Journal of Diabetes

Journal of Diabetes **6** (2014) 167–175

ORIGINAL ARTICLE

Oxidants, antioxidants and mitochondrial function in non-proliferative diabetic retinopathy

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Received 29 May 2013; accepted 14 July 2013.

doi: 10.1111/1753-0407.12076

Abstract

Background: Diabetic retinopathy (DR) is a preventable cause of visual disability. The aims of the present study were to investigate levels and behavior oxidative stress markers and mitochondrial function in non-proliferative DR (NPDR) and to establish the correlation between the severity of NPDR and markers of oxidative stress and mitochondrial function.

Methods: In a transverse analysis, type 2 diabetes mellitus (T2DM) patients with mild, moderate and severe non-proliferative DR (NPDR) were evaluated for markers of oxidative stress (i.e. products of lipid peroxidation (LPO) and nitric oxide (NO) catabolites) and antioxidant activity (i.e. total antioxidant capacity (TAC), catalase, and glutathione peroxidase (GPx) activity of erythrocytes). Mitochondrial function was also determined as the fluidity of the submitochondrial particles of platelets and the hydrolytic activity of F_0/F_1-ATPase.

Results: Levels of LPO and NO were significantly increased in T2DM patients with severe NPDR (3.19 ± 0.05 μmol/mL and 45.62 ± 1.27 pmol/mL, respectively; $P < 0.007$ and $P < 0.0001$ vs levels in health volunteers, respectively), suggesting the presence of oxidative stress. TAC had significant decrease levels with minimum peak in severe retinopathy with 7.98 ± 0.48 mEq/mL ($P < 0.0001$). In contrast with TAC, erythrocyte catalase and GPx activity was increased in patients with severe NPDR (139.4 ± 4.4 and 117.13 ± 14.84 U/mg, respectively; $P < 0.0001$ vs healthy volunteers for both), suggesting an imbalance between oxidants and antioxidants. The fluidity of membrane submitochondrial particles decreased significantly in T2DM patients with mild, moderate, or severe NPDR compared with that in healthy volunteers ($P < 0.0001$ for all). Furthermore, there was a significant increase in the hydrolytic activity of the F_0/F_1-ATPase in T2DM patients with mild NPDR (265.07 ± 29.55 nmol/PO_4; $P < 0.0001$ vs healthy volunteers), suggesting increased catabolism.

Conclusions: Patients with NPDR exhibit oxidative deregulation with decreased membrane fluidity of submitochondrial particles and increased systemic catabolism (mitochondrial dysfunction) with the potential for generalized systemic damage in T2DM.

Keywords: diabetes mellitus, diabetic retinopathy, membrane fluidity, nitrosative stress, oxidative stress.

Significant findings of the study: There were significant increases in lipid peroxidation (LPO) and nitric oxide (NO) from the early stages of non-proliferative diabetic retinopathy (NPDR). Progressive increases in LPO and NO levels are associated with the severity of NPDR, with an imbalance between oxidants, antioxidants and mitochondrial function.
What this study adds: Decreased fluidity of membrane submitochondrial particles of platelets and increased F_0/F_1-ATPase hydrolytic activity are sensitive markers of mitochondrial dysfunction that indicate a catabolic state from the early stages of NPDR.

Introduction

Diabetic retinopathy (DR) results in progressive visual deterioration, ranging from mild, moderate, severe to very severe non-proliferative DR (NPDR), with a risk of developing proliferative DR (PDR).[1,2] The hyperglycemia in diabetes mellitus (DM) is implicated in the apoptosis of vascular and neuronal cells of the retina, because the rate of DR is high among individual with long-term type 2 DM (T2DM).[3] Increases in HbA1c levels are associated with risk of DR, with decreases in HbA1c (achieved through a combination of effective clinical management and a healthy lifestyle), demonstrated to reduce the risk of microvascular complications of the retina.[4] Reactive oxygen species (ROS) are contributors to oxidative stress and include superoxide anion (O_2^-) and hydroxyl radical ($OH\cdot$).[5] The O_2^- is converted to hydrogen peroxide by superoxide dismutase (SOD), and can be converted to singlet oxygen with the capacity to react with polyunsaturated fatty acids of the lipid layer in the cellular membrane, resulting in lipid peroxidation (LPO).[6] At physiological concentrations, nitric oxide (NO) moderates cellular respiration.[7] However, at increased concentrations, NO produces nitrosative stress by oxidation of adducts of nitric oxide synthase (NOS) where peroxynitrite ($OONO^-$) favors the appearance of reactive nitrogen species (RNS). Overproduction of ROS and RNS impedes the correct cellular homeostasis that is associated with DM.[8] Increases in NO levels in T2DM are associated with the pathogenesis of DR.[9] Mitochondrial dysfunction in DR and the disruption of cellular homeostasis in DM play critical roles in retinal cell death due to changes in mitochondrial morphology, potential heterogeneity of mitochondrial membranes, the rate of oxygen consumption, and poor protein folding promote or favor the disappearance of vascular cells in the retina.[10] The aim of the present study was to investigate levels of oxidative stress markers and mitochondrial function in NPDR.

Methods

The present study was a transverse analytical study on 270 T2DM patients (aged between 30 and 75 years) who were unaware of their DR status, with HbA1c ≤12%. Of these patients, 12, 18, and 21 were found to have mild, moderate, and severe NPDR, respectively. Healthy age- and sex-matched volunteers without DM were included as a control group.

Laboratory tests were performed to determine levels of LPO and NO as oxidative stress markers, as well as the total antioxidant capacity (TAC) and catalase and glutathione peroxidase (GPx) activity of erythrocytes. Mitochondrial function was evaluated as the fluidity of submitochondrial particles of platelets and the hydrolytic activity of F_0/F_1-ATPase.

Blood samples

Peripheral blood (10 mL) samples were obtained from all study participants after an 8-h overnight fast. Serum and platelets were separated and stored at –80°C until processing.

Products of LPO and NO

Levels of LPO were evaluated using a commercially available kit (FR12; Oxford Biomedical Research, Oxford, MI, USA) according to the manufacturer's instructions. This assay is based on the reaction of the chromogenic reagent *N*-methyl-2-phenylindole with malondialdehyde (MDA) and 4-hydroxyalkenals (4HDA). Briefly, 200 μL serum was added to 455 μL of *N*-methyl-2-phenylindol in acetonitrile (Reagent 1) diluted with ferric acid in methanol. Samples were agitated before 105 μL methanesulfonic acid was added, with the mixture incubated at 45°C for 60 min. Samples were then centrifuged at 15 000*g* for 10 min at room temperature. Then, 200 μL supernatant was placed in a microplate and the absorbance of the sample was measured at 586 nm using a Synergy HT Multi-detection microplate reader (BioTek Instruments, Winooski, VT, USA). Standard curves using known concentrations of the standard 1,1,3,3-tetramethoxypropane in Tris-HCl were constructed.

To determine levels of NO catabolites, serum samples were deproteinized by the addition of 6 mg zinc sulfate to a 400-μL aliquot of each sample, agitation for 1 min and centrifugation at 10 000 *g* for 10 min at 4°C. The supernatant was removed and stored at –80°C.[11] Levels of NO

catabolites were determined using a colorimetric method (Nitric Oxide Assay Kit, user protocol 482650; Calbiochem, a brand of EMD Chemicals Inc., an affiliate of Merck, Darmstadt, Germany), according to the manufacturer's instructions. Briefly, 85 μL standard or sample was plated onto a microplate and then 10 μL nitrate reductase and 10 μL of 2 mmol/L NADH were added. Plates were agitated for 20 min at room temperature, after which 50 μL Colorant 1 was added and the samples agitated again, followed by the addition of 50 μL Colorant 2 and agitation at room temperature. The absorbance of the samples was determined at 540 nm using a Synergy HT Multi-detection microplate reader (BioTek Instruments).

Total antioxidant capacity

To determine TAC, a commercially available colorimetric kit was used (Total Antioxidant Power Kit, No. TA02.090130, Oxford Biomedical Research) according to the manufacturer's instructions. Standards and samples were diluted 1:40 using dilution buffer provided in the kit, and 200 μL was placed in each well of a microplate. A reference value was obtained by determining the absorbance of the plates at 450 nm, after which 50 μL copper solution was added and samples were incubated for 3 min at room temperature. Then, 50 μL stop solution was added and absorbance determined again at 450 nm. Results for TAC are expressed as mmol/L uric acid equivalents. The dilution factor was considered in the final results.

Catalase

To determine catalase activity, 500 μL substrate (10 mmol/L of H_2O_2) was added to 30 μL diluted standard or sample. Erythrocytes were incubated for 1 min at room temperature before the addition of 500 μL stop reagent (Bioxytech Catalase-520, catalogue no. 21042; OXIS International, Foster City, CA, USA). Assay tubes were and mixed by inversion and then 20 μL of each reaction mixture was placed in another assay tube. Finally, 2 mL horseradish peroxidase-reactive chromogen was added to the samples, which were mixed by inversion, and incubated for 10 min at room temperature. The absorbance of the samples was determined at 520 nm using a Synergy HT Multi-detection microplate reader (BioTek Instruments).

Glutathione peroxidase

Glutathione peroxidase activity was determined using a commerically available kit (Bioxytech GPx-340, catalogue no. 21017; OXIS International). Determination of GPx activity was based on the oxidation of reduced glutathione in presence of *tert*-butyl hydroperoxide, glutathione reductase, and NADPH. The decrease in absorbance at 340 nm following substrate addition was recorded, with the rate of decrease in absorbance directly proportional to GPx activity in the samples.

Fluidity of the submitochondrial membrane in platelets

Submitochondrial membranes of platelets were obtained by centrifuging blood samples at 2980*g* for 15 min at 4°C. The supernatant was removed and 200 μL cold buffer was added (composition [in mmol/L]: NaCl 140; KCl 4.7; $MgCl_2$ 1.2; KH_2PO_4 1.2; dextrose 11; HEPES 15) to the pellet and the samples homogenized. An aliquot of 70-μL of the samples, containing platelets, was stored at –80°C. Membrane fluidity was evaluated by incorporation of 1,3 dipyrenylpropane (DPyP) into the biological membranes.[12] Briefly, 2 mL Tris-HCl buffer (pH 7.8; 10 mmol/L) was added to a 35-μL aliquot of the submitochondrial membranes and mixed with a pipette at 24°C. Using a fluorescence spectrophotometer (L550B; Perkin-Elmer, Waltham, MA, USA), the fluorescence intensity of the monomer (I_m) and excimer (I_e) were determined at 395 and 494 nm, respectively. Then, 5 μL (0.1 μg) DPyP was added to the samples, which were incubated at room temperature in the dark for 3 h to enable incorporation of DPyP into the membranes. Fluorescence intensity second was again determined at 395 and 494 nm, and the I_e/I_m ratio calculated. The ratio I_e/I_m is directly related to membrane fluidity.

Hydrolysis of ATP

The hydrolytic activity of mitochondrial F_0/F_1-ATPase was measured as the liberation of inorganic phosphate from platelet mitochondria. Briefly, 3 mmol/L $MgCl_2$, 30 μL sample and 20 μL ATP (100 mmol/L) were added to 1 mL ATPase buffer (125 mmol/L KCl, 40 mmol/L of 3-[*N*-morpholino]propanesulfonic acid [MOPS], pH 8) and agitated. The tubes were incubated at 40°C for 10 min to permit the reaction to occur. The activity of the ATPase was stopped by the addition of 200 μL of 30% trichloroacetic acid. Samples were centrifuged at 1160*g* for 10 min at room temperature. Then, 800 μL supernatant was mixed with 1 mL of 3.3% ammonium molybdate and 100 μL of 10% ferrous sulfate. Samples were incubated for 20 min at room temperature before absorbance was determined at 660 nm. Results are expressed as nmol phosphate.

Statistical analysis

Results are expressed as the mean ± SEM (standard error of the mean). The significance of differences between groups was evaluated using Student's *t*-test or

Table 1 Demographic data and results of laboratory tests

	Non-proliferative diabetic retinopathy			
	Mild	Moderate	Severe	*P*-value
No. (%) men/women	7 (58%)/5 (42%)	12 (67%)/6 (33%)	19 (48%)/11 (52%)	NS
Age (years)	61.58 ± 1.81	58.72 ± 1.92	58.61 ± 2.09	NS
Duration of DM (years)	14.17 ± 1.66	14.22 ± 1.46	15.00 ± 1.27	NS
BMI (kg/m^2)	27.32 ± 1.00	29.32 ± 0.66	28.00 ± 1.00	NS
SBP (mmHg)	122.83 ± 4.86	130.05 ± 3.57	140.00 ± 4.05	NS
DBP (mmHg)	77.58 ± 2.80	78.72 ± 2.12	81.00 ± 1.92	NS
RIOP (mmhg)	15.75 ± 0.78	15.33 ± 0.47	15.00 ± 0.45	NS
LIOP (mmHg)	15.80 ± 0.72	15.80 ± 0.48	15.00 ± 0.45	NS
HbA1c (%)	8.21 ± 0.50	8.98 ± 0.40	9.00 ± 0.46	NS
Glucose (mg/dL)	123.66 ± 6.29	148.77 ± 11.76	142.00 ± 13.54	NS
Urea (mg/dL)	27.75 ± 2.24	38.44 ± 3.50	37.00 ± 2.58	NS
Creatinine (mg/dL)	0.81 ± 0.05	0.89 ± 0.06	1.00 ± 0.06	NS
Uric acid (mg/dL)	5.06 ± 0.32	5.78 ± 0.20	6.00 ± 0.27	NS
AST (IU/L)	28.08 ± 3.04	28.11 ± 1.96	31.00 ± 2.49	NS
ALT (IU/L)	31.00 ± 3.09	32.00 ± 3.43	31.00 ± 2.34	NS
Total bilirubin (mg/dL)	0.56 ± 0.06	0.54 ± 0.04	0.46 ± 0.03	NS
Direct bilirubin (mg/dL)	0.24 ± 0.03	0.26 ± 0.03	0.18 ± 0.03	NS
TC (mg/dL)	167.41 ± 8.93	199.66 ± 9.69	202.81 ± 7.87	NS
LDL (mg/dL)	90.55 ± 8.93	103.70 ± 6.86	123.75 ± 16.93	NS
HDL (mg/dL)	43.08 ± 2.62	42.38 ± 1.61	46.10 ± 3.56	NS
TG (mg/dL)	168.91 ± 25.22	271.22 ± 32.46	244.15 ± 30.81	NS

Unless indicated otherwise, data are the mean ± SEM (standard error of the mean).
DM, diabetes mellitus; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; RIOP, right intraocular pressure; LIOP, left intraocular pressure; AST, aspartate amino transferase; ALT, alanine amino transferase; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides.

the Mann–Whitney test, as appropriate. Multivariate analysis (ANOVA) or the Kruskal-Wallis test were used to compare values among patient groups with mild, moderate and severe NPDR. Frequencies were determined per category, as well as percentages and the χ^2 (or Fisher's exact) test. Spearman's correlation test was performed to establish the non-parametric measure of statistical dependence between variables. $P < 0.05$ was considered significant; the confidence interval was 95%.

Ethical considerations

This study was performed in accordance with the updated Declaration of Helsinki (http://www.wma.net/es/30publications/10policies/b3/17c.pdf, accessed January 2011) and in agreement with local and national laws (Mexico). Coded numbers were assigned to ensure patient confidentiality. The study protocol and informed consent were reviewed and approved by the Ethics and Research Committee of the Social Security Institute of Mexico (registration no. R-2012-785-040).

Results

As indicated in Table 1, patients with mild NPDR were significantly older than the other patient groups and control. However, there were no significant differences among patients with mild, moderate, and severe NPDR in terms of demographics, metabolic and hemodynamic parameters, intraocular pressures, liver enzymes, renal function, and lipid profiles (Table 1). Uric acid levels increased with the severity of the retinopathy ($P < 0.05$). Glucose and HbA1c levels were high in all three groups with NPDR compared with levels in healthy volunteers, which could indicate incomplete assistance by health services or inadequate self-management. All patients were obese and had dyslipidemia, with decreased high-density lipoprotein–cholesterol and increased low-density lipoprotein, total cholesterol and triglyceride levels.

Lipid peroxidation products

Levels of LPO products in healthy controls were 0.98 ± 0.13 μmol/L. These increased significantly with the severity of retinopathy from 1.42 ± 0.08 to 2.49 ± 0.05, and 3.29 ± 0.05 μmol/L (mild, moderate, and severe NPDR, respectively; $P < 0.017$; Fig. 1a), suggesting the presence of oxidative stress in NPDR. Because LPO is a well-established mechanism of cellular lesions, these findings suggest cellular and systemic oxidative stress.

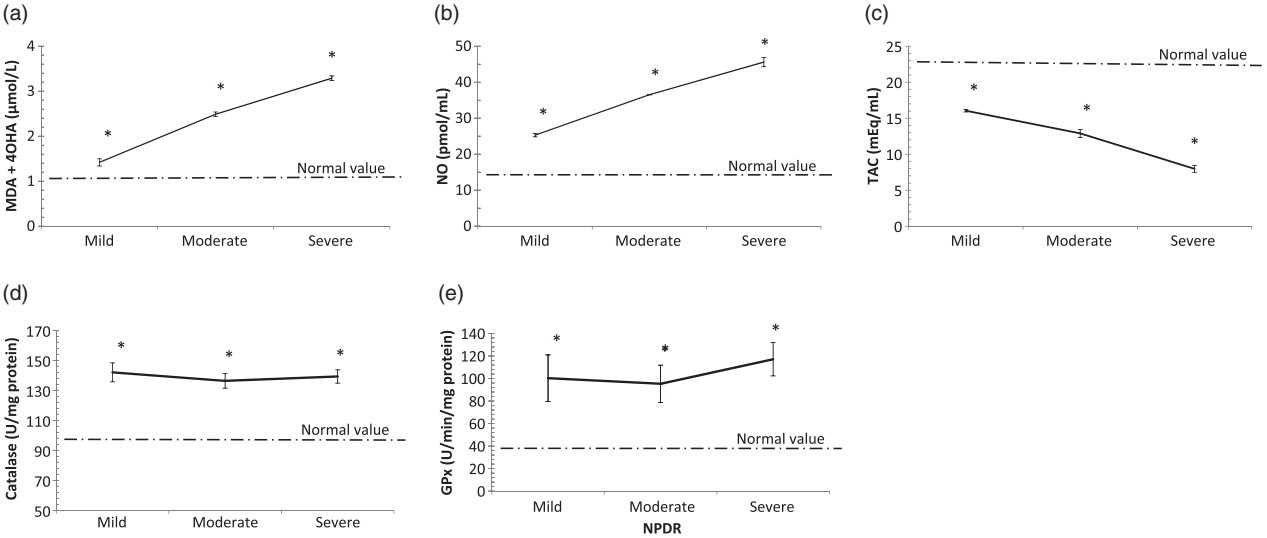

Figure 1 Oxidants (a,b) and antioxidants (c–e) in type 2 diabetes mellitus patients with mild, moderate, or severe non-proliferative diabetic retinopathy (NPDR). (a) Products of lipid peroxidation (LPO), given as malondialdehyde (MDA) and 4-hydroxyalkenals (4HDA), were highest in patients with severe NPDR (3.29 ± 0.05 µmol/L; $P < 0.017$ compared with control), as were levels of (b) nitric oxide (NO) catabolites (45.62 ± 1.27 pmol/mL; $P < 0.0001$ compared with control). (c) Total antioxidant capacity (TAC) was significantly decreased in all three groups of NPDR, with the greatest decrease seen in patients with severe NPDR (7.98 ± 0.48 mEq/mL; $P < 0.0001$ compared with control). (d,e) Erythrocyte catalase and glutathione peroxidase (GPx) activity was higher in patients with NPDR, with maximum catalase activity seen in patients with mild retinopathy (142 ± 6 U/mg protein; $P < 0.0001$ compared with control), whereas GPx activity was highest in those with severe retinopathy (117 ± 15 U/min/mg protein; $P < 0.0001$ compared with control). Data are the mean ± SE. *$P < 0.05$ compared with healthy volunteers.

Nitrites/nitrates

Levels of the NO catabolites nitrites/nitrates (NO_x) in healthy controls were 12.31 ± 1.15 pmol/mL. The NO_x levels increased significantly with the severity of retinopathy from 25.32 ± 0.46 to 36.56 ± 0.05, and 45.62 ± 1.27 pmol/mL (mild, moderate, and severe NPDR, respectively; $P < 0.0001$; Fig. 1b). These findings suggest nitrosative stress in NPDR.

Total antioxidant capacity

The TAC offers a complete picture of the function of cellular antioxidant systems. In healthy controls, uric acid values were 22.41 ± 1.10 mEq/mL, which decreased significantly with the severity of retinopathy from 16.05 ± 0.17 to 12.90 ± 0.55, and 7.98 ± 0.48 mEq/mL (mild, moderate, and severe NPDR, respectively; $P < 0.0001$; Fig. 1c).

Catalase activity

Erythrocyte catalase activity increased significantly in all three NPDR groups (142.12 ± 6.30, 136.45 ± 4.83, and 139.40 ± 4.42 U/mg protein for mild, moderate, and severe NPDR, respectively) compared with the healthy controls (94.17 ± 1.58 U/mg protein; $P < 0.0001$; Fig. 1d).

Glutathione peroxidase activity

In the healthy controls, GPx activity was 35.13 ± 2.74 U/min/mg protein. As for catalase activity, GPx activity increased significantly in T2DM patients with mild, moderate, and severe NPDR (100.32 ± 20.68, 95.32 ± 16.54, and 117.13 ± 14.84 U/min/mg protein, respectively; $P < 0.0001$ vs control; Fig. 1e). It is possible that the greater catalase and GPx activity in NPDR could be due to a compensatory response to the oxidative stress caused by a persistent metabolic disorder as a result of uncontrolled pharmacological treatment of T2DM.

Submitochondrial membrane fluidity

The I_e/I_m ratio in the healthy control group was 0.24 ± 0.01. The I_e/I_m ratio decreased significantly in T2DM patients with mild, moderate, and severe NPDR (0.12 ± 0.01, 0.16 ± 0.02, and 0.13 ± 0.01, respectively; $P < 0.0001$). The decrease in fluidity compared with the control group could be related to the effect that the fluidity of the membrane has on membrane potential induced by the increase in HbA1c, dyslipidemia, oxidative stress or their combination (Fig. 2a).

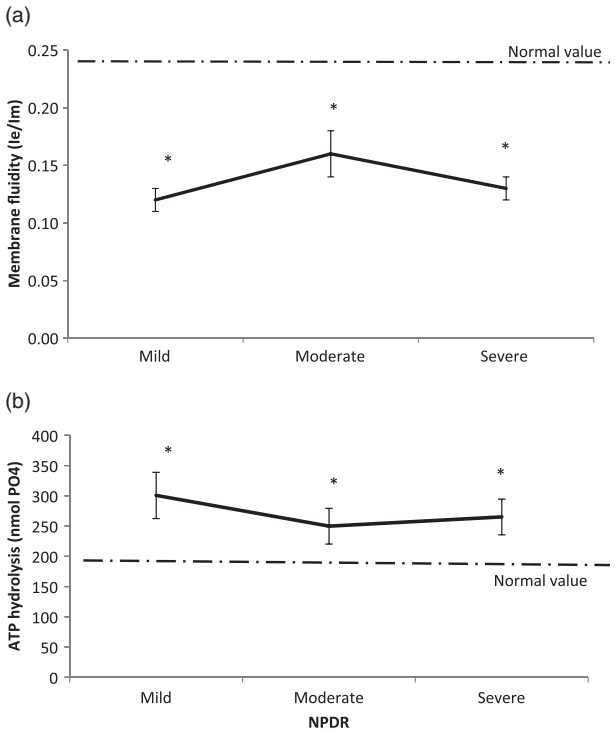

Figure 2 Mitochondrial function in type 2 diabetes mellitus patients with mild, moderate, or severe non-proliferative diabetic retinopathy (NPDR). (a) The fluorescence ratio of the excimer (I_e) to monomer (I_m) decreased significantly in patients with NPDR, with the greatest decrease seen in patients with mild retinopathy (I_e/I_m 0.12 ± 0.01; $P < 0.0001$ compared with control). (b) Significant increases were seen in the hydrolytic activity of F_0/F_1-ATPase in all three groups of NPDR, with the greatest increase seen in patients with mild retinopathy (300 ± 38 nmol PO_4; $P < 0.0001$ compared with control). This could mean lower ATP synthesis with excessive production of cellular catabolism. Data are the mean ± SE. *$P < 0.05$ compared with healthy volunteers.

Hydrolysis of F_0/F_1-ATPase

Significant increases were observed in the hydrolytic activity of F_0/F_1-ATPase in T2DM patients with mild, moderate, and severe NPDR (301 ± 38, 250 ± 29, and 265 ± 30 nmol PO_4, respectively) compared with the control group (185 ± 8 nmol PO_4; $P < 0.001$; Fig. 2b). These results suggest a mitochondrial catabolic state, associated with oxidative stress.

Spearman's correlation test

We found a positive correlation between elevated levels of LPO and NO, demonstrating the presence of oxidative and/or nitrosative stress that could contribute to the pathogenesis and progression of DR (Fig. 3a). In addition, there was a positive correlation between uric acid and LPO levels, which may reflect a compensatory mechanism for the oxidative state caused by the persistent hyperglycemia (Fig. 3c). Furthermore, there were positive correlations between LPO and NO_x with a decrease in TAC (Fig. 3b,e). There was an increased consumption of TAC defenses with progressive increases in LPO and NO due to lack of metabolic control. There was also a positive correlation between increased GPx and catalase activity. This could be related to a compensatory mechanism to prevent tissue damage via an increase in these two antioxidant enzyme systems (Fig. 3d).

Discussion

The time to evolution of DM in the present study was >14 years, which could have favored the oxidative deregulation found. The chronicity of DM is an important risk factor for the development of DR.[13] All patients had hyperglycemia despite close medical attention; this lack of metabolic control allows us to consider the biological posibility of finding diverse alterations related to hyperglycemia and the duration of DM. Chronic hyperglycemia alters the homeostasis of vascular endothelial cells and causes changes in the expression of genes that incite DR,[14] favoring early functional changes that augment the permeability of the basal membrane of the retina, with endothelial cell dysfunction and increased oxidative stress.[15]

Increases in MDA + 4HDA in poorly controlled and long-term T2DM can cause DR.[16] In the present study, we found LPO levels increased progressively with the severity of retinopathy. Periodic evaluation of LPO in DM could contribute to the early identification of oxidative stress and possibly diminish cell death as a result of the application of therapeutic interventions that tend to modify and control the metabolic state and oxidative stress. The NO_x levels showed the same pattern as LPO, with progressive increases seen with the increasing severity of retinopathy. Apparently, the biochemical changes induced by hyperglycemia resulted in augmentation of the oxidative and/or nitrosative stress,[17] which is supported by evidence that increased levels of markers of oxidative stress are key factors in the development of DR, directly affecting the retinal microvasculature in hyperglycemia.[18,19]

In contrast, we found progressive decreases in TAC with the severity of NPDR. It has been proposed that oxidative stress and diminished antioxidant defenses are associated with the progression of DR and PDR.[20] If concomitant oxidant deficiency is produced, oxidative stress can result in tissue damage; thus, it may be of use to conduct clinical trials to evaluate the benefit of the administration of complementary antioxidants to patients in controlling oxidative stress and the progression of DR.

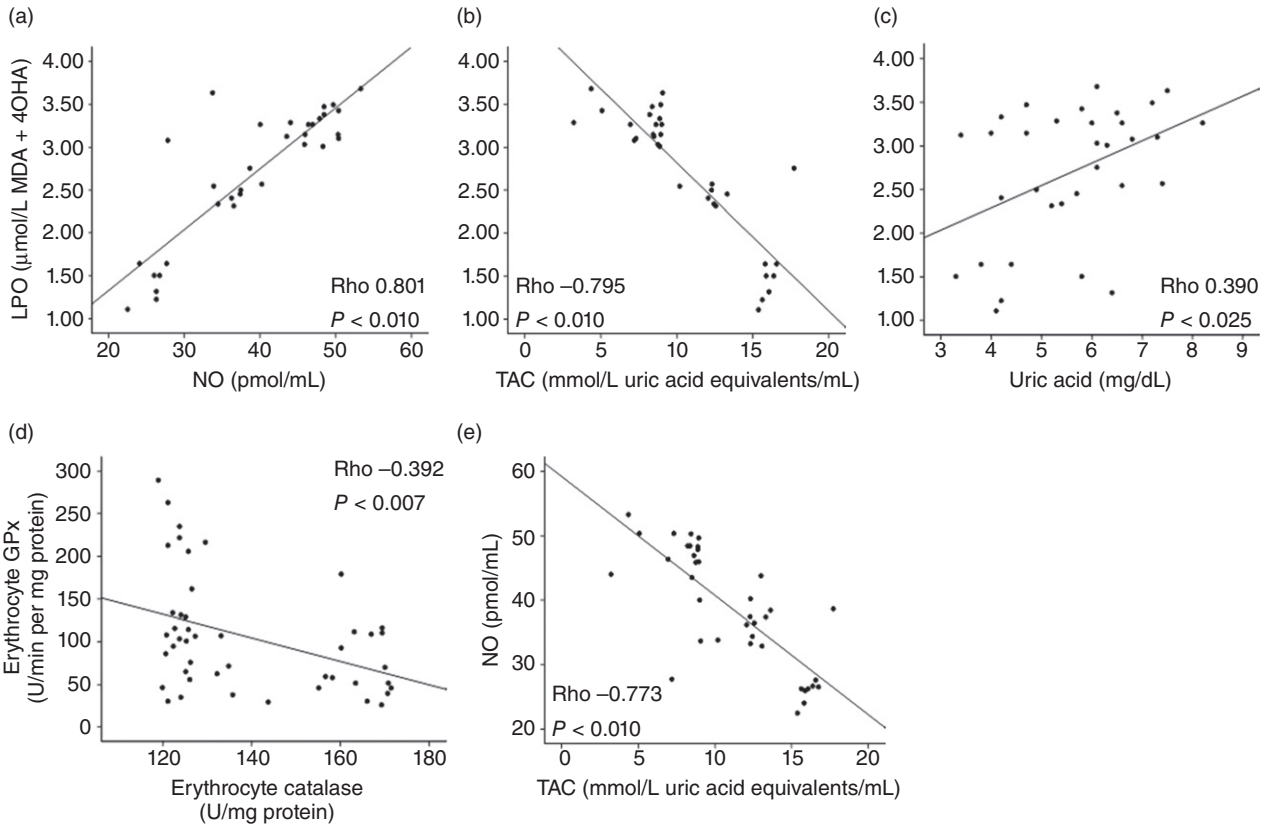

Figure 3 Results of Spearman's correlation tests. Significant positive correlations were found between (a) the products of lipid peroxidation (LPO) and nitric oxide (NO), as well as between (b) LPO and diminished total antioxidant capacity (TAC; $P < 0.010$). In addition, significant positive correlations were found between (c) LPO and uric acid ($P < 0.025$), (d) increased catalase and glutathione peroxidase (GPx) activity ($P < 0.007$), and (e) increased NO_x levels and TAC ($P < 0.010$). We can assume that there is oxidant, antioxidant, and mitochondrial dysregulation in chronic type 2 diabetes mellitus.

Uric acid is the final product of oxidation in the metabolism of purines. In the present study there was a progressive increase in uric acid levels with the severity of retinopathy, and a positive correlation between uric acid levels and increases in LPO. Increases in uric acid could have a compensatory role in the oxidative state caused by hyperglycemia,[21] because the uric acid has antioxidant activity by participating in the detoxification of lipid hydroperoxides into non-toxic alcohols.[22,23]

Erythrocyte catalase activity was significantly and inversely increased in relation to the TAC, depending on the severity of the retinopathy ($P < 0.0001$). It has been reported that SOD and catalase activity in leukocytes from T2DM patients with DR were inferior compared with leukocytes from healthy subjects and diabetic patients without DR.[24] In contrast with the decrease in GPx and SOD activity found in diabetic patients with microvascular complications,[25] our results demonstrate progressive increases in erythrocyte GPx activity with the severity of NPDR, possibly indicating a compensatory role in the oxidative and/or nitrosative state. However, we consider that the deregulation among the oxidants and/or antioxidants caused by persistent hyperglycemia could contribute to the appearance of microvascular complications present in T2DM. A possible explanation for the significant increase in the two antioxidant systems (GPx and catalase) and the significant decrease in TAC in the present study could be related to compensatory mechanisms for the oxidative state, as reported previously.[26]

The fluidity of the submitochondrial particles of platelets was significantly diminished in patients with mild, moderate, and severe NPDR, suggesting that the membranes are very sensitive to oxidative and/or nitrosative stress, as has been previously reported in Alzheimer's disease.[12] In animal models, it has been reported that under hyperglycemic conditions the mitochondria of the retinal pericytes show significant fragmentation and metabolic dysfunction; these changes could play an important role in accelerating the death, by apoptosis, of

the retinal pericytes in T2DM.[27,28] Possible pathogenic mechanisms involved in the platelet dysfunction include increased viscosity of the blood associated with changes in cellular deformity and the pathological interaction between leukocytes and endothelia. Chronic hyperglycemia affects cellular function and the extracellular matrix by: (i) increasing the activity of the polyol pathway; (ii) changing the intracellular redox state; (iii) activating protein kinase C; (iv) non-enzymatic glycation; and (v) increasing free radicals.[29]

The hydrolytic activity of F_0/F_1-ATPase is normally quite low. In the present study, F_0/F_1-ATPase activity in healthy individuals was 185 ± 8 nmol PO_4, and this increased significantly in all three NPDR groups, with the greatest activity seen in patients with mild NPDR. This suggests an important deregulation between the production and consumption of cellular energy (catabolism), possibly caused by the persistent hyperglycemic state and chronicity of DM. In Alzheimer's disease, it has been suggested that functional changes in mitochondrial F_0/F_1-ATPase are caused by: (i) the physical separation of F_0 and F_1 segments; (ii) changes in the assembly of the regulatory subunits of the enzyme; and (iii) an increase in the fluidity of the internal mitochondrial membrane.[30] In addition, we believe that excess in LPO and NO_x could also contribute to the increased F_0/F_1-ATPase hydrolytic activity.

One must consider that the adequate control of T2DM should include maintenance of normal glucose levels, thus preventing or delaying the appearance of microvascular complications of the retina. We believe that understanding the distinct molecular mechanisms responsible for mitochondrial damage could help identify management alternatives to postpone or prevent the DR that threatens the vision of people who suffer from DM.

Acknowledgement

This work was supported by National Chamber of the Pharmaceutical Industry 2012/Camara Nacional de la Industria Farmacéutica (CANIFARMA 2012; for research projects that have the potential to include medication).

Disclosure

The authors declare no conflict of interests.

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