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Original Contribution

Increased endoplasmic reticulum stress and Nrf2 repression in peripheral blood mononuclear cells of patients with stable coronary artery disease

Chiara Mozzini*, Anna Fratta Pasini, Ulisse Garbin, Chiara Stranieri, Andrea Pasini, Paola Vallerio, Luciano Cominacini

Section of Internal Medicine, Department of Medicine, University of Verona, 37134 Verona, Italy

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ABSTRACT

Endoplasmic reticulum (ER) stress is involved in the pathophysiology of atherosclerosis. Insults interfering with ER function lead to the accumulation of unfolded and misfolded proteins in the ER that initiates the unfolded protein response (UPR). When the UPR fails to control the level of unfolded and misfolded proteins, ER-initiated apoptotic signaling is induced. We evaluated: (1) the UPR and ER-initiated apoptotic signaling in peripheral blood mononuclear cells (PBMCs) of stable coronary artery disease (CAD) patients; (2) PBMC content of oxidation products of phospholipid 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine (oxPAPC); (3) the possible origin of oxPAPC in PBMCs; and (4) the expression of nuclear erythroid-related factor 2 (Nrf2)/antioxidant-related element (ARE), a cellular defense mechanism. Twenty-nine CAD patients and 28 matched controls were enrolled. Expression of glucose-regulated protein 78 kDa (GRP78/BiP), as a representative of the UPR, and of C/EBP homologous protein (CHOP), as a representative of ER apoptosis, was significantly higher in CAD than in controls ($p < 0.01$). Concentrations of oxPAPC in PBMCs, in plasma, and in low-density lipoprotein (LDL) were significantly higher in CAD compared to controls ($p < 0.01$). The oxPAPC in PBMCs may derive from circulating ox-LDL. Nrf2/ARE gene expression and circulating and cellular glutathione were significantly lower in CAD compared to controls ($p < 0.01$). In in vitro studies, increasing amounts of oxPAPC induced a dose-dependent increase in CHOP and apoptosis-related protein expression ($p < 0.01$) and a progressive decrease in Nrf2/ARE gene expression ($p < 0.01$). In PBMCs of CAD patients there is an activation of the UPR and ER-initiated apoptotic signaling, possibly related to an abnormal concentration of oxPAPC in PBMCs.

Coronary artery disease is a common complex atherosclerotic pathology associated with substantial morbidity and mortality [1,2].

Work over the past decade has revealed that endoplasmic reticulum (ER)[1] stress is an important event during the initiation, progression, and clinical progression of atherosclerosis [3]. It is known that a variety of insults can interfere with ER function, leading to the accumulation of unfolded and misfolded proteins in the ER [4,5]. In particular, it has been shown that oxidation products of phospholipid 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine (oxPAPC) leads to ER stress and activation of the unfolded protein response (UPR) in human aortic endothelial cells [6]. When ER stress occurs, three ER transmembrane sensors are activated to initiate adaptive responses [4,5,7]. These sensors include protein kinase-like ER kinase (PERK), inositol-requiring kinase 1, and the transcriptional factor activating transcription factor 6. All three sensors are maintained in an inactive state through the interaction of their N-terminus with glucose-regulated protein 78 kDa (GRP78) [8]. When unfolded proteins accumulate in the ER, GRP78 releases these sensors to allow their oligomerization and thereby initiates the UPR [9]. Once the UPR fails to control the level of unfolded and misfolded proteins in the ER, ER-initiated apoptotic signaling is induced with the activation of the death factor, CCAAT/enhancer binding protein homologous protein (CHOP) [4,5,7–9]. In this context, reduced apoptosis and plaque necrosis have been shown in mice lacking CHOP [10], providing direct evidence for a causal link between the ER-stress effector CHOP and plaque progression.

The UPR also generates excess levels of reactive oxygen species (ROS), a process that is driven by proteins involving new protein disulfide isomerase and ER oxidoreduction [11]. One of the most

Abbreviations:: ER, endoplasmic reticulum; UPR, unfolded protein response; PBMC, peripheral blood mononuclear cell; CAD, coronary artery disease; oxPAPC, oxidation products of phospholipid 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine; PEIPC, 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-*sn*-glycero-3-phosphorylcholine; POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine; PGPC, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine; Nrf2/ARE, nuclear erythroid-related factor 2/antioxidant-related element; GRP78/BiP, glucose-regulated protein 78 kDa; CHOP, C/EBP homologous protein; GSH, glutathione

* Corresponding author. Fax: +39 0458027496.
E-mail address: chiaramozzini@libero.it (C. Mozzini).

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important cellular defense mechanisms against excess ROS is regulated by nuclear erythroid-related factor 2 (Nrf2), a PERK-dependent master transcriptional activator, which regulates many of the antioxidant defense genes [12,13].

Because we have previously demonstrated a progressive increase in oxidative–inflammatory markers and monocyte activation from control subjects to stable and to unstable angina patients [14–16], in this study we evaluated: (1) the UPR and the ER-initiated apoptotic signaling in peripheral blood mononuclear cells (PBMCs) of stable coronary artery disease (CAD) patients; (2) the PBMC content of oxPAPC; (3) the possible origin of oxPAPC in PBMCs; and (4) the PBMC expression of Nrf2/antioxidant-related element (ARE).

Material and 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 enrolment.

Major requirements for the enrolment in all the groups were absence of infectious or acute/chronic inflammatory diseases or known malignancy and absence of acute/chronic renal failure, hepatic failure, or diabetes mellitus.

Two groups of subjects were studied: 29 patients with stable CAD and 28 control healthy (C) subjects. The CAD patients were enrolled on the basis of previously established criteria [14–17]. For CAD patients exclusion criteria were prior coronary artery bypass grafting, recent (<6 months) myocardial infarction (MI), recent (<6 months) percutaneous coronary intervention (PCI) or congestive heart failure, and coronary tree free of significant coronary artery disease (defined as at least one stenosis with minimal luminal diameter $>70\%$ of the arterial lumen by visual estimate) detected at coronary angiography.

The 28 C subjects were nurses or physicians from the Department of Internal Medicine, University of Verona Medical School, who had no cardiovascular risk factors (hypertension, cigarette smoking, diabetes mellitus, hypercholesterolemia, obesity, family history of premature cardiovascular disease), no continuative drug therapy, and no history of cardiovascular disease or valvular heart disease. The following data were obtained from all the patients: age, sex, presence of cardiovascular risk factors (hypertension, cigarette smoking, diabetes mellitus, hypercholesterolemia, family history of ischemic coronary artery disease), use of medications, previous MI, previous PCI.

Blood samples, low-density lipoprotein (LDL), and PBMC isolation

Venous blood samples were obtained from C subjects and CAD patients after 12 h fasting. 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 the phenolic antioxidant 2,6-di-*tert*-butyl-4-methylphenol (BHT; 10 mM; Sigma, Milan, Italy) to inhibit lipid peroxidation, and stored at $-80\,^{\circ}$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. PBMCs were isolated as previously described [16]. Total cholesterol, high-density lipoprotein (HDL) cholesterol, LDL cholesterol, triglycerides, and glucose were measured with standard methods. C-reactive protein (CRP) was measured using a commercially available high-sensitivity turbidimetric method (Syncron-PCR, Beckman Coulter, Brea, CA, USA). Plasma for LDL separation was frozen and stored as previously reported [18]. Samples were kept frozen for no longer than 12 months, with an average of 7 months, and thawed only once. LDL was separated by sequential flotation as described [14].

Evaluation of oxPAPC in PBMCs, plasma, and LDL from C subjects and CAD patients

OxPAPC in PBMCs and plasma of controls and CAD patients were measured on an Agilent mass spectrometer equipped with an electrospray source (Agilent Technologies, Milan, Italy) as previously described [19]. The following different oxPAPC 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 on an HPLC system (HP1100, Agilent Technologies). Quantification of the peak areas was performed by single-ion monitoring in the elution time range of 10–20 min using appropriate software. Authentic 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (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 [20].

Glutathione (GSH) measurement in plasma and in PBMCs

The detailed procedures for the measurement of cellular and plasma GSH have been previously described [19]. Samples were collected directly into specially prepared tubes containing the preservative BHT (10 mM), to reduce autoxidation, and frozen at $-80\,^{\circ}$C. Samples were analyzed using high-performance liquid chromatography with fluorescence detection of 7-fluorobenzo-2-oxa-1, 3-diazol-4-sulfonic acid at excitation 385 nm and emission 515 nm.

Oxidized (ox) LDL measurement in plasma

Circulating plasma ox-LDL levels were measured with the Mercodia oxidized LDL enzyme-linked immunosorbent assay (Mercodia AB) as previously described [14]. Cu^{2+}-modified LDL ranging from 50 to 500 ng/ml was used as a standard solution.

In vitro analysis

To verify whether ox-LDL or serum is a possible source of cellular oxPAPC, THP-1 monocytoid cells were incubated with increasing amounts (from 0 to 600 μg of LDL protein/ml) of native (n) and ox-LDL and of serum (from 0 to 50% in culture medium) derived from CAD patients and healthy C in the presence of BHT (10 mM) for 6 h, and cellular PGPC and POVPC were measured as described. LDL and Cu^{2+}-modified LDL were prepared as reported [14]. Immediately before the incubation with THP-1 cells, LDL was separated from diffusible low-molecular-mass compounds by gel filtration on a PD-10 Sephadex G-25 M gel in 10 mM phosphate-buffered saline. As for serum, THP-1 cells were incubated without and with increasing amounts (10, 30, and 50% of culture medium) of serum derived from CAD patients and healthy C for 6 h. THP-1 cells were also incubated with the corresponding amounts (10, 30, and 50% of culture medium) of lipoprotein-depleted serum (LPDS) derived from CAD patients, from which all the lipoproteins were taken away by ultracentrifugation at a density >1.21 g/ml, as previously described [16].

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 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 diphenyliodonium (DPI; 50 μM) for 6 h, and ROS, GSH, GRP78/BiP, CHOP and Nrf2/ARE gene expression was evaluated.

Intracellular ROS production was quantified through the oxidation of 2′,7′-dichlorofluorescin diacetate as previously described [21].

Endotoxin contamination of cell cultures involving the use of LDL and serum was routinely excluded with the chromogenic *Limulus* amoebocyte lysate assay (Sigma). Early apoptosis and cell viability were determined using the Annexin V–FITC Kit (Bender MedSystems, Vienna, Austria) and 7-amino-actinomycin D (BD Biosciences, Buccinasco, Italy) by flow cytometry.

Quantitative real-time PCR

Quantitative real-time PCR analysis was performed as previously described [16]. Total RNA was extracted from PBMCs with an RNeasy Mini Kit (Qiagen, Milan, Italy) and was reverse transcribed using an IScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The relative expression levels of mRNA encoding GRP78/BiP, CHOP, Nrf2, heme oxygenase-1 (HO-1), glutamate–cysteine ligase catalytic (GCLC) subunit, p53, BAX, BID, caspase 3, and caspase 9 were measured by an iCycler (Bio-Rad), using IQSYBR Green PCR SuperMix (Bio-Rad) and 300 nM each primer pair. Primers were designed by Beacon Design 4.0 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by MWG Biotech AG (Ebersberg, Germany) [16]. Normalized gene expression levels are 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]. GRP78/BiP, CHOP, Nrf2, HO-1, GCLC subunit, p53, BAX, BID, caspase 3, and caspase 9 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 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). GRP78/BiP, CHOP, Nrf2, HO-1, GCLC subunit, and p53 were detected by probing immunoprecipitates with rabbit polyclonal antibodies (Santa Cruz Biotechnology), followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Reactive antigens were visualized with Supersignal chemiluminescence substrate (Pierce) and quantified by densitometric analysis with ChemiDoc XRS (Bio-Rad). Protein expression data were quantified with Quantity One Software (Bio-Rad).

Statistical analysis

Data are expressed as mean ± standard deviation (SD) values if normally distributed. Differences between groups were analyzed by a two-tailed unpaired Student *t* test and by one-way ANOVA and post hoc multiple comparison using Student–Newman–Keuls′ test. A probability value (p) <0.05 was considered statistically significant.

Results

Baseline characteristics of the patients

Baseline clinical characteristics and laboratory data of CAD patients and healthy C are described in Table 1.

There were no statistical differences in clinical and metabolic characteristics between the two groups.

Plasma levels of high-sensitivity (hs)-CRP and ox-LDL were significantly higher in CAD patients than in healthy C ($p<0.05$), whereas plasma and PBMC concentrations of GSH were significantly lower in CAD patients than in healthy C ($p<0.05$).

Drug therapies were similar in the CAD group (acetylsalicylic acid, angiotensin-converting enzyme inhibitor, β-blocker, and statin).

UPR activation and Nrf2/ARE response in PBMCs and oxPAPC concentrations in plasma, LDL, and PBMCs of CAD patients and healthy C

Figs. 1a and b show the mRNA and protein expression of GRP78/BiP, CHOP, Nrf2, HO-1, and GCLC subunit in PBMCs of all CAD patients and healthy C considered in this study. GRP78/BiP and CHOP were significantly higher in CAD patients than in healthy C ($p<0.01$). Despite UPR activation there was no Nrf2/ARE gene response in CAD patients and the mRNA and protein expression of Nrf2, HO-1, and GCLC subunit was significantly lower than in healthy C ($p<0.01$).

Figs. 1c and d also show the concentrations of oxPAPC in plasma, LDL, and PBMCs of all CAD patients and healthy C considered in this study. The plasma, LDL, and PBMC concentrations of oxPAPC measured in this study (PEIPC, POVPC, PGPC) were significantly higher in CAD patients than in healthy C ($p<0.01$).

OxLDL and serum as a source oxPAPC in THP-1 cells

To verify whether ox-LDL or serum is a possible source of cellular oxPAPC, THP-1 cells were incubated with increasing amounts of n- and ox-LDL and of serum derived from CAD patients and healthy C in the presence of BHT (10 mM) for 6 h, and cellular oxPAPC were measured as described. Figs. 2a and b show that the incubation of

Table 1
Clinical characteristics and values of metabolic, inflammatory, and oxidative parameters in C and CAD.C, healthy controls; CAD, stable coronary artery disease patients; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HsCRP, high-sensitivity C-reactive protein; GSH, glutathione; Ox-LDL, oxidized low-density lipoprotein; NS, not significantly different; data are expressed as the mean ± SD.

	C ($n=28$)	CAD ($n=29$)	p
Age (years)	64 ± 10	66 ± 10	NS
Male/female	14/14	15/14	NS
BMI (kg/m^2)	25.5 ± 2	25 ± 2	NS
SBP (mm Hg)	130 ± 5	128 ± 5	NS
DBP (mm Hg)	80 ± 5	79 ± 3	NS
Heart rate (bpm)	67 ± 5	66 ± 5	NS
Total cholesterol (mg/dl)	163 ± 5	155 ± 5	NS
LDL cholesterol (mg/dl)	100 ± 5	100 ± 6	NS
HDL cholesterol (mg/dl)	52 ± 5	49 ± 5	NS
Triglycerides (mg/dl)	124 ± 6	120 ± 7	NS
Plasma glucose (mg/dl)	98 ± 8	101 ± 8	NS
HsCRP (mg/dl)	0.06 ± 0.006	0.3 ± 0.03	<0.01
Plasma GSH (μmol/L)	3.97 ± 1.23	2.49 ± 0.94	<0.01
PBMC GSH (ng/mg cell protein)	2.64 ± 0.15	1.65 ± 0.75	<0.01
Ox-LDL (μg/ml)	14.21 ± 0.79	25.60 ± 1.12	<0.01

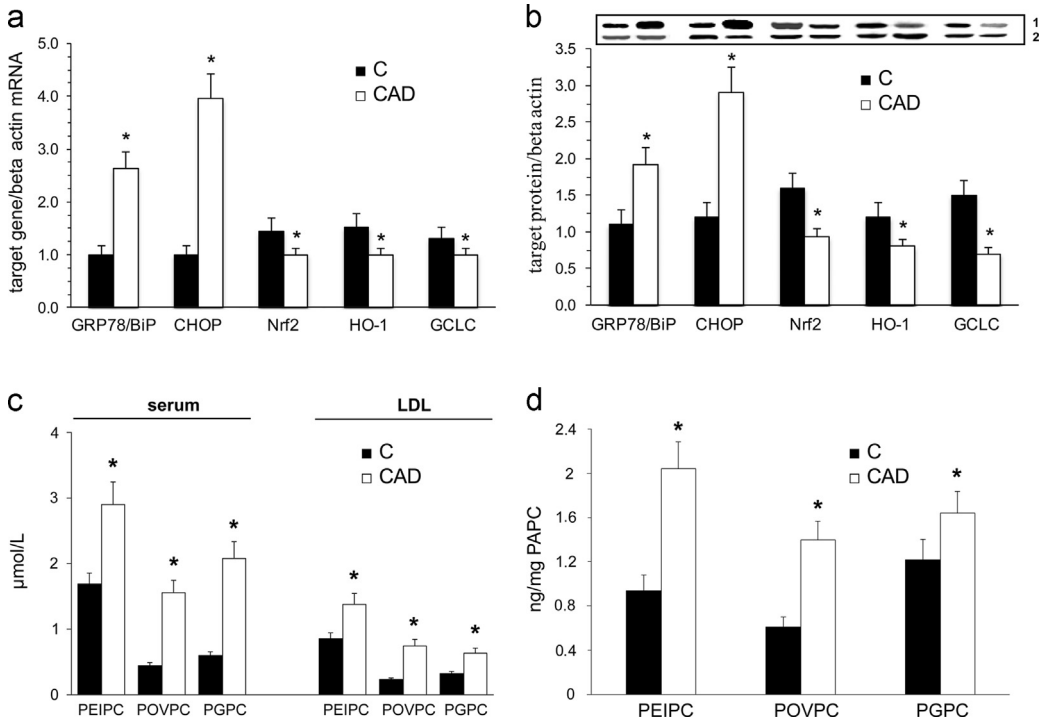

Fig. 1. GRP78/BiP, CHOP, Nrf2, HO-1, and GCLC mRNA and protein expression in PBMCs and oxPAPC concentrations in circulation and in PBMCs of all stable CAD patients and healthy controls (C). (a) mRNA of GRP78/BiP, CHOP, Nrf2, HO-1, and GCLC was analyzed by quantitative real-time PCR. Normalized gene expression levels are given as the ratio between the mean value for the target gene and that for the β-actin in each sample. (b) Representative Western blot analysis for the indicated proteins and the average quantification obtained by densitometric analysis of all the samples derived from healthy C and CAD patients. Data from the Western blot analysis are expressed as the density ratio of target protein (row 1) to control (β-actin) (row 2) in arbitrary units. (c) Serum and LDL concentrations of oxPAPC (PEIPC, POVPC, and PGPC) in healthy C and CAD patients. (d) OxPAPC (PEIPC, POVPC, and PGPC) concentrations in PBMCs of healthy C and CAD patients. Data represent the mean ± SD of measurements performed in triplicate in all CAD patients and healthy C. *$p < 0.001$ vs healthy C.

ox-LDL but not of n-LDL with THP-1 cells determined a dose-dependent increase in cellular content of oxPAPC (POVPC and PGPC; $p < 0.01$).

The incubation of THP-1 cells with increasing amounts of serum derived from CAD patients but not from healthy C for 6 h in the presence of BHT resulted in a dose-dependent significant increase in oxPAPC (POVPC and PGPC) in THP-1 cells (p from < 0.05 to < 0.001; Figs. 2c and d). The concentrations of POVPC and PGPC in THP-1 cells were in a range similar to those found in circulating PBMCs (from 0.5 to 2.5 ng of POVPC or PGPC/mg PAPC). Because the majority of circulating oxPAPC is carried by lipoproteins, we used LPDS to eliminate their effect from the CAD patients' sera. The incubation of THP-1 cells with LPDS almost completely abolished the increase in cellular oxPAPC (Figs. 2c and d), indicating that the serum lipoproteins may be one of the major sources of oxPAPC of circulating cells.

GRP78/BiP, CHOP, and Nrf2/ARE gene expression in THP-1 cells enriched with serum and LPDS from CAD patients and healthy C

Figs. 3a and b show the mRNA and protein expression of GRP78/BiP, CHOP, Nrf2, OH-1, and GCLC subunit in THP-1 cells enriched with serum and LPDS of CAD patients and healthy C after 6 h of incubation. The expression of GRP78/BiP and CHOP was higher in THP-1 cells enriched with serum derived from CAD patients than from healthy C ($p < 0.001$). In contrast, there was no response of Nrf2, HO-1, or GCLC mRNA and protein expression in THP-1 cells enriched with the serum of CAD patients, whereas it was significantly higher in the cells derived from healthy C ($p < 0.001$). When THP-1 cells were incubated with LPDS derived from CAD patients, only small, and not significant, variations in GRP78/BiP and Nrf2/ARE gene expression were observed.

Effects of PGPC on ROS generation and GRP78/BiP, CHOP, and Nrf2/ARE gene expression

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 ($p < 0.01$; Fig. 4a). The generation of ROS in THP-1 cells was greatly reduced in the presence of the NADPH oxidase inhibitor DPI ($p < 0.01$). At variance with ROS, GSH dose-dependently increased only at 0.4 and 0.8 ng PGPC/mg PAPC, whereas at higher concentrations it significantly decreased (Fig. 4b; $p < 0.01$). As shown in Fig. 4b, DPI reversed almost completely the reduction in GSH induced by the highest concentrations of PGPC ($p < 0.01$).

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 ($p < 0.01$; Figs. 5a and b). 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, HO-1, and GCLC ($p < 0.01$), whereas at higher concentrations, it reduced Nrf2/ARE gene expression ($p < 0.01$; Figs. 5c and d). As shown in Figs. 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 for the first time demonstrated that the expression of GRP78/BiP, as a representative of UPR, and of CHOP, as a representative of ER-initiated apoptotic signaling, was significantly higher in PBMCs of CAD patients compared to those of healthy C. ER stress and UPR activation markers have been

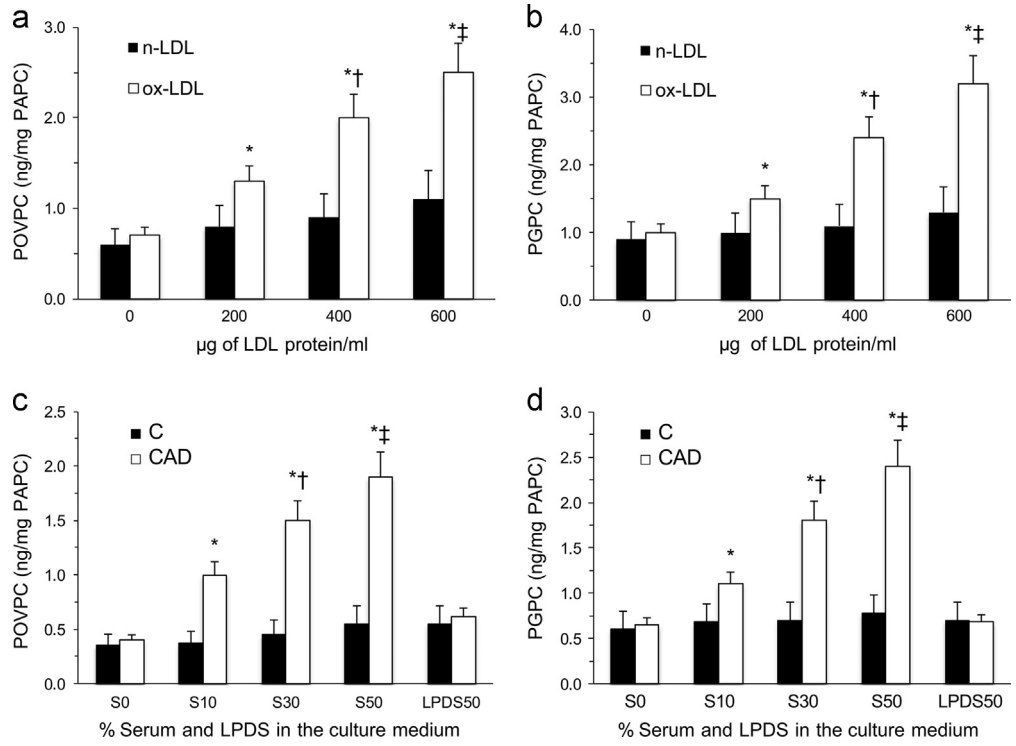

Fig. 2. OxPAPC in THP-1 incubated with native and oxidized LDL and with serum and LPDS. Increasing amounts of n-LDL and Cu^{2+}-modified LDL were incubated with THP-1 cells for 6 h at 37 °C and the following cellular oxPAPC were measured: (a) POVPC and (b) PGPC. Increasing amounts of serum (10, 30, and 50%) derived from stable CAD patients and healthy controls (C) were also incubated with THP-1 cells for 6 at 37 °C and (c) POVPC and (d) PGPC were measured. Finally THP-1 cells were incubated with 50% of LPDS derived from CAD patients. (a) and (b): $*p < 0.01$ vs 0; $\dagger p < 0.01$ vs 200; $\ddagger p < 0.01$ vs 400. (c) and (d): $*p < 0.01$ vs S0; $\dagger p < 0.01$ vs S10; $\ddagger p < 0.01$ vs S30.

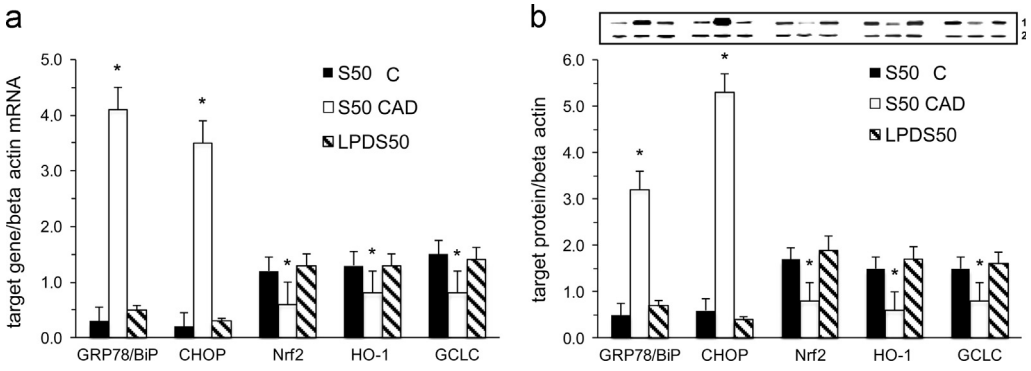

Fig. 3. Gene expression in THP-1 cells enriched with serum and LPDS derived from CAD and C. THP-1 cells were incubated without and with 50% serum and 50% LPDS derived from stable CAD patients and healthy controls (C) for 6 h at 37 °C. (a) mRNA of GRP78/BiP, CHOP, Nrf2, HO-1, and GCLC was analyzed by quantitative real-time PCR. Normalized gene expression levels are given as the ratio between the mean value for the target gene and that for the β-actin in each sample. (b) Representative Western blot analysis for the indicated proteins and the average quantification obtained by densitometric analysis of all the samples derived from healthy controls and CAD patients. Data from the Western blot analysis are expressed as the density ratio of target protein (row 1) to control (β-actin, row 2) in arbitrary units. Data represent the mean ± SD of measurements performed in triplicate. $*p < 0.01$ vs S50 healthy controls (C).

observed in both human and animal atherosclerotic lesions. Myoishi et al. [22] examined histological sections of human atherosclerotic coronary artery lesions obtained at autopsy or after directional coronary atherectomy [22]. Both smooth muscle cells and macrophages exhibited a markedly increased expression of the ER chaperones GRP78 and GRP94 and CHOP in thin-cap atheroma and ruptured plaques compared with intimal thickening, fibrous plaques, and thick-cap atheroma. Actually advanced atherosclerotic plaques have a pathophysiological environment that causes ER stress and activates the UPR owing to the presence of oxidized lipids, inflammation, and metabolic stress [23,24]. The fact that UPR and CHOP are activated in PBMCs of CAD patients indicates also that the circulating environment may be somehow altered in CAD patients. In this study we have shown that inflammation, altered redox state of PBMCs, and abnormal levels of ox-LDL are chronically present in patients with CAD, over the acute event, despite achievement of the correct targets for glucose, lipid, and blood pressure values. CAD patients, in fact, presented higher plasma levels of hs-CRP and ox-LDL and lower circulating GSH than healthy C. These results are substantially in line with previous studies [25–27] and in particular with our previous reports [14–16] in which hs-CRP levels and ox-LDL concentrations were higher in stable angina patients than in controls. In this context, ox-LDL has already been shown to trigger ER stress in vascular cells in vitro [28]. In addition, it has been established that oxidized lipids can induce ER stress and apoptosis in human aortic smooth muscle cells [28] and cause the death of macrophages via a CHOP-dependent pathway, with this process being inhibited by antioxidants [22]. In particular it has been shown that oxPAPC leads to ER stress and activation of the UPR in human aortic

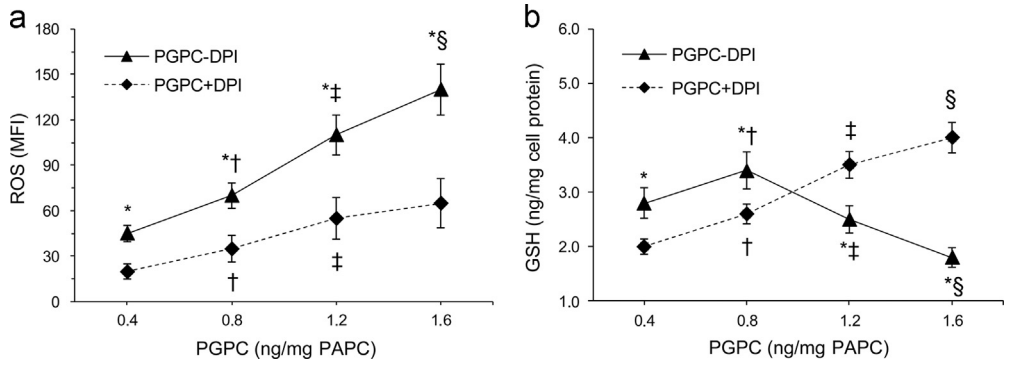

Fig. 4. Effects of PGPC on ROS generation and on GSH in THP-1 cells. Increasing amounts of PGPC with or without the addition of DPI (50 μM) were incubated with THP-1 cells, and ROS and GSH were measured respectively after 15 min and 6 h of incubation at 37 °C. Data on ROS and GSH are expressed respectively as mean fluorescence intensity (MFI) in arbitrary units and as ng/mg cell protein. The values of ROS and GSH represent the mean ± SD of experiments performed in triplicate on six different occasions. *$p < 0.01$ vs PGPC+DPI; †$p < 0.01$ vs PGPC 0.4 ng/mg PAPC; ‡$p < 0.01$ vs PGPC 0.8 ng/mg PAPC; §$p < 0.01$ vs PGPC 1.2 ng/mg PAPC.

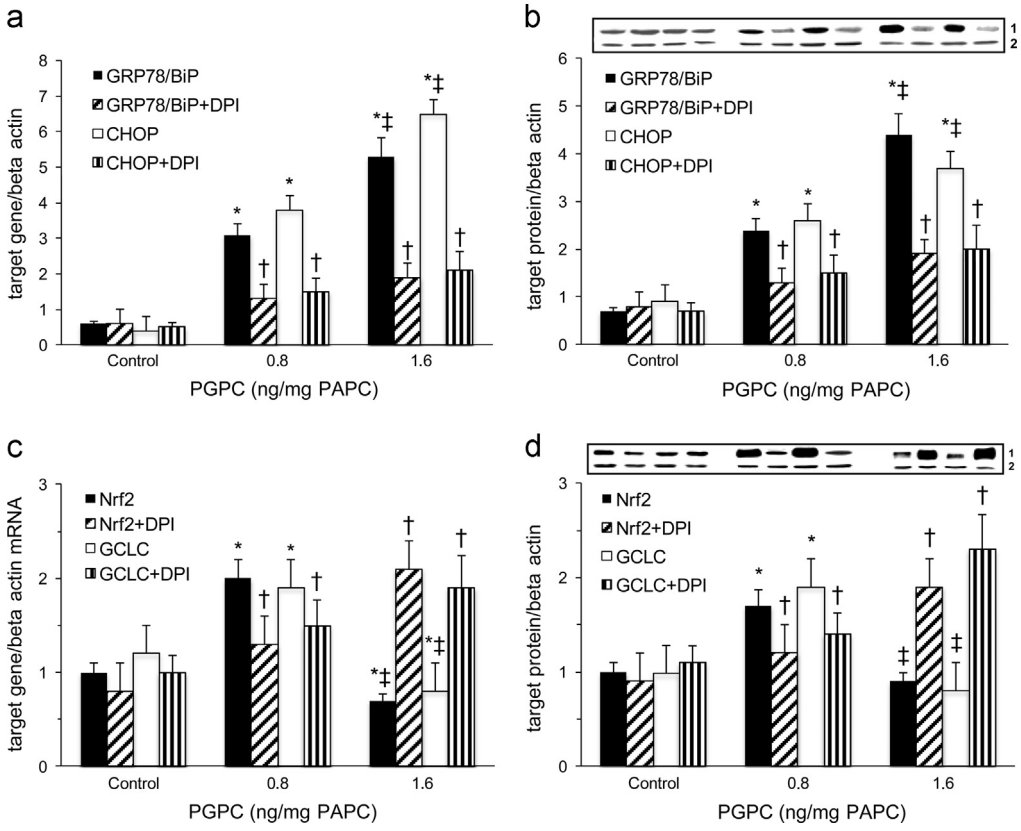

Fig. 5. Effects of PGPC on GRP78/BiP, CHOP, and Nrf2/ARE gene expression in THP-1 cells. Increasing amounts of PGPC with or without the addition of DPI (50 μM) were incubated with THP-1 cells and mRNA of (a) GRP78/BiP and CHOP and (c) Nrf2 and GCLC was analyzed by quantitative real-time PCR. Normalized gene expression levels are given as the ratio between the mean value for the target gene and that for the β-actin in each sample. (b, d) Representative Western blot analysis of the indicated proteins and the average quantification obtained by densitometric analysis of all the samples. Data from Western blot analysis are expressed as the density ratio of target protein (row 1) to control (β-actin, row 2) in arbitrary units. Data represent the mean ± SD of measurements performed in triplicate on six different occasions. *$p < 0.01$ vs control; †$p < 0.01$ vs GRP78/BiP and CHOP; ‡$p < 0.01$ vs PGPC 0.8 ng/mg PAPC.

endothelial cells [28]. In this study we found that plasma and PBMC concentrations of oxPAPC were significantly higher in CAD patients compared to healthy C. The reason plasma oxPAPC is higher in CAD patients than in healthy C may merely reflect the concentration of circulating ox-LDL. Ox-LDL, in fact, has previously been shown to contain significant amounts of oxPAPC [29]. The fact that in our study oxPAPC were higher in LDL of CAD patients than in that of healthy C directly confirms that the increase in plasma oxPAPC in CAD patients may, at least partially, be related to the levels of circulating ox-LDL. In contrast. the reason PBMCs of CAD patients have a higher concentration of oxPAPC than healthy C is currently unclear. However, it is known that there is a persistent interchange and mobility between oxPAPC in cell membranes [30–34]. We have hypothesized that there may be an interchange also between circulating ox-LDL and PBMCs. As a matter of fact, when the monocytoid THP-1 cells were incubated with ox-LDL, but not with n-LDL, there was a significant exchange of oxPAPC from ox-LDL to PBMCs, meaning that ox-LDL may be a source of oxPAPC for circulating cells. More interestingly, we also found that incubation of THP-1 cells with increasing amounts of serum derived from CAD patients, but not from healthy C, resulted in a dose-dependent significant increase in cellular oxPAPC. To eliminate the possible interchange between circulating lipoproteins and THP-1, we then incubated the cells with LPDS, i.e., a

serum deprived of lipoproteins. The fact that the incubation of THP-1 cells with LPDS almost completely abolished the increase in cellular oxPAPC suggests that the serum lipoproteins and in particular ox-LDL may be a major source of oxPAPC for circulating cells. In THP-1 cells enriched with oxPAPC, the expression of GRP78/BiP and CHOP was higher in cells incubated with serum derived from CAD patients compared to that from healthy C. When THP-1 cells were incubated with LPDS only small, and not significant, variations in GRP78/BiP and CHOP expression were observed, suggesting that the interchange of oxPAPC between ox-LDL and PBMC may have a role in inducing UPR and ER-initiated apoptotic signaling. However, it is well known that ox-LDL contains a plethora of lipid oxidation products that may exert proapoptotic effects [35]. On the basis of the present results, therefore, we cannot exclude that, under our experimental conditions, other oxidized lipids interchanged between ox-LDL and PBMCs and contributed to ER stress observed in CAD. Furthermore it has to be emphasized that growing evidence suggests that signaling pathways in the UPR and inflammation are interconnected through various mechanisms and in particular through the activation of nuclear factor κB [36], a master transcription factor with numerous functions including regulation of the inflammatory response. In this context, we have previously reported that ox-LDL induced an inflammatory response via specific receptors in both stable and unstable CAD patients, the phenomenon, however, being much more evident in the latter than in the former [14–16]. Provided that the slight increase in hs-CRP we found in this study in stable CAD patients is related to a mechanism other than ER stress, on the basis of the present results we cannot exclude that inflammation may have, at least partially, taken an active part in the induction of UPR. Further studies are needed to explore in more detail how ER stress can be affected by the extent of inflammatory response.

Unexpectedly, our results also show that oxPAPC-induced UPR and ER-initiated apoptotic signaling observed in PBMCs of CAD patients were not associated with an adequate expression of the genes correlated to the activity of the Nrf2/ARE system, one of the most important cellular defense mechanism against excess of ROS [12,13]. The inadequate response of the Nrf2/ARE system was observed both in PBMCs of CAD patients and in oxPAPC-enriched THP-1 cells. Our results are in line with a series of studies in which the same inadequacy was reported in other chronic pathologies such as chronic renal failure [37] and chronic obstructive pulmonary disease [38]. Although the precise mechanism of decreased Nrf2/ARE expression in chronic pathologies remains to be fully determined, a partial explanation of these results may derive from a recent paper from our group [19] in which monocytes from heavy smokers with the highest production of ROS did not appropriately react in terms of Nrf2/ARE activation. Actually when in our study we analyzed the dose-dependent effect of PGPC on ROS generation in THP-1 cells, in agreement with previously published data [19], we found that PGPC dose-dependently increased ROS formation and that this effect was dependent on the activation of NADPH oxidase because it was inhibited by DPI, a specific inhibitor of the enzyme. GRP78/BiP and CHOP expression induced by PGPC in THP-1 cells paralleled the dose-dependent increase in ROS, and their expression was inhibited by DPI. In contrast, 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 GCLC, whereas at higher concentrations, it reduced Nrf2/ARE gene expression. The repression of Nrf2/ARE gene activation was reversed at the highest concentrations of PGPC by DPI, suggesting that the amount of intracellular ROS may be one of the determinants of the inadequate response of Nrf2/ARE genes. So it is likely that it is an excess of oxidative stress that orchestrates the switch from the protective UPR and Nrf2/ARE gene expression to ER-initiated apoptotic signaling.

Conclusions

So, in conclusion, the results of this study show that PBMCs of stable CAD patients are characterized by the activation of UPR and of ER-initiated apoptotic signaling without an adequate Nrf2/ARE response. An excess of systemic oxidative stress with an interchange of oxPAPC between ox-LDL and PBMC may markedly contribute to the condition of ER stress observed in CAD.

References

[1] Mathers, C. D.; Loncar, D. Projection of global mortality and burden of disease from 2002 to 2030. *PLoS Med* **3:**2011–2030; 2006.
[2] Go, A. S.; Mozaffarian, D.; Roger, V. L.; Benjamin, E. J.; Berry, J. D.; Borden, W. B.; Bravata, D. M.; Dai, S.; Ford, E. S.; Fox, C. S.; Franco, S.; Fullerton, H. J.; Gillespie, C.; Hailpern, S. M.; Heit, J. A.; Howard, V. J.; Huffman, M. D.; Kissela, B. M.; Kittner, S. J.; Lackland, D. T.; Lichtman, J. H.; Lisabeth, L. D.; Magid, D.; Marcus, G. M.; Marelli, A.; Matchar, D. B.; McGuire, D. K.; Mohler, E. R.; Moy, C. S.; Mussolino, M. E.; Nichol, G.; Paynter, N. P.; Schreiner, P. J.; Sorlie, P. D.; Stein, J.; Turan, T. N.; Virani, S. S.; Wong, N. D.; Woo, D.; Turner, M. B. Executive summary: heart disease and stroke statistics—2013 update: a report from the American Heart Association. *Circulation* **127:**143–152; 2013.
[3] Tabas, I. The role of endoplasmic reticulum stress in the progression of atherosclerosis. *Circ. Res.* **7:**839–850; 2010.
[4] Kaufman, R. J. Orchestrating the unfolded protein response in health and disease. *J. Clin. Invest.* **110:**1389–1398; 2002.
[5] Ron, D.; Walter, P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell. Biol.* **8:**519–529; 2007.
[6] Gargalovic, P. S.; Gharavi, N. M.; Clark, M. J.; Pagnon, J.; Yang, W. P.; He, A.; Truong, A.; Baruch-Oren, T.; Berliner, J. A.; Kirchgessner, T. G.; Lusis, A. J. The unfolded protein response is an important regulator of inflammatory genes in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **26:**2490–2496; 2006.
[7] Kim, I.; Xu, W.; Reed, J. C. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat. Rev. Drug Discovery* **7:**1013–-1030; 2008.
[8] Kansanen, E.; Jyrkkanen, H.; Levonen, A. Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids. *Free Radic. Biol. Med.* **52:**973–982; 2012.
[9] Bertolotti, A.; Zhang, Y.; Hendershot, L. M.; Harding, H. P.; Ron, D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat. Cell Biol.* **2:**326–332; 2000.
[10] Thorp, E.; Li, G.; Seimon, T.; Kuriakose, G.; Ron, D.; Tabas, I. Reduced apoptosis and plaque necrosis in advanced atherosclerotic lesions of Apoe−/− and Ldlr−/− mice lacking CHOP. *Cell Metab.* **9:**474–481; 2009.
[11] McCullough, K. D.; Martindale, J. L.; Klotz, L. O.; Aw, T. Y.; Holbrook, N. J. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol. Cell. Biol.* **21:**1249–1259; 2001.
[12] Cullinan, S. B.; Diehl, J. A. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J. Biol. Chem.* **279:**20108–20117; 2004.
[13] Singh, S.; Vrishni, S.; Singh, B. K.; Rahman, I.; Kakkar, P. Nrf2/ARE stress response mechanism: a control point in oxidative stress mediated dysfunction and chronic inflammatory disease. *Free Radic. Res.* **44:**1267–1288; 2010.
[14] Cominacini, L.; Anselmi, M.; Garbin, U.; Fratta Pasini, A.; Stranieri, C.; Fusaro, M.; Nava, C.; Agostoni, P.; Keta, D.; Zardini, P.; Sawamura, T.; Lo Cascio, V. Enhanced plasma levels of oxidized low-density lipoprotein increase circulating nuclear factor-kappa B activation in patients with unstable angina. *J. Am. Coll. Cardiol.* **46:**799–806; 2005.
[15] Anselmi, M.; Garbin, U.; Agostoni, P.; Fusaro, M.; Pasini, A. F.; Nava, C.; Keta, D.; Turri, M.; Zardini, P.; Vassanelli, C.; Lo Cascio, V.; Cominacini, L. Plasma levels of oxidized-low-density lipoproteins are higher in patients with unstable angina and correlated with angiographic coronary complex plaque. *Atherosclerosis* **185:**114–120; 2006.
[16] Fratta Pasini, A.; Anselmi, M.; Garbin, U.; Franchi, E.; Stranieri, C.; Nava, M. C.; Boccioletti, V.; Vassanelli, C.; Cominacini, L. Enhanced levels of oxidized low-density lipoprotein prime monocytes to cytokine overproduction via up-regulation of CD14 and toll-like receptor 4 in unstable angina. *Arterioscler. Thromb. Vasc. Biol.* **27:**1991–1997; 2007.
[17] Braunwald, E. Unstable angina: a classification. *Circulation* **80:**410–414; 1989.
[18] Evans, K.; Mitcheson, J.; Laker, M. F. Effect of storage at −70 degrees C on lipid, lipoprotein and apolipoprotein concentrations. *Clin. Chim. Acta* **258:**219–229; 1997.
[19] Garbin, U.; Fratta Pasini, A.; Stranieri, C.; Cominacini, M.; Pasini, A.; Manfro, S.; Lugoboni, F.; Mozzini, C.; Guidi, G.; Faccini, G.; Cominacini, L. Cigarette smoking blocks the protective expression of Nrf2/ARE pathway in peripheral

mononuclear cells of young heavy smokers favouring inflammation. *PLoS One* 4; 2009*e8225* 4; 2009.
[20] Watson, A. D.; Leitinger, N.; Navab, M.; Faull, K. F.; Hörkkö, S.; Witztum, J. L.; Palinski, W.; Schwenke, D.; Salomon, R. G.; Sha, W.; Subbanagounder, G.; Fogelman, A. M.; Berliner, J. A. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *J. Biol. Chem.* **272:**13597–13607; 1997.
[21] Cominacini, L.; Fratta Pasini, A.; Garbin, U.; Davoli, A.; Tosetti, M. L.; Campagnola, M.; Rigoni, A.; Pastorino, A. M.; Lo Cascio, V.; Sawamura, T. Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. *J. Biol. Chem.* **275:**12633–-12638; 2000.
[22] Myoishi, M.; Hao, H.; Minamino, T.; Watanabe, K.; Nishihira, K.; Hatakeyama, K.; Asada, Y.; Okada, K.; Ishibashi-Ueda, H.; Gabbiani, G.; Bochaton-Piallat, M. L.; Mochizuki, N.; Kitakaze, M. Increased endoplasmic reticulum stress in atherosclerotic plaques associated with acute coronary syndrome. *Circulation* **116:**1226–1233; 2007.
[23] Hansson, G. Inflammation, atherosclerosis and coronary artery disease. *N. Engl. J. Med.* **352:**1685–1695; 2005.
[24] Garbin, U.; Baggio, E.; Stranieri, C.; Pasini, A.; Manfro, S.; Mozzini, C.; Vallerio, P.; Lipari, G.; Merigo, F.; Guidi, G.; Cominacini, L.; Fratta Pasini, A. Expansion of necrotic core and shedding of Mertk receptor in human carotid plaques: a role for oxidized polyunsaturated fatty acids? *Cardiovasc. Res.* **97:**125–133; 2013.
[25] Ridker, P. M.; Cannon, C. P.; Morrow, D.; Rifai, N.; Rose, L. M.; McCabe, C. H.; Pfeffer, M. A.; Braunwald, E. C-reactive protein levels and outcomes after statin therapy. *N. Engl. J. Med.* **352:**20–28; 2005.
[26] Cavalca, V.; Veglia, F.; Squellerio, I.; Marenzi, G.; Minardi, F.; De Metrio, M.; Cighetti, G.; Boccotti, L.; Ravagnani, P.; Tremoli, E. Glutathione, vitamin E and oxidative stress in coronary artery disease: relevance of age and gender. *Eur. J. Clin. Invest.* **39:**267–272; 2009.
[27] Elesber, A.; Best, P.; Lennon, R.; Mathew, V.; Rihal, C. S.; Lerman, L. O.; Lerman, A. Plasma 8-iso-prostaglandin F2α, a marker of oxidative stress, is increased in patients with acute myocardial infarction. *Free Radic. Res.* **40:**385–391; 2006.
[28] Sanson, M.; Augé, N.; Vindis, C.; Muller, C.; Bando, Y.; Thiers, J. C.; Marachet, M. A.; Zarkovic, K.; Sawa, Y.; Salvayre, R.; Nègre-Salvayre, A. Oxidized low-density lipoproteins trigger endoplasmic reticulum stress in vascular cells: prevention by oxygen-regulated protein 150 expression. *Circ. Res.* **104:**328–336; 2009.
[29] Ishii, H.; Tezuka, T.; Ishikawa, H.; Takada, K.; Oida, K.; Horie, S. Oxidized phospholipids in oxidized low-density lipoprotein down-regulate thrombomodulin transcription in vascular endothelial cells through a decrease in the binding of RARbeta-RXRalpha heterodimers and Sp1 and Sp3 to their binding sequences in the TM promoter. *Blood* **101:**4749–4765; 2003.
[30] Stemmer, U.; Dunai, Z.; Koller, D.; Koller, D.; Purtsinger, G.; Zenzmaier, E.; Deigner, H. P.; Aflaki, E.; Kratky, D.; Hermetter, A. Toxicity of oxidized phospholipids in cultured macrophages. *Lipids Health Dis.* **11:**110; 2012.
[31] Stemmer, U.; Ramprecht, C.; Zenzmaier, E.; Stojčić, B.; Rechberger, G.; Kollroser, M.; Hermetter, A. Uptake and protein targeting of fluorescent oxidized phospholipids in cultured RWA 264.7 macrophages. *Biochim. Biophys. Acta* **1821:**706–718; 2012.
[32] Stemmer, U.; Hermetter, A. Protein modification by aldehydophospholipids and its functional consequences. *Biochim. Biophys. Acta* **1818:**2436–2445; 2012.
[33] Friedman, P.; Horkko, S.; Steinberg, D.; Witztum, J. L.; Dennis, E. A. Correlation of antiphospholipid antibody recognition with the structure of synthetic oxidized phospholipids: importance of Schiff base formation and aldol condensation. *J. Biol. Chem.* **277:**7010–7020; 2002.
[34] Rhode, S.; Grurl, R.; Brameshuber, M.; Hermetter, A.; Schutz, G. J. Plasma membrane fluidity affects transient immobilization of oxidized phospholipids in endocytotic sites for subsequent uptake. *J. Biol. Chem.* **284:**2258–2265; 2009.
[35] Parthasarathy, S.; Raghavamenon, A.; Garelnabi, A. M.; Santanam, N. Oxidized low-density lipoprotein. *Methods Mol. Biol.* **610:**403–417; 2010.
[36] Hotamisligil, G. S. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* **140:**900–917; 2010.
[37] Kim, H.; Vaziri, N. Contribution of impaired Nrf2–Keap1 pathway to oxidative stress and inflammation in chronic renal failure. *Am. J. Physiol. Renal Physiol.* **298:**F662–F671; 2010.
[38] Suzuki, M.; Betsuyaku, T.; Ito, Y.; Nagai, K.; Nasuhara, Y.; Kaga, K.; Kondo, S.; Nishimura, M. Down-regulated NF-E2 related factor 2 in pulmonary macrophages of aged smokers and patients with chronic obstructive pulmonary disease. *Am. J. Respir. Cell Mol. Biol.* **39:**673–682; 2008.