studies, the hypertrophic adipose tissue will secrete enough inflammatory factors to induce insulin resistance only in the presence of macrophagic infiltration [30]. Because the macrophages that infiltrate the adipose tissue are derived from peripheral blood monocytes, characterizing these cells in the blood of obese subjects is an important condition for obtaining substantial advance in the understanding of the phenomena that govern adipose tissue inflammation.

A few previous attempts to characterize peripheral blood monocytes from obese subjects have generated some controversial results. Boschmann and colleagues [13]
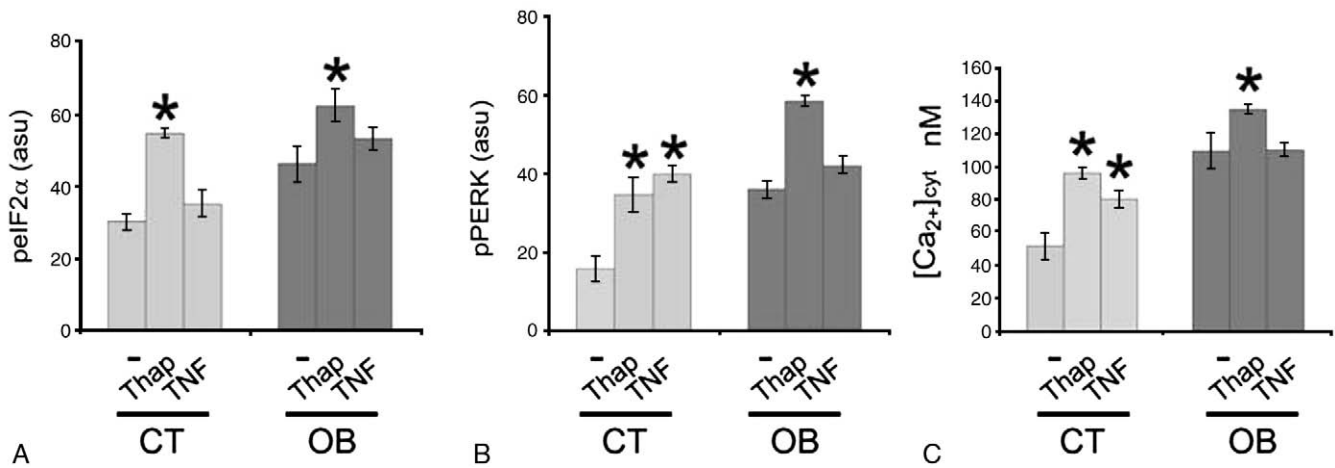
Fig. 5. Phosphor-eIF2α (A) and phosphor-PERK (B) expressions were evaluated by IB analysis of total protein extracts of monocytes obtained from peripheral blood of CT and OB subjects. The cells were treated with saline (−), thapsigargin, or TNF-α; results are expressed as asu. Cytosolic calcium (C) was determined in monocytes obtained from the peripheral blood of CT and OB subjects; the cells were treated with saline (−), thapsigargin, or TNF-α . In all experiments, n = 7 for CT and n = 6 for OB; *$P < .05$ vs (−) of the respective condition. Thap indicates thapsigargin.
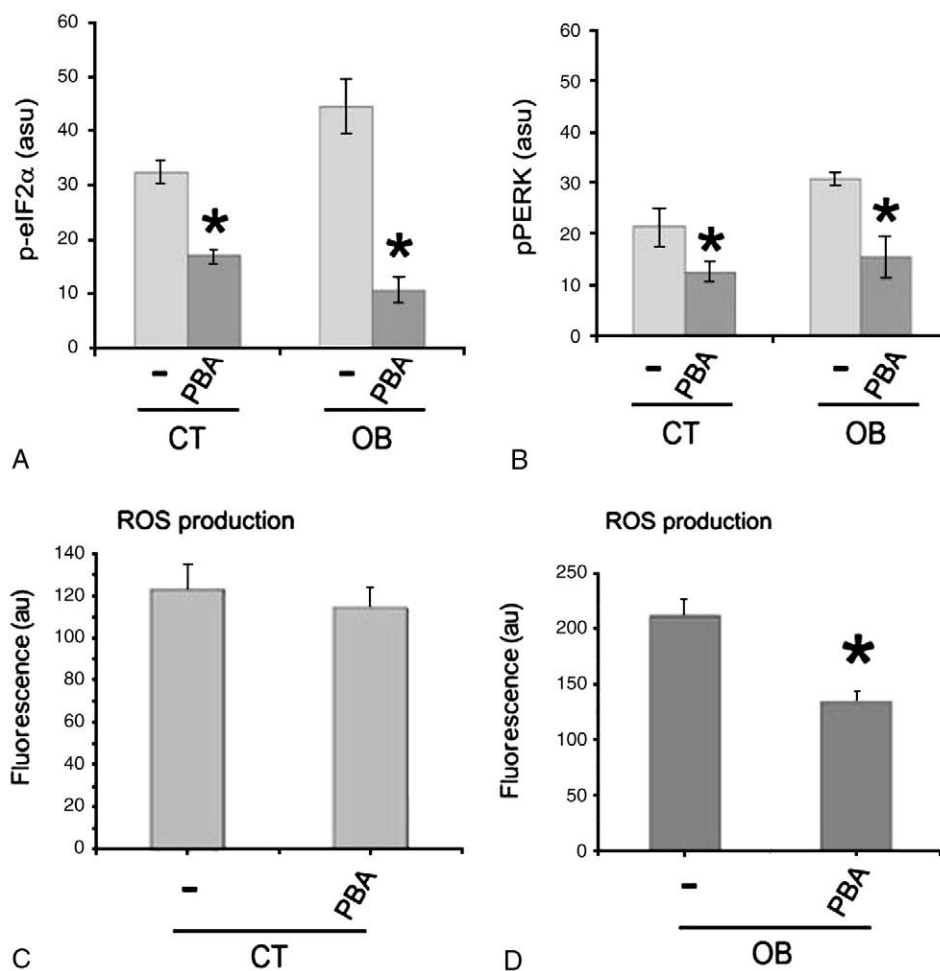


Fig. 6. Phosphor-eIF2α (A) and phosphor-PERK (B) expressions were evaluated by IB analysis of total protein extracts of monocytes obtained from peripheral blood of CT and OB subjects. The cells were treated with saline (−) or PBA; results are expressed as asu. Reactive oxygen species were determined by the H$_2$DCF-DA method in freshly isolated monocytes from CT (C) and OB (D) treated with saline (−) or PBA. In all experiments, n = 7 for CT and n = 6 for OB; *$P < .05$ vs (−) of the respective condition.

found that monocytes from obese subjects express significantly higher amounts of the integrin CD11b than controls. This membrane protein is involved in diapedesis of active or recruited phagocytes and is taken as an early marker of phagocytic activation [31]. In addition, Ghanim and coworkers [12] observed that monocytes from obese patients have increased nuclear factor–$\kappa$B binding to DNA and reduced levels of I$\kappa$B. This was accompanied by increased levels of TNF-$\alpha$, migration inhibitor factor, and matrix metalloproteinase–9 mRNA, suggesting that not only incipient but also intermediate process of monocytic activation can be detected in peripheral blood monocytes form obese subjects. However, Makkonen and coworkers [14] evaluated the expression of TNF-$\alpha$, 11$\beta$-hydroxysteroid dehydrogenase–1, resistin, and CD68 in monocytes from obese women and found no difference as compared with controls. Tumor necrosis factor–$\alpha$ and resistin may be considered as intermediate markers of activation; CD68 is constitutively expressed in monocytes and macrophages, and changes in its expression do not necessarily reflect activation. Finally, the role for 11$\beta$-hydroxysteroid dehydrogenase–1 in monocytes/macrophages is virtually unknown; thus, the finding that it did not differ between monocytes of lean and obese subjects has little informative value.

Nevertheless, as an attempt to provide further insights into the characterization of peripheral blood monocytes from obese patients, we evaluated 2 early markers of inflammatory commitment: ROS production and ERS. Generation of ROS is a very early event in the activation of phagocytic cells [32]. Here, using H$_2$DCF-DA for ROS determination and immunoblotting of 2 important antioxidant enzymes, SOD and CAT, we show that monocytes from nondiabetic obese subjects produce higher amounts of ROS than cells from lean controls. In addition, the monocytes from obese patients are more responsive to 2 different stimuli, thapsigargin and TNF-$\alpha$, producing increased amounts of ROS. Thus, it is clear that the monocytes in the peripheral blood of obese subjects are already committed to an inflammatory phenotype. Although this is the first time that increased ROS production by monocytes from obese patients is reported, in a recent study, increased production of ROS by monocytes was detected as early as 2 hours after the consumption of a high-fat/high-carbohydrate meal [33], which may support our findings.

When immune cells become active, a number of proteins are rapidly synthesized, ranging from adhesion molecules and signaling proteins to cytokines and proteins involved in the control of ROS accumulation [34]. A recent study has shown that, when macrophages are stimulated by lipopolysaccharide, there is an activation of ERS that is paralleled by ROS production [16]. As a rule, ERS installs when a given cell is submitted to a challenge that requires a prompt response, involving a change in the rate of protein synthesis [35]. Here, we hypothesized that active monocytes, upon production of high amounts of ROS, require increased synthesis of antioxidant enzymes; and this could contribute

to ERS installation. To test this hypothesis, we initially performed immunoprecipitation experiments that revealed an increased association of SOD and CAT with the ER chaperone GRP78/BiP in the monocytes of obese subjects. This was accompanied by an increased expression of markers of ERS, which could be further enhanced by treatment with an inhibitor of Ca$^{2+}$-ATPase, thapsigargin (providing a mitochondrial insult), but not with TNF-$\alpha$, suggesting that the immune stimulus was already in operation and could not be boosted by an additional inflammatory stimulus. The evaluation of cytosolic Ca$^{2+}$ as a marker of ERS relies on the fact that the ER, as the main site of storage for cellular Ca$^{2+}$, may nonspecifically release this ion as a consequence of a harmful stimulus.

To further explore the connection between ERS and ROS, we treated isolated monocytes with a chemical chaperone, PBA, known to reduce ERS [36]. In a recent study, PBA was used to treat insulin-resistant diabetic mice; and upon reduction of ERS, the metabolic parameters were much improved [2]. Here, we obtained a significant reduction in ERS in isolated monocytes; and this was accompanied by a reduction in ROS production, establishing a link between ERS and ROS in active human peripheral blood monocytes.

Thus, the present data reinforce the concept that monocytes in peripheral blood from obese subjects are already committed to an inflammatory phenotype. Two new markers of early activation were characterized, and the demonstration that reduction of ERS with a chemical chaperone inhibits ROS production opens exciting therapeutic possibilities for controlling the inflammatory setup of obese subjects.

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