Free Radical Research, 2015; Early Online: 1–9

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715762.2014.997229

informa
healthcare

ORIGINAL ARTICLE

Endoplasmic reticulum stress and Nrf2 repression in circulating cells of type 2 diabetic patients without the recommended glycemic goals

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Abstract

Endoplasmic reticulum (ER) stress plays a role in the pathogenesis of type 2 diabetes mellitus (T2DM), with activation of the unfolded protein response (UPR) and ER apoptosis in β-cells. The aim of the study is investigating the role of the prolonged glycemic, inflammatory, and oxidative impairment as possible UPR and ER apoptosis inductors in triggering the ER stress response and the protective nuclear erythroid-related factor 2 (Nrf2)/antioxidant-related element (ARE) activation in peripheral blood mononuclear cells (PBMC) of T2DM patients without glycemic target.

Oxidative stress markers (oxidation product of phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine [oxPAPC], and malondialdehyde [MDA]), the UPR and ER apoptosis, the activation of the pro-inflammatory nuclear factor-kappa B (NF-kB) with its inhibitory protein inhibitor-kBα, and the expression of the protective Nrf2 and heme oxygenase-1 (HO-1) were evaluated in PBMC of 15 T2DM patients and 15 healthy controls (C).

OxPAPC concentrations (in PBMC and plasma), MDA levels (in plasma), the expressions of the glucose-regulated protein 78 kDa (or BiP) as representative of UPR, and of the CCAAT/enhancer-binding protein homologous protein as representative of ER apoptosis were significantly higher ($p < 0.01$) in T2DM with respect to C. IkBα expression was significantly lower ($p < 0.01$) in T2DM as well as Nrf2 and HO-1.

In vitro experiments demonstrated that hyperglycemic conditions, if prolonged, were NF-kB inductors, without a corresponding Nrf2/ARE response.

In PBMC of T2DM without glycemic target achievement, there is an activation of the UPR and of the ER apoptosis, which may be related to the chronic exposure to hyperglycemia, to the augmented inflammation, and to the augmented oxidative stress, without a corresponding Nrf2/ARE defense activation.

Keywords: apoptosis, hyperglycemia, NF-kB, oxidative stress, UPR

Abbreviations: ATF6, activating transcription factor 6; CAT, catalase; CHOP, CCAAT/enhancer-binding protein; DPI, diphenyliodonium; ER, endoplasmic reticulum; GRP78/BiP, glucose-regulated protein 78 kDa; HO-1, heme oxygenase 1; IkBα, inhibitor-kBα; IRE1, inositol-requiring kinase 1; HOMA, homeostatic model of insulin resistance; MAGE, mean amplitude of glycemic excursion; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate; NF-kB, nuclear factor-kB; Nrf2, nuclear erythroid-related factor 2; ARE, antioxidant-related element; OxPAPC, oxidation products of phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine; PBMC, peripheral blood mononuclear cells; PEIPC, 1-palmitoyl-2-(5, 6-epoxyisoprostane E2)-sn-glycero-3-phosphorylcholine; PERK, protein kinase-like ER kinase; PGPC, 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine; POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus patients; UPR, unfolded protein response

Background

A growing body of evidence has suggested in the last years that the endoplasmic reticulum (ER) stress is involved in the pathogenesis of type 2 diabetes mellitus (T2DM), contributing to loss of pancreatic β-cells and resistance to insulin [1].

If the most convincing emphasis for the role of ER stress in β-cells failure was initially found by studying rare genetic disorders [1,2], evidence for animal and human pancreatic islets ER stress has been proven in conditions where β-cells were exposed to high glucose concentrations, which has been seen to overwhelm the ER folding capacity, causing an imbalance in homeostasis and leading to the unfolded protein response (UPR) and to the ER apoptosis [3,4]. Nevertheless, conditions of glucose deprivation can also cause ER stress, with proven activation of the UPR and the ER apoptosis [5].

Briefly, in all conditions perturbing the ER, three ER trans-membrane sensors are activated to initiate adaptive responses [6,7]. These sensors include protein kinase-like ER kinase (PERK), inositol-requiring kinase 1, and the activating transcription factor 6. All three sensors are maintained in an inactive state through the interaction of their N-terminus with the glucose-regulated protein 78 kDa (GRP78, also called BiP). When unfolded proteins accumulate in the ER, BiP releases these sensors to allow their oligomerization and thereby initiates the UPR [1]. Once the UPR fails to control the levels of the unfolded and misfolded proteins in the ER, the ER-initiated

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(Received date: 6 August 2014; Accepted date: 6 December 2014; Published online: 5 February 2015)

apoptotic signaling is induced, with the activation of the death factor CCAAT/enhancer-binding protein homologous protein (CHOP) [1,6].

Oxidative stress plays a key role both in the initiation and in the progression of T2DM, which is accompanied by increased production of the reactive oxygen species (ROS), contributing to the insulin resistance and to the activation of pro-inflammatory signaling pathways, mainly regulated by the pro-inflammatory nuclear factor-kappa B (NF-kB) [8,9].

Normally held in the cytoplasm in complex with the inhibitor-kBα (IkBα), canonical activation of NF-kB involves phosphorylation of IkBα and its proteasome degradation when inflammation occurs [10].

One of the most important cellular defense mechanisms against oxidative stress is regulated by the nuclear erythroid-related factor 2 (Nrf2)/antioxidant-related element (ARE), a PERK-dependent master transcriptional activator, which regulates many of the antioxidant enzymes [11–13].

The primary aim of this study is the evaluation of the UPR and the ER-initiated apoptotic signaling in circulating cells (peripheral blood mononuclear cells, PBMC) of T2DM patients without glycemic target achievement. In fact, if many studies have been conducted on pancreatic β-cells [3,4] consistent with our previous results in patients with stable coronary artery disease (CAD) [14], few but consistent data [15] suggest that the circulating cells are also vulnerable to ER stress in T2DM patients. It is known that they are likely susceptible to ER-stress-induced alterations in inflammatory/oxidative environment, as it occurs in atherosclerosis and diabetes [14,15], indicating that the circulating surrounding may also be somehow altered.

The second aim of this study is investigating the short and prolonged effect of glucose on UPR, on ER-initiated apoptotic signaling, on activation of pro-inflammatory signaling pathways, and on the protective Nrf2/ARE activation in cultured monocytoid THP-1 cells.

Methods

Study population

The study was approved by the hospital ethics committee in accordance with the ethical standard of the Declaration of Helsinki, and informed consent was obtained from all the patients before their enrollment.

The major requirements for the enrollment in all the groups were absence of infectious or acute/chronic inflammatory diseases, known malignancy, absence of acute/chronic renal failure, hepatic failure, ischemic heart disease, current smoking, hypertension, obesity, and pregnancy.

Two groups of subjects were studied: 15 patients with T2DM and 15 control healthy subjects (C). All the T2DM patients had T2DM diagnosed ≥ 6 months before the study entry, poorly controlled, and without regular treatment for diabetes at the time of the blood sample collection, recruited from the Department of Internal Medicine, University Hospital of Verona, Italy. All the C subjects were age-matched nurses or physicians working in the same department, who had no cardiovascular risk factors (hypertension, cigaret smoking, diabetes mellitus, hypercholesterolemia, obesity, and family history of premature cardiovascular disease), no continuative drug therapy, and no history of any acute or chronic disease.

Following a 12-hour overnight fast, all subjects were subjected to venous blood sampling.

Blood samples and PBMC isolation

Blood was collected from each subject and drawn into pyrogen-free blood collection tubes. Multiple aliquots of serum or plasma were placed into sterile 1-ml screw-capped polypropylene vials, containing phenolic antioxidant 2,6-di-tert-butyl-4-methylphenol (10 mM) (SIGMA, Milan, Italy) to inhibit lipid peroxidation, and stored at −80°C. Samples were kept frozen for no longer than 9 months with an average of 5 months. The samples were frozen and thawed only once. PBMC were isolated as previously described [16]. Total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, glucose, insulin, and glycated hemoglobin (HbA1%) levels were measured with standard methods.

C-reactive protein (CRP) was measured by a commercially available high-sensitivity turbidimetric method (Synhron-PCR, Beckman Coulter, Brea CA, USA).

Evaluation of phospholipid oxPAPC in plasma and in PBMC from C subjects and T2DM patients

1-Palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC) in plasma and in PBMC of C subjects and T2DM patients were measured on an Agilent mass spectrometer equipped with an electrospray source (Agilent Technologies, Milan, Italy) as previously described [17]. The following different oxPAPCs were taken into consideration: 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphorylcholine (PEIPC); 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine (POVPC); and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC). Flow injection experiments were performed using a high-performance liquid chromatography (HPLC) system (HP1100; Agilent Technologies, Milan, Italy). Quantification of the peak areas was performed by single ion monitoring in the elution time range of 10–20 min using appropriate software. Authentic PAPC, POVPC, and PGPC were obtained from Avanti Polar Lipids, Inc. (Delfzijl, The Netherlands). PEIPC was prepared and analyzed in our laboratory as previously described [18].

MDA detection

Malondialdehyde (MDA) detection in plasma and culture medium was achieved by HPLC with fluorescence detection [19].

Quantitative real-time PCR

Quantitative real-time PCR analysis was performed as previously described [16]. Total RNA was extracted from PBMC with an RNeasy Mini Kit (Qiagen, Milan, Italy) and was reverse-transcribed using IScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The relative expression levels of mRNA encoding GRP78/BiP, PERK, CHOP, IkBα, Nrf2, and HO-1 subunits were measured by iCycler (Bio-Rad, Hercules, CA, USA), using IQSYBR Green PCR SuperMix (Bio-Rad, Hercules, CA, USA) and 300 nM of each primer pair. Primers were designed using Beacon Design 4.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) and synthesized using MWG Biotech AG (Ebersberg, Germany) [16]. Normalized gene expression levels were given as the ratio between the mean value for the target gene and that for the β-actin in each sample.

Western blot analysis

Western blot analysis was performed as previously described [16]. In particular, PERK, GRP78/BiP, CHOP, IkBα, Nrf2, and HO-1 were immunoprecipitated from 1 mg of each PBMC protein lysate or nuclear lysate with mouse monoclonal antibodies, and anti-β-actin (Santa Cruz Biotechnology, Heidelberg, Germany). Immune complexes were captured with protein A/G-Sepharose beads (Pierce, Rockford, IL, USA) for 2 h, and the beads were washed four times with 100 mM of NaCl. To ensure the specificity of protein antibody interaction, lysates were incubated with beads in the absence of antibody as well as with an irrelevant immunoglobulin isotype control (Caltag, Little Balmer, UK). PERK, GRP78/BiP, CHOP, IkBα, Nrf2, and HO-1 were detected by probing immunoprecipitates with rabbit polyclonal antibodies (Santa Cruz Biotechnology, Heidelberg, Germany), followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA). Reactive antigens were visualized with SuperSignal chemiluminescence substrate (Pierce, Rockford, IL, USA) and quantified by densitometry analysis with ChemiDoc XRS (Bio-Rad). Protein expression data were quantified with Quantity One Software (Bio-Rad, Hercules, CA, USA).

In vitro analysis

THP-1 monocytoid cells were incubated for 2 h and overnight (ON) with different concentrations of glucose: 10 mM and 25 mM. At the times indicated, ROS, MDA, PGPC, and the expression levels of PERK, CHOP, IkBα, Nrf2, and HO-1 were evaluated with or without the presence of the inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase diphenyliodonium (DPI; 50 mM), superoxide dismutase (SOD, 150 U/ml), and catalase (CAT, 200 U/ml). Intracellular ROS production was quantified through the oxidation of 2,7-dichlorofluorescin diacetate as previously described [20]. To examine the intracellular esterase activity in basal and glucose-stimulated THP-1 cells, we used 5,6-carboxy-2,7-dichlorofluorescein diacetate (CF-DA); its hydrolysis produced a fluorescence product [21].

To study the relationship between the UPR and the ER-initiated apoptotic signaling with the Nrf2/ARE response and the cellular content of oxPAPC, THP-1 monocytoid cells were also incubated with crescent concentrations of PGPC (in a range from 0 to 1.6 ng/mg PAPC) with or without the presence of the inhibitor of NADPH oxidase DPI (50 mM) for 6 h; ROS, GRP78/BiP, CHOP, and Nrf2/ARE gene expression levels were quantified [14].

Apoptosis assay

Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC; Ex/Em = 488/519 nm; Invitrogen Corporation, Camarillo, USA).

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed by two-tailed unpaired Student's *t*-test and by one- or two-way analysis of variance for repeated measures followed by post-hoc Tukey's test for multiple comparisons using the "SYSTAT" program and statistical software manual (SYSTAT Inc., Evanston, Illinois) for Macintosh. Statistical significance was inferred at p values < 0.05.

Results

Baseline characteristics of the subjects

Baseline clinical characteristics and laboratory data of T2DM patients and C subjects are described in Table I.

T2DM patients did not present target fasting plasma glucose or HbA1% concentrations, according to the American Diabetes Association (ADA) 2014 Guidelines [22]. There was no difference in total and LDL cholesterol levels between the two groups. HDL level was significantly lower in T2DM than in C subjects. Plasma levels of high-sensitivity (hs)-CRP and MDA were significantly much higher in T2DM patients than in healthy C subjects ($p < 0.01$).

UPR, ER apoptosis, NF-kB activation, and Nrf2/ARE expression in PBMC of T2DM patients and healthy C subjects

Figure 1a and b shows the mRNA and protein expression of GRP78/BiP, CHOP, Nrf2, HO-1, and IkBα in PBMC of all T2DM patients and healthy C subjects considered in this study. GRP78/BiP and CHOP expression was significantly higher in T2DM patients than in healthy C subjects ($p < 0.01$). Furthermore in T2DM the mRNA and protein expression of Nrf2, HO-1, and IkBα were significantly lower in T2DM patients than in healthy C subjects ($p < 0.01$).

Table I. Clinical characteristics and values of metabolic, inflammatory, and oxidative parameters in T2DM patients and healthy C subjects.

	C ($n = 15$)	T2DM ($n = 15$)	p
Age (years)	62 ± 5	59 ± 5	ns
Male/Female	8/7	8/7	ns
BMI (Kg/m^2)	24 ± 1	28 ± 1	< 0.05
SBP (mmHg)	129 ± 5	130 ± 5	ns
DBP (mmHg)	82 ± 5	79 ± 3	ns
Heart rate (bpm)	67 ± 5	68 ± 5	ns
Total cholesterol (mg/dL)	163 ± 5	177 ± 9	ns
LDL cholesterol (mg/dL)	89 ± 8	98 ± 7	ns
HDL cholesterol (mg/dL)	52 ± 5	39 ± 4	< 0.05
Triglycerides (mg/dL)	126 ± 16	144 ± 18	ns
FPG (mmol/L)	5.4 ± 0.1	15.8 ± 1	< 0.01
HbA1%	5.5 ± 0.5	12 ± 3	< 0.01
HOMA	nd	5 ± 0.5	nd
Insulin (mU/L)	nd	7.1	nd
hs CRP (mg/L)	0.6 ± 0.06	5 ± 0.02	< 0.01
MDA (μmol/L)	0.8 ± 0.05	3.5 ± 0.09	< 0.01

C, healthy control subjects; T2DM, type 2 diabetes mellitus patients; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HbA1%, glycated hemoglobin; HOMA, homeostasis model of insulin resistance (fasting blood glucose × fasting insulin)/22.5; hs CRP, high-sensitivity C-reactive protein; MDA, malondialdehyde; ns, not significantly different; nd, not determined; data are expressed as mean ± SD.

OxPAPC concentrations in plasma and PBMC of T2DM patients and healthy C subjects

Figure 2a and b shows the concentrations of oxPAPC in plasma and PBMC of all T2DM patients and healthy C subjects considered in this study.

The plasma and PBMC concentrations of oxPAPC measured in this study (PEIPC, POVPC, and PGPC) were significantly higher in T2DM patients than in healthy C subjects ($p < 0.01$).

In vitro analysis: ROS, MDA, and PGPC generation and PERK, CHOP, IkBα, Nrf2, and HO-1 expression in THP-1 cells incubated with different concentrations of glucose for 2 h and ON

There was a dose-dependent effect of glucose concentration on ROS generation in THP-1 cells at different times ($p < 0.01$), which was inhibited by DPI ($p < 0.01$) and by SOD+ CAT (Figure 3a and b). The difference was not linked to glucose-stimulated variation of esterase activity since the fluorescence produced by CF-DA was not significantly different in basal and glucose-stimulated THP-1 cells (data not shown).

Glucose-induced ROS generation was associated to a dose- and time-dependent rise of MDA in the culture medium ($p < 0.01$) and of PGPC in THP-1 cell extracts, which was also inhibited by DPI ($p < 0.01$) (Figure 3c and d).

Figure 4a and b shows the mRNA and protein expression of PERK, CHOP, IkBα, Nrf2, and HO-1 after 2 h and ON incubation with 10 and 25 mM of glucose. The expression

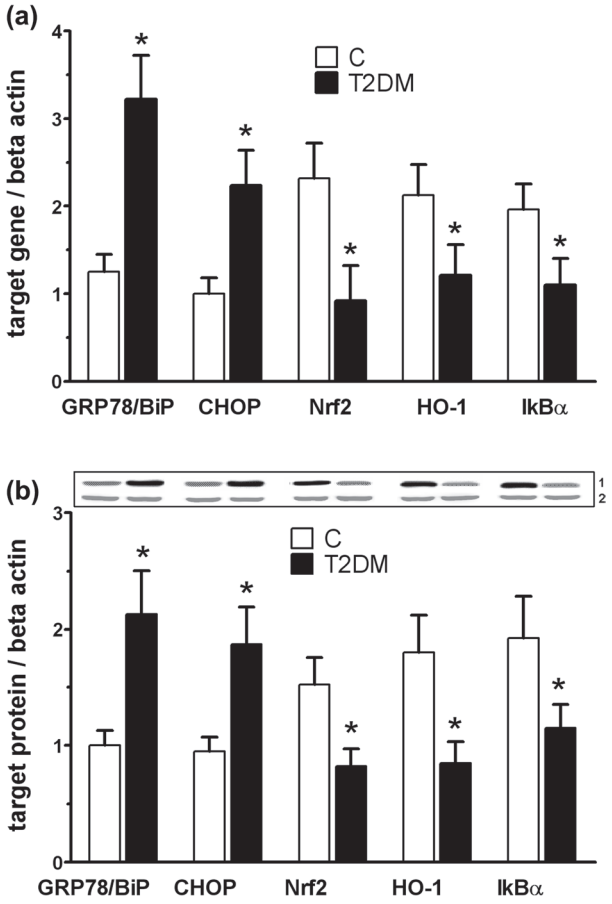

Figure 1. GRP78/BiP, CHOP, Nrf2, HO-1, and IkBα mRNA and protein expression in PBMC mRNA of GRP78/BiP, CHOP, Nrf2, HO-1, and IkBα were analyzed by quantitative real-time PCR (a). Normalized gene expression levels were given as the ratio between the mean value for the target gene and that for the β-actin in each sample. Representative Western blot analysis for the indicated proteins and the average quantification obtained by densitometric analysis of all the samples derived from healthy control subjects (C) and from T2DM patients (b). Data on Western blot analysis are expressed as the density ratio of target protein (1) to control (β-actin) (2) in arbitrary units. Data represent the mean ± SD of measurements performed in triplicate in all T2DM patients and healthy C subjects. *$p < 0.01$ versus C.

of PERK, chosen as representative of UPR and as Nrf2 transcriptional activator, was significantly higher ($p < 0.01$) at glucose concentration of 25 mM at 2 h, without increase at glucose concentration of 25 mM ON.

The expression of CHOP, as representative of ER apoptosis, was significantly higher ($p < 0.01$) only at glucose concentrations of 25 mM ON. Induction of apoptosis has been demonstrated with a significant increase of Annexin V$^+$ cells at glucose concentration of 25 mM ON. The expression of IkBα was significantly lower ($p < 0.01$) both at glucose concentrations of 10 and 25 mM at 2 h, and at glucose concentration of 25 mM ON. The expression of Nrf2 was significantly higher ($p < 0.01$) at glucose concentrations of 25 mM at 2 h, with a lack of increase at glucose concentration of 25 mM ON. The expression of HO-1 was even significantly lower ($p < 0.01$) at glucose concentrations of 25 mM ON with respect to all the other conditions.

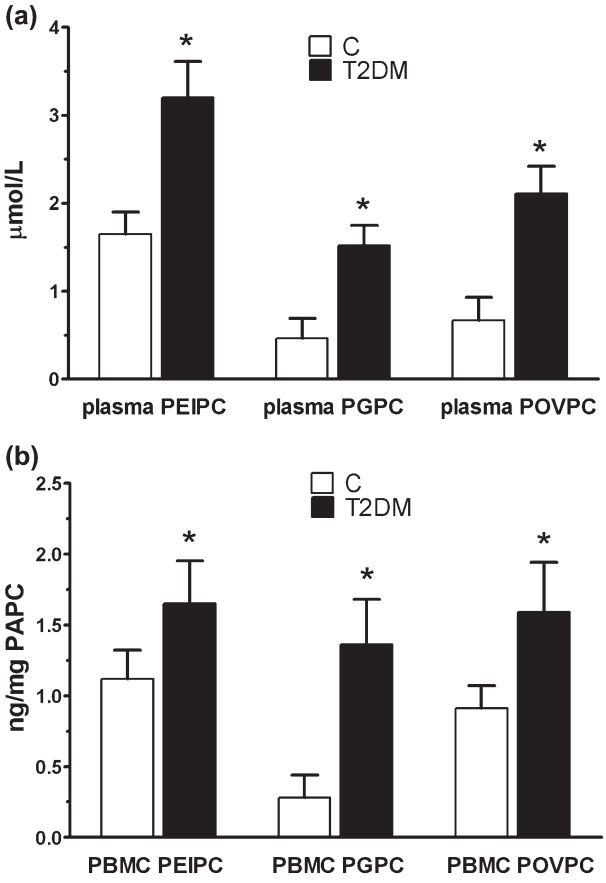

Figure 2. OxPAPC concentrations in serum and in PBMC of all T2DM patients and healthy C subjects. Serum concentrations of oxPAPC, PEIPC, POVPC, and PGPC in healthy subjects (C) and in T2DM patients (a). OxPAPC (PEIPC, POVPC, and PGPC) concentrations in PBMC of healthy C subjects and of T2DM patients (b). Data represent the mean ± SD of measurements performed in triplicate in all T2DM patients and healthy C subjects. *$p < 0.01$ versus C.

In vitro effects of PGPC on ROS generation and GRP78/BiP, CHOP, and Nrf2/ARE gene expression are indicated in Figure 5.

THP-1 cells incubated with increasing amounts of PGPC (in the range from 0 to 1.6 ng/mg PAPC) showed a dose-dependent increase in ROS generation ($p < 0.01$) (Figure 5a and b). The generation of ROS in THP-1 cells was greatly reduced in the presence of the NADPH oxidase inhibitor DPI ($p < 0.01$) and by SOD+ CAT.

We also found that PGPC dose-dependently increased the mRNA and protein expression of GRP78/BiP and CHOP ($p < 0.01$), which was reduced by DPI ($p < 0.01$) (Figure 5c and d). Otherwise, the behavior of Nrf2/ARE gene expression induced by PGPC was different depending on its concentration. At low concentrations, PGPC evoked a dose-dependent rise in Nrf2 and HO-1 ($p < 0.01$), whereas at higher concentrations, it reduced Nrf2/ARE gene expression ($p < 0.01$) (Figure 5c and d). As shown in Figure 5c and d, DPI reversed the reduction in Nrf2/ARE gene expression induced by the highest concentrations of PGPC ($p < 0.01$).

Discussion

In this study we have investigated the UPR and the ER-initiated apoptotic signaling in circulating cells of T2DM patients without the recommended glycemic goals and the role of the prolonged glycemic, inflammatory, and oxidative stress as possible UPR and ER apoptosis inductors in triggering and orchestrating the ER stress response and the Nrf2/ARE pathway activation.

We have first demonstrated that the expression of GRP78/BiP as representative of UPR and of CHOP as representative of ER-initiated apoptotic signaling was significantly higher in PBMC of T2DM patients than in healthy C subjects.

Animal and human studies have confirmed the activation of ER stress markers under condition of hyperglycemia. In fact, mouse pancreatic islets pretreated with different glucose concentrations induced an increase of ER stress markers expression with a correspondent inadequate insulin secretion at the highest glucose concentrations [3].

Moreover, in human studies, ER stress and UPR activation markers were increased in β-cells from pancreatic sections of T2DM patients compared with non-diabetic pancreatic tissue [23].

However, there are only few data about the ER stress involvement in circulating cells of T2DM patients and their vulnerability to hyperglycemia-induced ER stress and dysfunction [15]. In this study authors demonstrated that monocytes derived from patients with T2DM were more susceptible to apoptosis than those derived from healthy volunteers and that they showed an elevated expression of the markers of ER stress. However, there was a lack of knowledge about why and by what mechanisms circulating cells were vulnerable in T2DM. Our study has tried to better investigate this point: the fact that the UPR and the ER apoptosis are activated in PBMC of T2DM patients indicates that the circulating environment may also be somehow altered in T2DM patients, with analogies with our results in CAD patients [14]. Of course, in the case of T2DM the "primum movens" may be hyperglycemia, a situation that is supposed to start a series of reactions leading to an increased production of ROS via activation of NADPH oxidase [24,25]. As a matter of fact, in our in vitro studies we demonstrated that glucose dose-dependently increased ROS generation at different times and that the increase was inhibited by DPI, a specific inhibitor of NADPH oxidase. The glucose-induced ROS generation was associated, after prolonged incubation with high concentration of glucose, with a significant increase of CHOP. Otherwise, PERK expression was higher at 2nd hour of incubation with 25 mM of glucose. The fact that DPI inhibited the expression of PERK and CHOP, respectively, after 2 h and after overnight incubation indicates that ROS may play a major role in UPR and in ER-initiated apoptotic signaling.

The results of this study also show that MDA concentration was higher in T2DM than in healthy C. Our results concerning increased MDA levels in T2DM are consistent

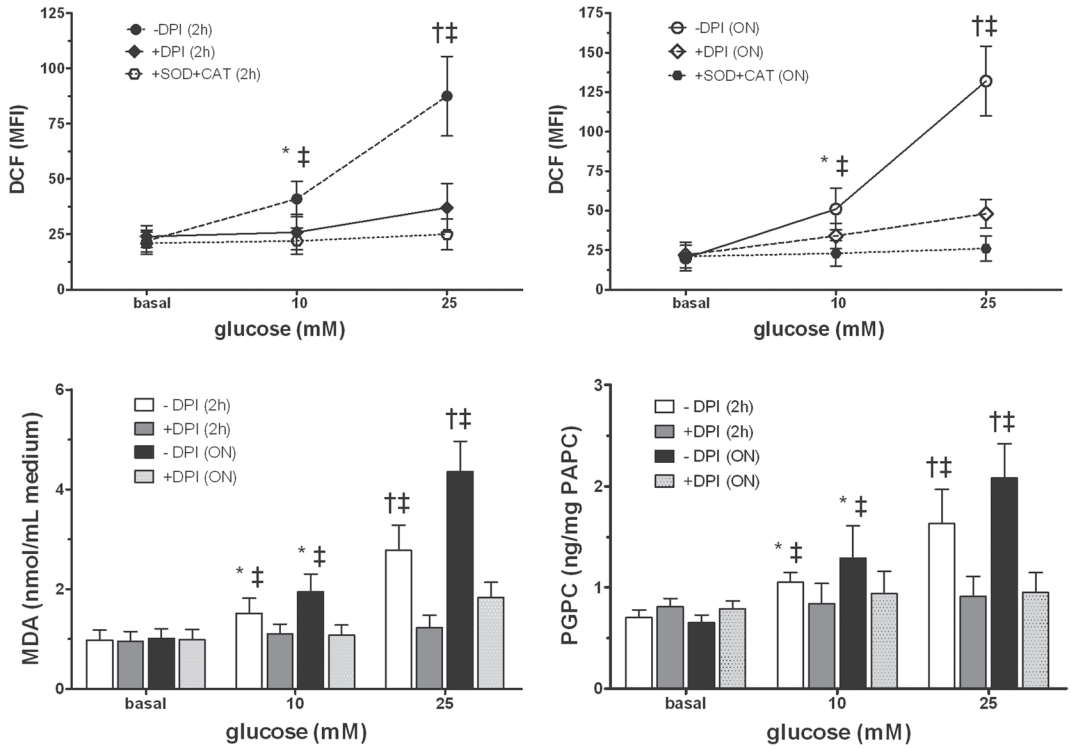

Figure 3. In vitro analysis: ROS, MDA, and PGPC generation in THP-1 cells incubated with different concentrations of glucose for 2 h and ON. Effect of glucose concentration on ROS generation in THP-1 cells at 2 h and ON, + or − DPI (50 μM) + SOD (150 U/ml) + CAT (200 U/ml) (a and b). Effect of glucose concentration on MDA and on PGPC in THP-1 cells at 2 h and ON + or − DPI (50 μM) (c and d). Data represent the mean ± SD of measurements performed in triplicate in six different occasions. *$p < 0.01$ versus basal, †$p < 0.01$ versus glucose 10 mM, ‡$p < 0.01$ versus + DPI. MFI = mean fluorescence intensity.

with previous data [26,27], also in patients with newly diagnosed T2DM [28]. Increased production of ROS in T2DM patients may therefore affect oxidation of fatty acids since MDA is a collection of end-products of various types of oxidized fatty acids, and it is used to estimate lipid peroxidation in vivo. These oxidized fatty acids may derive from oxidation of cellular membrane phospholipids, a primary target of superoxide generated by NADPH-oxidase [29]. The fact that in this study plasma and PBMC concentrations of oxPAPC were significantly higher in T2DM patients than in healthy C subjects agrees with this hypothesis. This view is also supported by the results we obtained in in vitro studies where incubation with high concentration of glucose induced an increase of MDA and oxPAPC that was inhibited by DPI, an inhibitor of NADPH oxidase. In turn the results of this study also show that oxPAPC further potentiate the generation of ROS via NADPH oxidase indicating that these compounds may contribute to generating ER stress.

This study was also aimed to examine the role of inflammation, NF-kB, and Nrf2 signaling in T2DM. NF-kB activation in the T2DM patients of this study (resulting in decreasing expression of total IkBα) confirms the growing evidence that considers the ER as a crucial site where metabolic and inflammatory pathways converge [30]. ER stress and inflammation have been shown to be interconnected through various mechanisms and in particular through the activation of NF-kB [31], a master transcription factor with numerous functions including the regulation of the inflammatory response, with proven involvement in T2DM [32]. It has been proposed that NF-kB activation during ER stress depends on UPR-induced IkBα reduction [33]. Of course, activation of NF-kB may also be a consequence of oxidative stress as previously demonstrated in other cells [34]. These conclusions are in line with the results we obtained in vitro. The glucose- and ROS-induced ER stress in THP-1 cells was associated with a reduction in IkBα and at any time at any concentration of glucose.

Despite the presence of ER stress and the augmented levels of oxidative stress and inflammation, we observed an inadequate response of Nrf2/ARE response in PBMC of T2DM patients. Our results are in agreement with a series of studies where the same inadequacy was reported in other chronic pathologies such as chronic kidney failure [35], chronic obstructive pulmonary disease [36], and chronic CAD, as we have recently demonstrated [14]. Also in vitro experiments confirmed that in hyperglycemic conditions, if prolonged (glucose concentration of 25 mM ON), there was no Nrf2/ARE response, as it conversely occurred in acute extreme conditions (glucose concentration of 25 mM at 2 h).

So it is likely that it is an excess of oxidative or metabolic stress that orchestrates the switch from the protective UPR and Nrf2/ARE gene expression to ER-initiated apoptotic signaling and this could explain the similar results in different pathologies, as we have already supposed in previous studies [14,17]. Similar impairments in

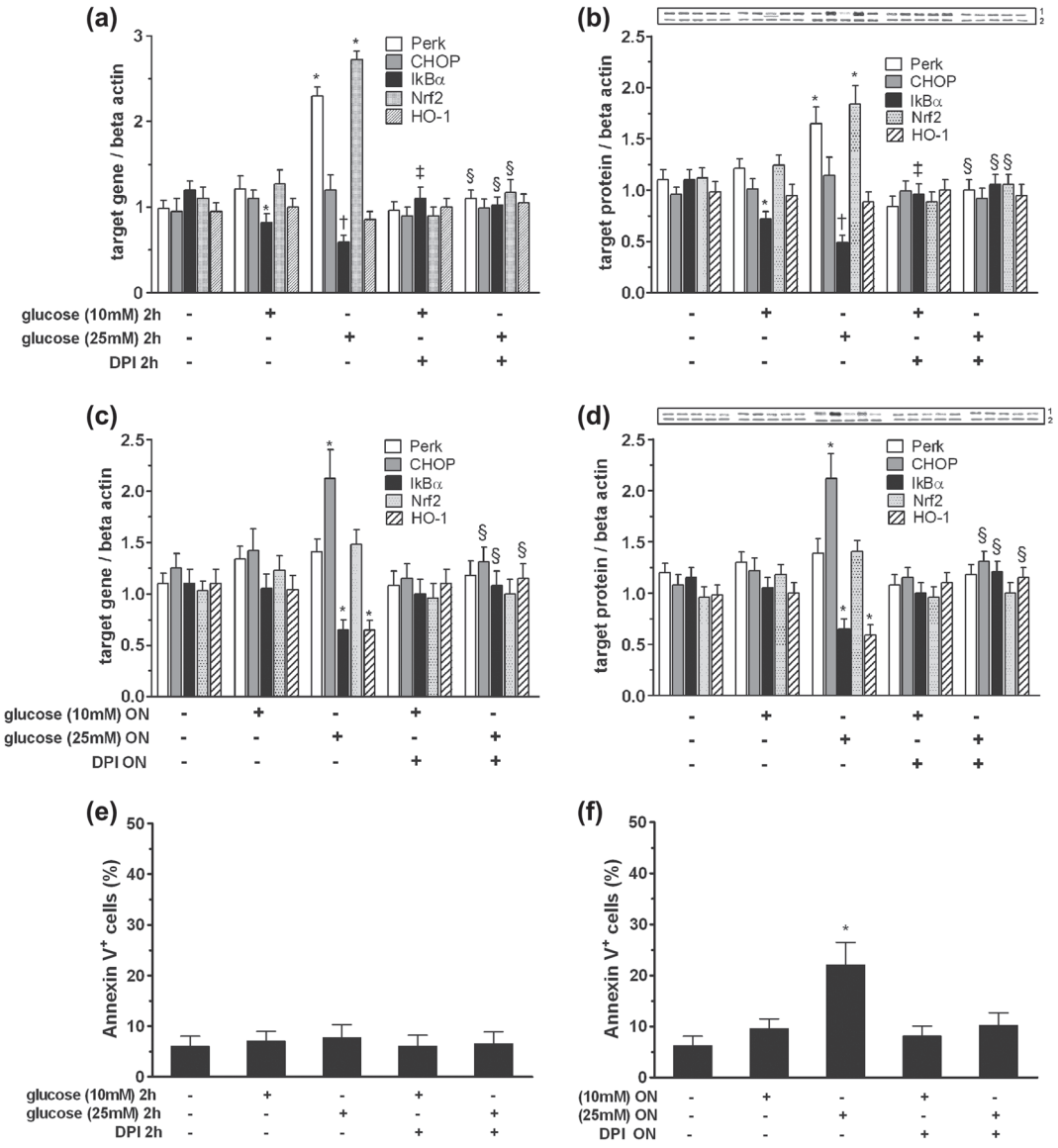

Figure 4. In vitro analysis: PERK, CHOP, IkBα, Nrf2, and HO-1 expression and annexin V-FITC assay in THP-1 cells incubated with different concentrations of glucose for 2 h and ON. mRNA and protein expression of PERK, CHOP, IkBα, Nrf2, and HO-1 after 2-h incubation with 10 and 25 mM of glucose (a and b). mRNA and protein expression of PERK, CHOP, IkBα, Nrf2, and HO-1 after ON incubation with 10 and 25 mM of glucose (c and d). The percentage of annexin V-FITC-positive cells after 2-h incubation with 10 and 25 mM of glucose (e) and after ON incubation with 10 and 25 mM of glucose (f). Representative Western blot analysis for the indicated proteins and the average quantification was obtained by densitometric analysis of all the samples. Data on Western blot analysis are expressed as the density ratio of target protein (1) to control (β-actin) (2) in arbitrary units. Data represent the mean ± SD of measurements performed in triplicate in six different occasions. $^{*}p < 0.01$ versus basal, $^{\dagger}p < 0.01$ versus glucose (10 mM)-DPI, $^{\ddagger}p < 0.01$ versus glucose (10 mM)-DPI, $^{\S}p < 0.01$ versus glucose (25 mM)-DPI.

Nrf2/ARE were observed in the endothelial cells exposed to high glucose in the retina from donors with diabetic retinopathy [37].

We are conscious that this study has several limitations: first of all the not wide sample of patients (that nevertheless it is principally due to the precise categorization of the patients enrolled, as explained in exclusion criteria section). Second, it should be important to evaluate the mean amplitude of glycemic excursions as estimated by continuous glucose monitoring systems, which remains certainly the most comprehensive index for assessing the intraday glycemic variability. In fact, it has been demonstrated that glucose fluctuations during postprandial periods and, more generally, during glucose swings, exhibited a more specific triggering effect on oxidative stress than chronic sustained hyperglycemia [38,39].

Conclusions

In conclusion, the present study has demonstrated that in PBMC of T2DM patients without glycemic target achievement, there is activation of the UPR and of the ER apoptosis, which may be related to the chronic exposure to hyperglycemia, to the augmented inflammation, and to the

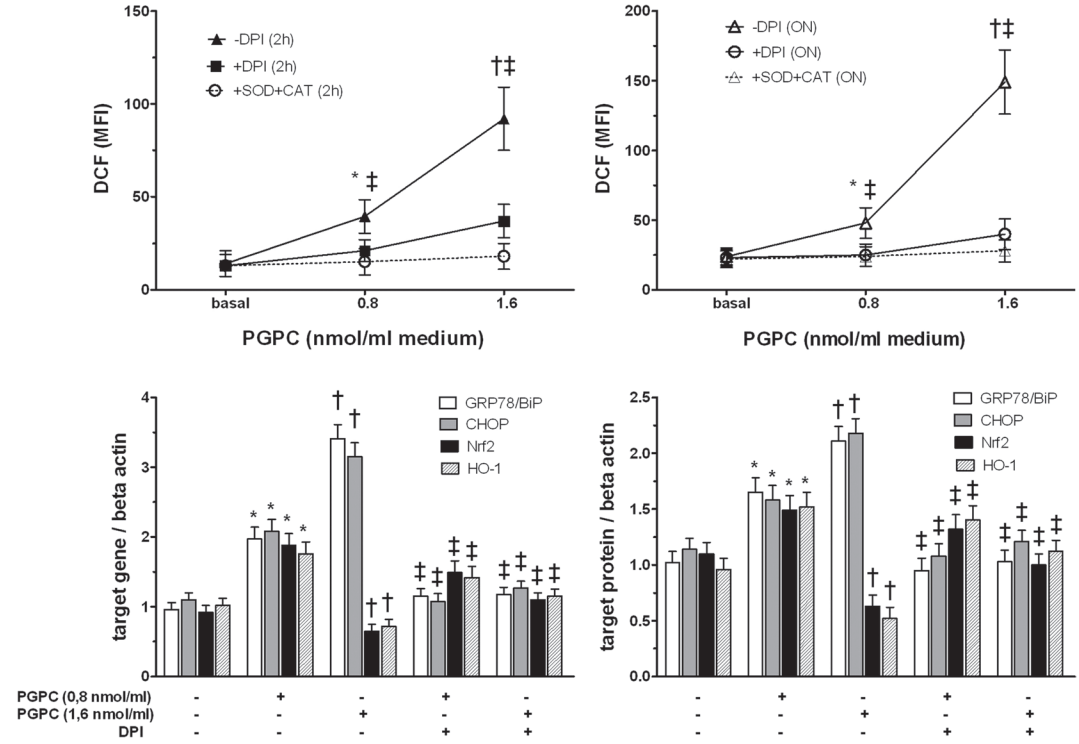

Figure 5. In vitro effects of PGPC on ROS generation and GRP78/BiP, CHOP, and Nrf2/ARE gene expression. Effect of PGPC concentration on ROS generation in THP-1 cells at 2 h and ON, + or − DPI (50 μM) and + SOD (150 U/ml) + CAT (200 U/ml) (a and b). mRNA and protein expression of PERK, CHOP, IkBα, Nrf2, and HO-1 after incubation with increasing amounts of PGPC (0.8–1.6 nmol/mL) (c and d). Representative Western blot analysis for the indicated proteins and the average quantification was obtained by densitometric analysis of all the samples. Data on Western blot analysis are expressed as the density ratio of target protein (1) to control (β-actin) (2) in arbitrary units. Data represent the mean ± SD of measurements performed in triplicate in six different occasions. *$p < 0.01$ versus basal, †$p < 0.01$ versus PGPC 0.8 nmol/mL basal, ‡$p < 0.01$ versus PGPC + DPI, §$p < 0.01$ versus PGPC 1.6 nmol/mL + DPI.

augmented oxidative stress, without a corresponding Nrf2/ARE defense activation, allowing new insight into the circulating environment of T2DM patients.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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