

Research Article

The Production of Nitric Oxide, IL-6, and TNF-Alpha in Palmitate-Stimulated PBMNCs Is Enhanced through Hyperglycemia in Diabetes

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We examined nitric oxide (NO), IL-6, and TNF- α secretion from cultured palmitate-stimulated PBMNCs or in the plasma from type 2 diabetes mellitus (T2MD) patients or nondiabetic (ND) controls. Free fatty acids (FFA) have been suggested to induce chronic low-grade inflammation, activate the innate immune system, and cause deleterious effects on vascular cells and other tissues through inflammatory processes. The levels of NO, IL-6, TNF- α , and MDA were higher in supernatant of palmitate stimulated blood cells (PBMNC) or from plasma from patients. The results obtained in the present study demonstrated that hyperglycemia in diabetes exacerbates *in vitro* inflammatory responses in PBMNCs stimulated with high levels of SFA (palmitate). These results suggest that hyperglycemia primes PBMNCs for NO, IL-6, and TNF-alpha secretion under *in vitro* FFA stimulation are associated with the secretion of inflammatory biomarkers in diabetes. A combined therapy targeting signaling pathways activated by hyperglycemia in conjunction with simultaneous control of hyperglycemia and hypertriglyceridemia would be suggested for controlling the progress of diabetic complications.

1. Introduction

Circulating free fatty acids (FFAs) are elevated in patients with type 2 diabetes mellitus (T2DM), obesity, metabolic syndrome, and dyslipidemia [1–4]. FFAs represent a complex group of structurally variable molecules stored in the body as triglycerides and released through lipolysis [3, 5]. FFAs are classified according to the carbon chain length in short-, medium-, and long-chain fatty acids, the presence or absence of double bonds as saturated (SFA) and unsaturated fatty acids, respectively, and the number of double bonds as mono- or polyunsaturated (PUFA) [6, 7]. The effect of FFA on cellular signaling pathways depends on the chemical structure. It has been reported that chronic exposure to SFA

increases oxidative stress and inflammation, leading to the development of cardiovascular diseases and insulin resistance [8–12].

Oxidative stress, reflecting an imbalance between prooxidant and antioxidant effectors, plays an important role in diabetic vascular complications [13]. Superoxide, nitric oxide, and lipid peroxidation are indicators of oxidative stress in the body. Despite the number of studies concerning FFA-induced superoxide overproduction [14–22], there are few reports concerning FFA-induced nitric oxide (NO) production. NO is a highly diffusible and unstable gas that acts as a modulator of vascular tone, glucose transport in skeletal muscle cells and adipocytes, blood flow, force generation in skeletal muscle, cytotoxicity, and inflammation [23–26].

FFA also regulates the immune system through interactions with specific cell surface receptors, such as Toll-like receptors (TLR) and G-protein-coupled receptors (GPCR), thereby activating NF-kappaB and c-Jun amino-terminal kinase (JNK) pathways, which stimulate the secretion of proinflammatory cytokines (IL-1beta, IL-6 and TNF-alpha) and chemokines [27–30].

It is well known the effects of hyperglycemia and hyperlipidemia on peripheral blood mononuclear cells (PBMNCs) by activation of NADPH oxidase system leading to reactive oxygen species production, TLR expression, enhancing NF-kappaB activity, and inducing proinflammatory cytokines, chemokines, and circulating adhesion molecules secretion [8, 21, 31–41].

Thus, elevated plasma FFA levels act as inflammatory inducers, which potentially contribute to vascular disorders [27–30, 42, 43]. Thus, the aim of the present study was to investigate the *in vitro* effects of palmitate (C16:0), the major SFA in plasma [44, 45], on the modulation of oxidative stress and inflammation in T2DM patients. Nitric oxide, with or without palmitate induction, was quantified and correlated with proinflammatory cytokines secreted in the cultured supernatant of PBMNCs from type 2 diabetes patients. The association among plasmatic triglycerides, NO, proinflammatory cytokines (IL-6 and TNF-alpha), and oxidative stress (malondialdehyde) is discussed.

2. Material and Methods

This study was approved through the Ethical Committee of Santa Casa Hospital (Belo Horizonte-MG, Brazil) and written informed consent was obtained from all participants prior to the study.

2.1. Subjects. T2DM patients ($n = 29$), diagnosed according to the criteria of the American Diabetes Association [46], and nondiabetic controls ($n = 16$), ranging from 45 to 70 years of age, were recruited from the Endocrinology Department of Santa Casa Hospital. Type 2 DM patients were treated with statins and beta-blockers in addition to hypoglycemic drugs. Prior to the study, all volunteers received complete physical examinations, and detailed evaluations of medical histories and laboratory analyses were performed (Table 1). Pregnant women and individuals suffering from alcoholism, infection, inflammation, dementia, or malignant diseases and smoking addictions were excluded from this study.

2.2. Preparation of Fatty Acids. Palmitate and low-endotoxin bovine serum albumin (BSA, FFA-free) were purchased from Sigma-Aldrich Co. FFA was dissolved in 0.1 M NaOH at 70°C and subsequently complexed with 10% BSA at 55°C for 10 min to obtain a final FFA concentration of 500 μ M (molar ratio 2.4:1) [42, 47]. A 10 mM fatty acid-albumin complex stock solution and a 0.5 μ M BSA control solution were freshly prepared, filtrated, and diluted prior to each experiment.

2.3. Preparation of Peripheral Blood Mononuclear Cells. PBMNCs were purified from 10.0 mL of heparinized venous

TABLE 1: Clinical and biochemical characteristics of the studied population.

Parameters	T2DM ($n = 29$)	ND ($n = 16$)	<i>P</i>
Female/Male ratio	19/10	11/5	NA
Age (years)	58.3 \pm 9.0	57.1 \pm 10.0	ns
Body mass index (kg/m ²)	30.8 \pm 9.8	24.6 \pm 4.1	<0.05
Disease duration (years)	6.7 \pm 6.4	NA	NA
Systolic pressure (mmHg)	127.9 \pm 14.5	122.3 \pm 15.9	ns
Diastolic pressure (mmHg)	86.6 \pm 8.6	88.9 \pm 7.9	ns
Fasting glucose (mg/dL)	147.0 \pm 40.7	89.0 \pm 9.0	<0.05
Glycated hemoglobin (%)	8.1 \pm 1.1	5.3 \pm 0.2	<0.05
Total cholesterol (mg/dL)	191.6 \pm 65.7	160.7 \pm 20.0	ns
Low density lipoprotein (mg/dL)	115.3 \pm 39.7	104.5 \pm 32.6	ns
High density lipoprotein (mg/dL)	45.6 \pm 10.6	50.2 \pm 14.0	ns
Triglycerides (mg/dL)	142.0 \pm 51.0	108.6 \pm 37.7	<0.05

Data as means \pm SD.

NA: not applicable; ns: not significant.

Significant differences between the groups were determined using Student's *t*-test ($P < 0.05$).

blood, using a Ficoll-Hypaque gradient as previously described [48], with slight modifications. The trypan blue exclusion test showed that the cell viability in all samples was >95%.

2.4. Preparation of Plasma. EDTA venous blood samples were collected using a standard venipuncture technique. The plasma was obtained through centrifugation (200 g for 15 min, at room temperature), and the samples were stored at -80°C until further analysis. Subsequent analyses were performed within 3 months from the day of storage.

2.5. Quantification of Proinflammatory Cytokines and NO in Supernatant of PBMNCs. Aliquots (100 μ L) of a PBMNC suspension ($1 \times 10^6/\text{mL}$) from T2DM patients and ND controls in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) were incubated in the presence or absence of BSA (0.5 μ M) or palmitate (500 μ M) for 72 hours at 37°C under 5% CO₂. The final volume was adjusted to 300 μ L in DMEM supplemented with 10% FBS. After incubation, the cells were centrifuged and the supernatant was collected. The interleukin-6 (IL-6 human EIA Kit—Enzo Life Sciences, Inc., New York, USA) and tumor necrosis factor-alpha (TNF- α human EIA Kit—Enzo Life Sciences, Inc., New York, USA) concentrations were determined through enzyme-linked immunosorbent assay (ELISA). Because NO is unstable, the quantitative of NO was indirectly determined based on the detection of the blood nitrite and nitrate levels. The NO concentration was measured using the Total Nitric Oxide Assay Kit (Assay Designs, Enzo Life Sciences, Inc., New York, USA).

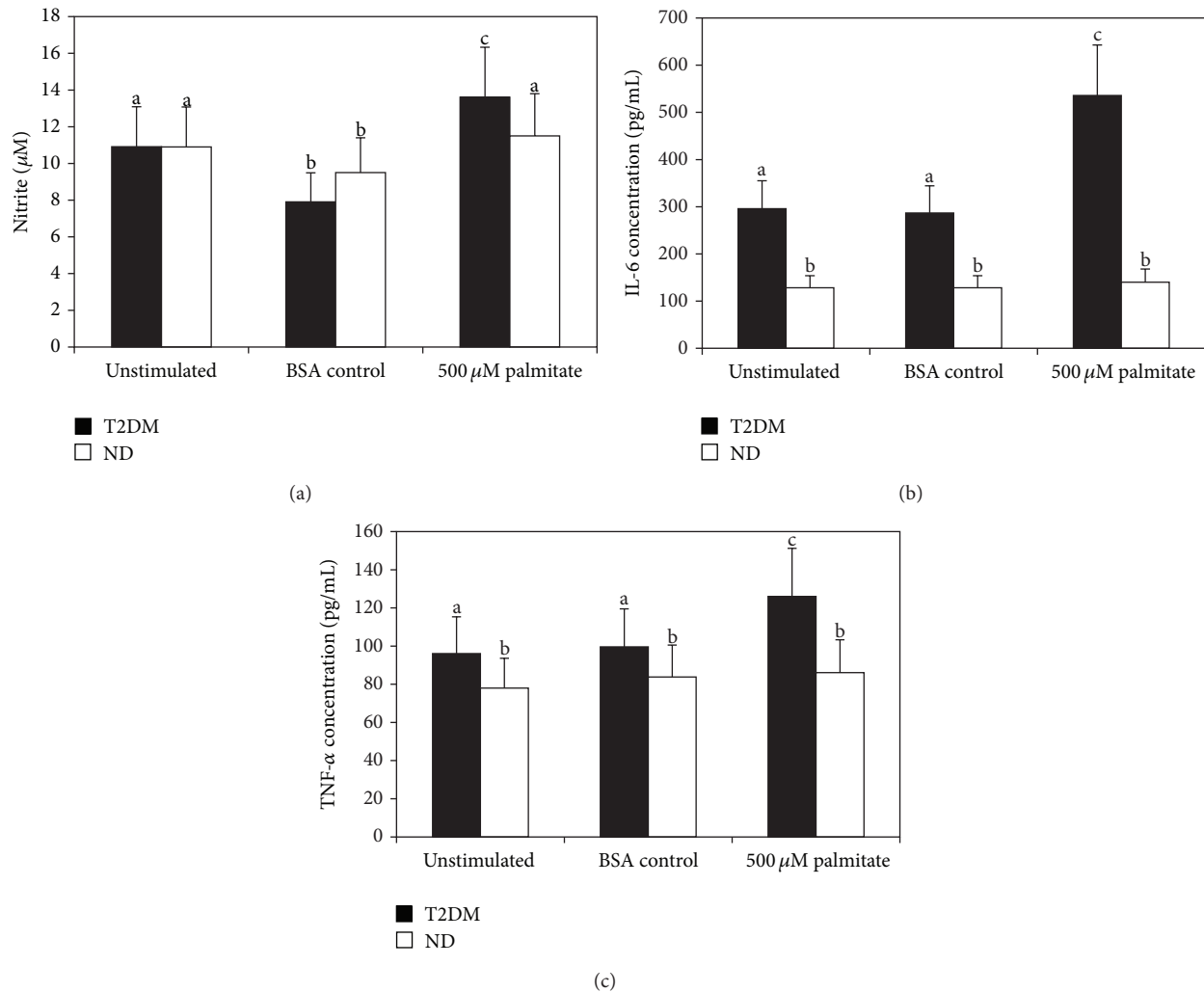


FIGURE 1: Palmitate induces NO, IL-6, and TNF- α secretion in peripheral blood mononuclear cells (PBMNC) from patients with type 2 diabetes. (a) Nitrite production; (b) IL-6 production; (c) TNF- α production. Different letters denote significance at $P < 0.05$ using Student's t -test. $n = 10$ for each group.

2.6. Quantification of NO, MDA, and Proinflammatory Cytokines in Plasma. The plasma levels of NO, IL-6, and TNF- α were determined as described above. The plasma MDA concentration was measured using the TBARS Assay Kit (ZeptoMetrix Corp., New York, USA) according to the manufacturer's instructions.

2.7. Statistical Analyses. The values are presented as the means \pm standard deviation (SD). The nonparametric Kolmogorov-Smirnov test was used to assess the normal distribution of the continuous variables. Comparisons between groups were performed using unpaired Student's t -tests. Within-group correlations were performed using Pearson's r correlation. All analyses were considered significant at P values < 0.05 using Origin 6.0 software (Microcal Software Inc., Northampton, MA, USA).

3. Result

3.1. PBMNCs from T2DM Patients Are More Sensitive to Palmitate Stimulation Than the Cells from ND Controls. As depicted in Figure 1, palmitate activated the secretion of NO, IL-6, and TNF- α in PBMNCs from T2DM patients compared with those from ND controls ($P < 0.05$). The results of the induced effect of palmitate on PBMNCs from T2DM patients and ND controls, expressed as the means \pm SD, were NO, 11.5 \pm 1.3 and 13.6 \pm 2.2; IL-6, 86.1 \pm 14.1 and 126.0 \pm 29.0; and TNF- α , 140.0 \pm 28.1 and 535.8 \pm 115, respectively. The results shown in Figure 1 also demonstrated that PBMNCs from T2DM patients secreted significantly ($P < 0.05$) higher amounts of IL-6 (256.7 \pm 81.1) and TNF- α (96.1 \pm 17.5) compared with the cells from ND controls (IL-6: 128.3 \pm 32.3, TNF- α : 78.0 \pm 13.6). No difference ($P > 0.05$) was observed in NO production in PBMNCs

from T2DM patients (10.9 ± 1.7) and ND controls (10.9 ± 1.2) without stimulation.

The production of NO and proinflammatory cytokines was not altered in the presence of BSA ($P > 0.05$) in T2DM patients and ND controls: NO, 11.5 ± 1.3 and 13.6 ± 2.2 ; IL-6, 86.1 ± 14.1 and 126.0 ± 29.0 ; and TNF-alpha, 140.0 ± 28.1 and 535.8 ± 115 , respectively.

3.2. Palmitate-Induced NO and IL-6 Production in PBMCs Are Associated in T2DM Patients, but Not in ND Controls. Figure 2 shows the Pearson's correlations between the levels of NO, IL-6, and TNF-alpha in PBMCs from T2DM patients and ND controls after palmitate stimulation. The correlation between NO and IL-6 were significantly strong in stimulated PBMCs from T2DM patients ($r = 0.63$, $P = 0.04$) and moderate in PBMCs from ND ($r = 0.47$, $P = 0.17$). No correlation was observed between NO and TNF-alpha in PBMCs from T2DM patients and ND controls.

3.3. The Plasma MDA and Proinflammatory Cytokine (IL-6 and TNF-Alpha) Concentrations Are Elevated in T2DM. Table 2 shows that T2DM patients had enhanced plasma concentrations of MDA, IL-6, and TNF-alpha compared with ND ($P < 0.05$). No difference was observed in NO levels between T2DM patients and ND ($P > 0.05$). The results, expressed as the means \pm SD, were MDA, 14.5 ± 3.5 and 8.7 ± 3.3 ; IL-6, 119.1 ± 23.3 and 97.6 ± 13.5 ; TNF-alpha, 78.7 ± 32.7 and 58.5 ± 29.5 ; NO, 53.5 ± 12.9 and 51.13 ± 8.7 , for T2DM patients and ND controls, respectively.

3.4. Plasmatic Nitric Oxide Levels Correlate with MDA and IL-6 Levels in the Plasma from T2DM Patients. Correlations between the levels of NO and IL-6 and TNF-alpha and MDA are shown in Figure 3. Strong positive correlation was observed between NO and IL-6 in T2DM patients ($r = 0.72$, $P < 0.0001$). The results also demonstrated a significantly negative correlation between NO and MDA in T2DM patients ($r = -0.47$, $P = 0.0093$).

3.5. Plasmatic Triglyceride Levels in T2DM Patients Correlate with the Plasma Levels of MDA, IL-6, and TNF-Alpha. Figure 4 shows the Pearson's correlations between the levels of triglyceride and NO and IL-6 and TNF-alpha in the plasma from T2DM patients and ND. The triglyceride levels were positively correlated with MDA ($r = 0.43$, $P = 0.018$), IL-6 ($r = 0.52$, $P = 0.003$), and TNF-alpha ($r = 0.37$, $P = 0.048$) in the plasma of T2DM patients.

3.6. Plasmatic Glucose Levels in T2DM Patients Correlate with the Plasma Levels of Triglycerides, MDA, IL-6, and TNF-Alpha. Figure 5 shows the Pearson's correlations between the levels of glucose and triglycerides, NO, MDA, and proinflammatory cytokines levels in the plasma from T2DM patients and ND controls. The glucose levels were positively correlated with triglycerides ($r = 0.40$, $P = 0.03$), MDA ($r = 0.60$, $P = 0.0006$), IL-6 ($r = 0.40$, $P = 0.04$), and TNF-alpha ($r = 0.35$, $P = 0.05$) in the plasma of T2DM patients.

TABLE 2: Plasma levels of oxidative stress biomarkers and proinflammatory cytokines.

Parameter	T2DM ($n = 29$)	ND ($n = 16$)	P
Nitric Oxide (μM)	53.5 ± 12.9	51.13 ± 8.7	ns
MDA (μM)	14.5 ± 3.5	8.7 ± 3.3	<0.05
IL-6 (pg/mL)	119.1 ± 23.3	97.6 ± 13.5	<0.05
TNF-alpha (pg/mL)	78.7 ± 32.7	58.5 ± 29.5	<0.05

Data as means \pm SD.

ns: not significant.

Significant differences between the groups were determined using Student's t -test ($P < 0.05$).

4. Discussion

The results obtained in the present study showed that hyperglycemia in diabetes primes PBMCs *in vivo*, inducing the *in vitro* upregulation of NO and proinflammatory cytokines in cells stimulated with palmitate. The plasmatic evaluation demonstrated greater levels of triglycerides, MDA, IL-6, and TNF-alpha in T2DM patients compared with ND. No difference was observed in the NO plasma levels between T2DM patients and ND. In addition, the results of this study revealed that the levels of NO were correlated with MDA and IL-6, and levels of triglycerides were correlated with MDA, IL-6, and TNF-alpha in the plasma from T2DM patients.

Diabetes is a multifactorial disease characterized by hyperglycemia and hyperlipidemia, which are important risk factors for endothelial dysfunction resulting in cardiovascular events [49]. FFAs, particularly SFA, have been shown to induce a proinflammatory profile associated with obesity, T2DM, insulin resistance, and dyslipidemia [4, 8–11]. The results presented herein show the inflammatory effects of the saturated fatty acid palmitate on PBMCs from T2DM patients but not in cells from ND (Figure 1), suggesting that hyperglycemia plays a role in palmitate-induced inflammation. Studies have shown that the combined effect of high glucose and FFA levels in human monocytes modulate macrophage proliferation involving glucose-dependent oxidation of LDL, potentiate cytotoxic effects via superoxide overproduction, and amplify inflammation via TLR [21, 50, 51]. However, Tripathy et al. [32] demonstrated that an increase in FFA concentration induces oxidative stress and inflammation in human leukocytes from ND subjects. These discrepancies might be associated with differences in the experimental protocols.

The inflammatory changes observed in the presence of palmitate could be associated with NF-kappaB activation [21, 28, 32, 52–55]. NF-kappaB is a key mediator that regulates immune and inflammatory responses and modulates multiple proinflammatory target genes in endothelial cells, vascular smooth muscle cells, and macrophages [56]. The activation of NF-kappaB leads to the increased production of adhesion molecules, leukocyte-attracting chemokines, various inflammatory cytokines, including TNF-alpha and IL-6, and NO through iNOS expression [57–60].

NO has anti- or proinflammatory properties [61]. NO plays an important role in vascular homeostasis, and in

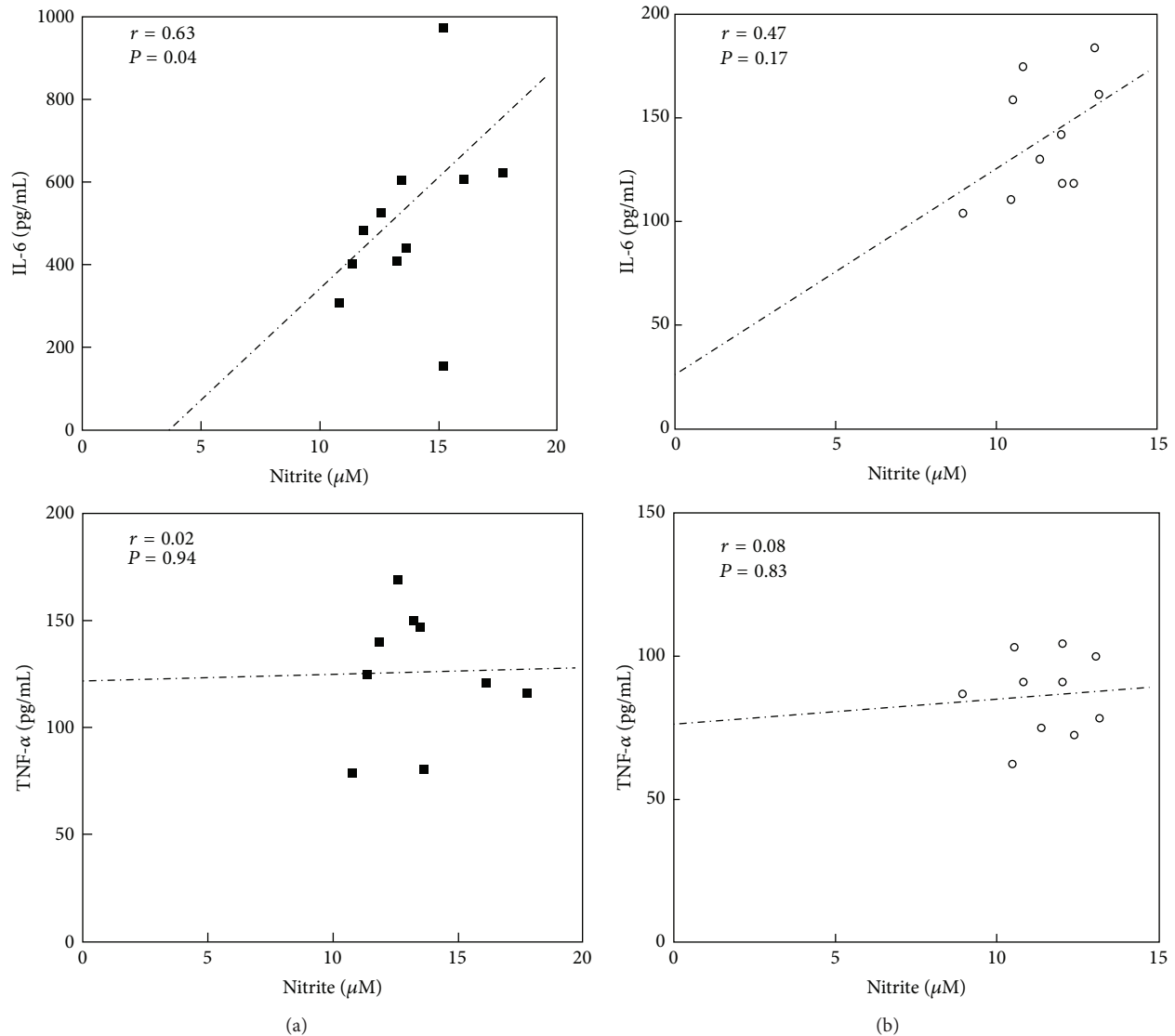


FIGURE 2: Pearson's correlation coefficients between NO and proinflammatory cytokines in PMBNCs from T2DM patients (a) and nondiabetic controls (b) after stimulation with palmitate. $n = 10$ for each group.

immune cells, NO regulates antimicrobial and antitumor activities, although excess NO production might cause tissue damage and is associated with acute and chronic inflammation [56, 62]. Nitric oxide synthase (NOS) synthesizes NO from L-arginine using NADPH and oxygen as cosubstrates [63]. Three isoforms of NO synthase have been described: neuronal (nNOS or NOS 1), inducible (iNOS or NOS 2), and endothelial (eNOS or NOS 3) [64]. Activated macrophages and neutrophils produce large amounts of NO through iNOS activity [65, 66]. The results of this study demonstrated increased NO production and a positive correlation between NO and IL-6 levels in palmitate-stimulated PMBNCs from T2DM patients, suggesting that iNOS expression can be elevated through palmitate-induced proinflammatory cytokine secretion. No differences were observed in the cells from ND controls (Figures 1 and 2). Unbound palmitic acid treatment increased NO production in skeletal muscle [67]. However,

in endothelial cells, FFA induced the inhibition of eNOS, thereby attenuating NO production [68–71].

To evaluate *in vivo* inflammation, we quantified the plasma levels of NO, the oxidative stress biomarker (MDA), and proinflammatory cytokines (IL-6 and TNF- α) in T2DM patients and ND controls. Consistent with other studies [72–93], the results of the present study demonstrated elevated levels of IL-6 and TNF- α , reflecting the activation of innate immune cells, and high levels of MDA, indicating the presence of oxidative stress in T2DM patients compared with ND controls. Diabetic conditions (hyperglycemia and hyperlipidemia) increase proinflammatory and oxidative stress levels, culminating in endothelium dysfunction [1, 27, 42, 56, 90, 94, 95]. Oxidative stress reduced NO production through eNOS [56], and the increased levels of superoxide could react with NO to produce peroxynitrite, a highly toxic product [23, 96]. Peroxynitrite nitrates

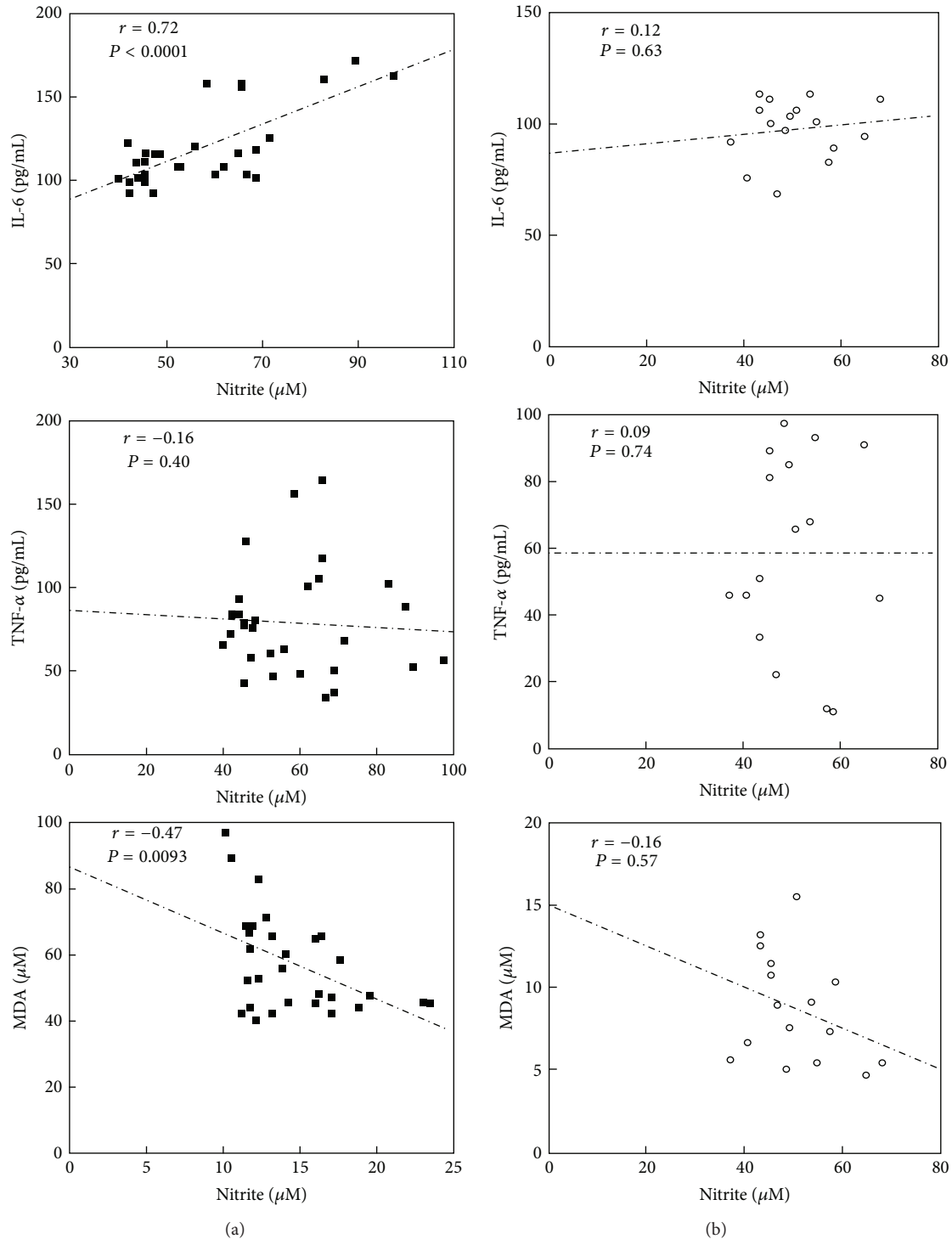


FIGURE 3: Pearson's correlation coefficients between nitric oxide and proinflammatory cytokines and MDA in the plasma from T2DM patients (a) and nondiabetic controls (b). $n = 29$ for T2DM patients and 16 for nondiabetic controls.

the tyrosine residues in a number of proteins and modulates their functions [97, 98]. The results in the present study did not show any differences in the plasma NO levels between the studied groups (Table 2). However, we observed a negative association between NO and MDA levels in the plasma from T2DM patients, suggesting that increased oxidative

stress could affect NO bioavailability, leading to endothelial dysfunction in diabetes (Figure 3).

The results obtained in the present study also demonstrated high levels of triglycerides in the plasma from T2DM patients compared with ND controls (Table 1). FFAs are stored in the body in the form of triglycerides and are released

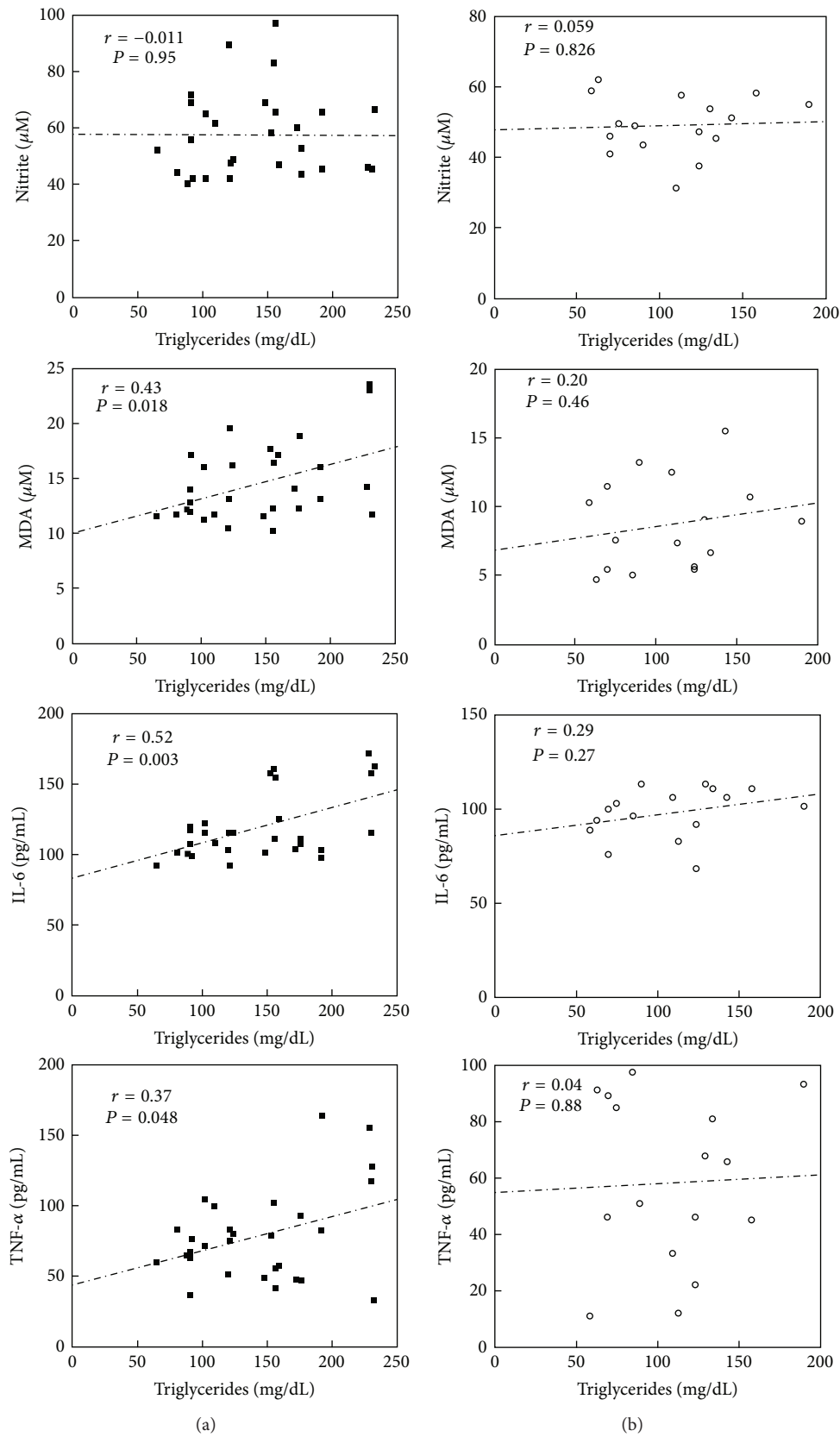


FIGURE 4: Pearson's correlation coefficients between triglycerides and proinflammatory cytokines and MDA in the plasma of T2DM patients (a) and nondiabetic controls (b). $n = 29$ for T2DM patients and 16 for nondiabetic controls.

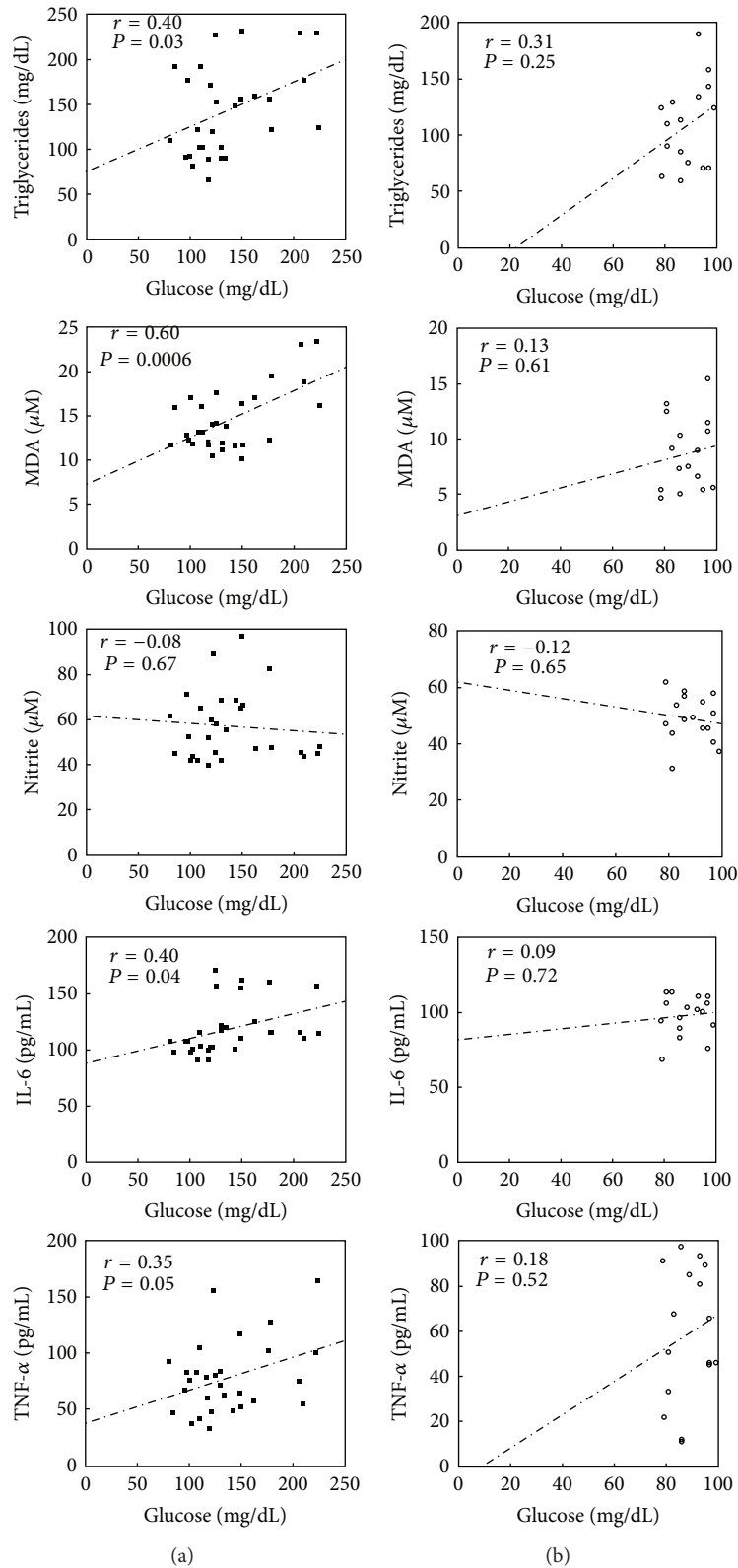


FIGURE 5: Pearson's correlation coefficients between glucose and triglycerides, MDA, nitric oxide, and proinflammatory cytokines in plasma of T2DM (a) patients and nondiabetic controls (b). $n = 29$ for T2DM and 16 for nondiabetic controls.

into tissues through lipolysis, a process regulated through insulin [99]. Impaired insulin signaling increases lipolysis, resulting in increased FFA levels [100, 101]. The results of the present study showed that triglycerides levels are positively associated with the MDA, IL-6, and TNF-alpha levels in the plasma from T2DM patients, but this correlation was not observed in the plasma from ND controls. No correlation was observed between triglycerides and NO in the plasma from the studied groups (Figure 4). Glucose levels are positively correlated with the triglycerides, MDA, IL-6, and TNF-alpha levels in the plasma from T2DM patients, but not in the plasma from ND controls (Figure 5).

Accumulating evidence has shown that the regulation of dyslipidemia is of equal importance for the regulation of hyperglycemia and hypertension in the care of patients with T2DM. Hyperlipidemia represents a major risk factor for the development of vascular dysfunction and atherosclerosis [27–30, 42, 43]. Most T2DM patients are obese and have elevated plasma FFA levels [102, 103]. Moreover, high-fat diets might induce metabolic dysfunction and inflammation through the release of FFA through lipolysis and proinflammatory cytokines through downstream signaling [104, 105].

FFAs have been suggested to induce chronic low-grade inflammation, activate the innate immune system, and cause deleterious effects on vascular cells and other tissues through inflammatory processes. The results obtained in the present study demonstrated that hyperglycemia in diabetes exacerbates *in vitro* inflammatory responses in PBMNCs stimulated with high levels of SFA (palmitate). Furthermore, the results suggest that the endothelium levels of NO could be regulated through oxidative stress and high levels of triglycerides are correlated with oxidative stress and proinflammatory cytokine secretion in T2DM patients. Endothelial dysfunction is associated with several pathophysiological conditions in diabetes [56]. Combined therapy targeting the intracellular mechanisms underlying metabolic alterations leading to endothelial dysfunction is an important issue in the prevention of vascular complications associated with diabetes. The simultaneous control of hyperglycemia and hypertriglyceridemia is necessary to ameliorate the progression to diabetic vasculopathy.

Conflict of Interests

The authors confirm that there is no conflict of interests.

Acknowledgments

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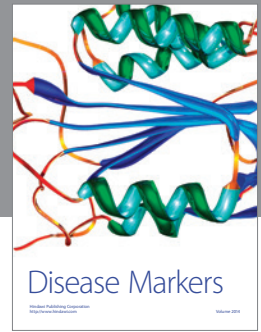
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