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Oxidative stress, glutathione status, sirtuin and cellular stress response in type 2 diabetes ☆

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ABSTRACT

Oxidative stress has been suggested to play a main role in the pathogenesis of type 2 diabetes mellitus and its complications. As a consequence of this increased oxidative status a cellular adaptive response occurs requiring functional chaperones, antioxidant production and protein degradation. This study was designed to evaluate systemic oxidative stress and cellular stress response in patients suffering from type 2 diabetes and in age-matched healthy subjects. Systemic oxidative stress has been evaluated by measuring plasma reduced and oxidized glutathione, as well as pentosidine, protein carbonyls lipid oxidation products 4-hydroxy-2-nonenal and F2-isoprostanes in plasma, and lymphocytes, whereas the lymphocyte levels of the heat shock proteins (HSP) HO-1, Hsp72, Sirtuin-1, Sirtuin-2 and thioredoxin reductase-1 (TrxR-1) have been measured to evaluate the systemic cellular stress response. Plasma GSH/GSSG showed a significant decrease in type 2 diabetes as compared to control group, associated with increased pentosidine, F2-isoprostanes, carbonyls and HNE levels. In addition, lymphocyte levels of HO-1, Hsp70, Trx and TrxR-1 ($P<0.05$ and $P<0.01$) in diabetic patients were higher than in normal subjects, while sirtuin-1 and sirtuin-2 protein was significantly decreased ($p<0.05$). In conclusion, patients affected by type 2 diabetes are under condition of systemic oxidative stress and, although the relevance of downregulation in sirtuin signal has to be fully understood, however induction of HSPs and thioredoxin protein system represent a maintained response in counteracting systemic pro-oxidant status. This article is part of a Special Issue entitled: Antioxidants and Antioxidant Treatment in Disease.

1. Introduction

Oxidative stress is caused by an unbalance between a relative overload of oxidants and a depletion of antioxidants, and it is implicated in the pathogenesis of several chronic diseases, including atherosclerosis, ischaemia/reperfusion injury, chronic inflammatory diseases, renal failure and diabetes mellitus [1–4]. As a consequence of the increased burden of oxidative stress severe damage to DNA, proteins and lipids occur [1]. With regard to the role of oxidative stress in the pathogenesis and in the clinical history of diabetes mellitus, compelling evidences have been provided that both Type 1 and Type 2 diabetic patients are under conditions of an increased oxidative stress and that the complications of diabetes mellitus are partially mediated by oxidative stress [4–6]. Several mechanisms seem to be involved in the genesis of oxidative stress in diabetic patients. Among these, glucose autoxidation, non enzymatic protein glycation as well as the formation of advanced-glycation end-products (AGEs) have been demonstrated in patients with diabetes and a direct relationship with the circulating blood glucose levels and glucose variability has been repeatedly demonstrated [5,7,8]. In particular, it has been shown that two subclasses of AGEs, such as N-ε(carboxymethyl)lysine and pentosidine, accumulate in various tissues of poorly controlled diabetic patients [9]. The interplay between oxidative stress and AGEs is very complex. In fact, reactive oxygen species (ROS) accelerate the formation of AGEs, which in turn, as glycated proteins, are also able to produce ROS via complex biochemical mechanisms [10–12]. During these reactions, protein modifications take place and compounds with a carbonyl or dicarbonyl moieties are formed [5].

In addition to the above mentioned AGEs and protein carbonyls, various other products derived from lipid peroxidation accumulate in biological fluids and tissues of diabetic patients. 4-hydroxy-2-nonenal (HNE) is considered an important marker of lipid peroxidation, but at the same time it is directly involved in both citotoxicity and mutagenic activity. In fact, 4-HNE further reacts with protein

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residues, such as histidine, to generate stable Michael adducts [13,14]. Interestingly, HNE-modified proteins have been identified in the serum and in renal tissues of type 2 diabetic patients [13,15]. The free radical-based oxidation of arachidonic acid [16,17] is one of the most relevant biochemical pathways that generate isoprostanes. Increased levels of F2-isoprostanes can be found in the plasma or urine of patients affected by several chronic inflammatory or degenerative diseases, including diabetes, and are currently used as *in vivo* indicators of lipid peroxidation [5,18–21]. It is of interest that in addition to their well known role as in indicator of the oxidative stress status, several lines of evidences demonstrate that F2-isoprostanes are directly involved in vascular function and endothelial mediated platelet aggregation [22–24].

In order to adapt to environmental changes and survive to different types of injuries, eukaryotic cells have developed a complex networks of cellular stress response mechanisms which detect and control different forms of stress. These responses include the heat shock protein synthesis [25–29] as well as the stimulation of the thioredoxin system [30,31]. Together, they form a powerful system involved in many intracellular and extracellular processes, including cell proliferation, the redox regulation of gene expression and signal transduction, protection against oxidative stress, anti-apoptotic functions, growth factor and cytokine-like effects, and the regulation of the redox state of the extracellular environment [27]. The nicotinamide adenine dinucleotide (NAD)-dependent deacetylase, named sirtuin, are a complex of proteins that modulate the transcriptional silencing of selected region of the genome via the regulation of proteins' acetylation–deacetylation pathways. They play a significant role in the aging @@process and in the pathogenesis of various chronic and degenerative diseases [32]. The present study was designed to evaluate the presence of systemic oxidative stress, glutathione status and cellular stress response in plasma and lymphocytes of patients with type 2 diabetes. Furthermore, we demonstrated in the same patients, for the first time in literature to our knowledge, that changes in the redox state of glutathione are associated with activation of cellular stress response, evaluated as the induction at the circulating lymphocyte level of heme oxygenase-1, Hsp70 as well as the antioxidant thioredoxin protein and the related enzyme thioredoxin reductase-1 (TrxR-1) and with a significant reduction of Sirtuin expression.

2. Methods

2.1. Patients

All the procedures on human subjects have been conducted in accordance with the guidelines in the Declaration of Helsinki and the experimental protocol has been formally approved by the Ethical Committee of the University of Catania. All the patients gave also their informed consent prior to undergo any procedure. Fifteen adults with type 2 diabetes which were followed on a regular basis in the outpatient clinic of the Department of Internal Medicine of the Policlinico di Catania joined this study. Clinical data of the participating subjects are summarized in Table 1. Patients were referred by the primary care physicians or by one of the outpatient Diabetes Centers of the Catania metropolitan area. Exclusion criteria were the following: renal insufficiency secondary to diabetes or to any other cause, ischemic heart disease or heart failure (stage 2 or above according to NYHA classification), hepatic failure, endocrine disease other than diabetes mellitus, any other clinical or laboratory evidence of major organ disease. All patients underwent to a run in period of 4 to 8 weeks designed to achieve a stabilization of blood pressure levels, blood glucose control. All the subjects were not taking antioxidants, no limitation was given to the type of pharmacological therapy employed to control diabetes or hypertension, medications were maintained constant throughout the entire duration of the study.

Table 1
Clinical parameters of diabetic patients with normal renal function.

Clinical parameters (unit)	Mean ± SEM (range)
Age (years)	61 ± 4 (35–71)
Male/female	8/7
Body weight (Kg)	82 ± 17 (65–94)
Height (cm)	165 ± 10 (152–175)
Body mass index	28 ± 7 (24–34)
Plasma creatinine (mg/dl)	1,04 ± 0.52 (0.84–1.37)
Creatinine clearance (ml/min)	76 ± 10 (66–96)
MDRD-estimated GFR (ml/min)	74 ± 6 (59–89)
24 h proteinuria (mg)	77 ± 16 (44–249)
Fasting plasma glucose (mg/dl)	133 ± 12 (120–243)
Glycated hemoglobin (%)	6.4 ± 2.2 (4.6–9.7)
Sistolic blood pressure (mm Hg)	155 ± 10 (140–165)
Diastolic blood pressure (mm Hg)	85 ± 6 (70–95)

2.2. Samples

Blood (6 ml) was collected after an overnight fast by venopuncture from an antecubital vein in EDTA-coated tubes. Immediately after sampling, 2 ml of blood were centrifuged at 10,000×*g* for 1 min at 4 °C to separate plasma from red blood cells and 4 ml were utilized for lymphocytes purification.

2.3. Lymphocytes purification

Lymphocytes from peripheral blood were purified by using the Ficoll Paque System following the procedure provided by the manufacturer (GE Healthcare, Piscataway, NJ).

2.4. Reduced and oxidized glutathione assay

Reduced glutathione (GSH) and glutathione disulfide (GSSG) were measured by the NADPH-dependent GSSG reductase method as described by Adams et al. [33]. For total glutathione (GSH + GSSG), aliquots (0.1 ml) of whole blood were immediately added to 0.1 ml of a cold solution containing 10 mM DTNB and 5 mM EDTA in 100 mM potassium phosphate, pH 7.5. The sample were mixed by tilting and centrifuged at 12,000 *g* for 2 min at 4 °C. An aliquot (50 μl) of the supernatant was added to a cuvette containing 0.5 U of GSSG reductase in 100 mM potassium phosphate and 5 mM EDTA, pH 7.5 (buffer 1). After 1 min of equilibration, the reaction was initiated with 220 nmol of NADPH in buffer 1 for a final reaction volume of 1 ml. The formation of a GSH-DTNB conjugate was then measured at λ = 412 nm. The reference cuvette contained equal concentrations of DTNB, NADPH and enzyme, but not sample. For the GSSG assay, aliquot (0.75 ml) of blood samples were immediately added to 0.75 ml of a solution containing 10 mM NEM and 5 mM EDTA in 100 mM potassium phosphate, pH 7.5. The sample were mixed by tilting and centrifuged at 12,000 *g* for 2 min at 4 °C. An aliquot (900 μl) of the supernatant was passed at one drop/s through a SEP-PAK C18 Column (Waters, Framingham, MA) that had been washed with methanol followed by water. The column was then washed with 0.1 ml of buffer 1. Aliquots (865 μl) of the combined eluates were added to a cuvette with 250 nmol of DTNB and 0.5 U of GSSG reductase. The assay then proceeded as in the measurement of total GSH. GSH was then obtained by subtracting GSSG values from total glutathione. GSH and GSSG standards in the ranges between 0 and 10 nmol and 0.010 and 10 nmol, respectively, added to control samples were used to

obtain the relative standard curves, and the results were expressed in nmol of GSH or GSSG, respectively, per ml of blood.

2.5. Western blot analysis

Plasma samples were ready to use, while the lymphocyte pellet was homogenized and centrifuged at 10,000×*g* for 10 min and the supernatant was used for analysis after dosage of proteins. The Aliquot (40 μg) of protein extract was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred overnight to nitrocellulose membrane and the non specific binding of antibodies was blocked with 3% non-fat dry milk in PBS. Membrane were then probed with a monoclonal mouse anti-Hsp70 antibody (SPA-810, Stressgen, Ann Arbor, MI, USA) that recognizes only the inducible form, while immunodetection of HO-1, DNPH, HNE, Trx, TrxR-1, Sirt-1 and Sirt-2 was performed using polyclonal rabbit antibodies: SPA-895 (Stressgen), V0401 (DAKO, Glostrup, Denmark), HNE11-S (Alpha Diagnostic International, San Antonio, TX, USA), 07–613 (Upstate Biotechnology, Charlottesville, VA), sc-15404 and sc-20966 (Santa Cruz Biotech. Inc., Santa Cruz, CA,USA), respectively. A goat polyclonal antibody specific for β-actin was used for loading control (1:1000). Blots were then incubated with secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (IgG) when probing DNPH, HNE, Trx, TrxR-1, sirtuin-1 and sirtuin-2, whereas a HRP-conjugated goat anti-mouse (IgG) was used in the case of Hsp70 and HRP-conjugated anti-goat (IgG) for detection of β-actin followed by ECL chemiluminescence (Amersham, GE Healthcare, Piscataway, NJ). Immunoreactive bands were quantified by scanning Western blot imaged films with a laser densitometer (LKB-Ultrascan, XL model, Pharmacia, American Instruments, Haverhill, MA, USA).

2.6. Measurement of F2-isoprostanes and pentosidine

The HPLC analysis of F2-isoprostanes was performed as described by Ritov et al. [34]. Values are expressed as nM for plasma F2-isoprostanes. Plasma pentosidine was measured according to Miyata et al. [35]. Values are expressed as pmol/mg protein.

2.7. Protein measurements

Sample protein concentrations were determined by the bicinchoninic acid (BCA) method [36].

2.8. Statistical analysis

All results are expressed as means ± S.E.M. Each experiment was performed, unless otherwise specified, in triplicate. Data were analyzed by one-way ANOVA, followed by inspection of all differences by Duncan's new multiple-range test. Differences were considered significant at $P<0.05$.

3. Results

Diabetes is a chronic inflammatory disease and this study was designed to evaluate systemic oxidative stress and cellular stress response in patients suffering from type 2 diabetes and in age-matched healthy subjects. Clinical data of patients and control subjects are summarized in Table 1.

The content of reduced and oxidized GSH in plasma of type 2 diabetic patients was determined as a measure of the antioxidant status and compared with the levels in the control group (Fig. 1). We found a significant decrease (to 63% of control levels) in the content of reduced GSH and a significant increase (by 46%) in the content of oxidized GSH (GSSG) in the diabetic samples compared with the control samples. These changes in absolute GSH and GSSG concentrations resulted in a 68% decrease in the GSH/GSSG ratio in type 2 diabetes compared with control patients (data not shown). Changes in the redox glutathione status were consistent with oxidative modifications of redox status in plasma proteins between the experimental and control groups investigated. Protein oxidation was evaluated by measuring the amount of protein carbonyls (DNPH). Protein carbonyls most often are detected by two methods, *i.e.*, derivatization with 2,4-dinitrophenylhydrazine (DNPH), followed by immunochemical detection with an antibody against the resulting protein hydrazone, or formation of the Schiff base by biotin hydrazide followed by detection of the protein-bound imine with enzyme- or fluorophore-linked avidin or streptavidin. Here we describe the DNPH-based detection method that is routinely used for detection of carbonylated proteins. In the DNPH method, samples are derivatized with DNPH. DNPH reacts with carbonyl groups to form protein-resident 2,4-dinitrophenylhydrazone (DNP), which is then detected using commercially available anti-DNP antibodies. As shown in Fig. 2A, protein carbonyls were found at a significantly ($p<0.05$) higher level in plasma of type 2 diabetic patients than in control subjects. Furthermore, one measure of oxidative marker is lipid oxidation, indexed by HNE, which can also occur in brain and peripheral tissues under oxidative stress [37,38]. HNE, formed from arachidonic acid or other unsaturated fatty acids following free radical attack, was bound by Michael in addition to proteins, particularly at cysteine, histidine, or lysine residues [39]. Examination of plasma content of HNE in diabetic patients showed a significant ($p<0.05$) elevation of protein-bound HNE as compared to control group (Fig. 2B). As previously reported, AGEs, such as pentosidine, are reliable markers of oxidative stress in diabetes and diabetic nephropathy, but they can trigger *per se* further inflammation thus creating a self-sustained vicious circle responsible for tissue damage [38,40]. In accordance with this theory, we demonstrated a significant increase ($P<0.01$) in plasma levels (Fig. 3A) of pentosidine in patients suffering from type 2 diabetes with respect to control subjects. The next

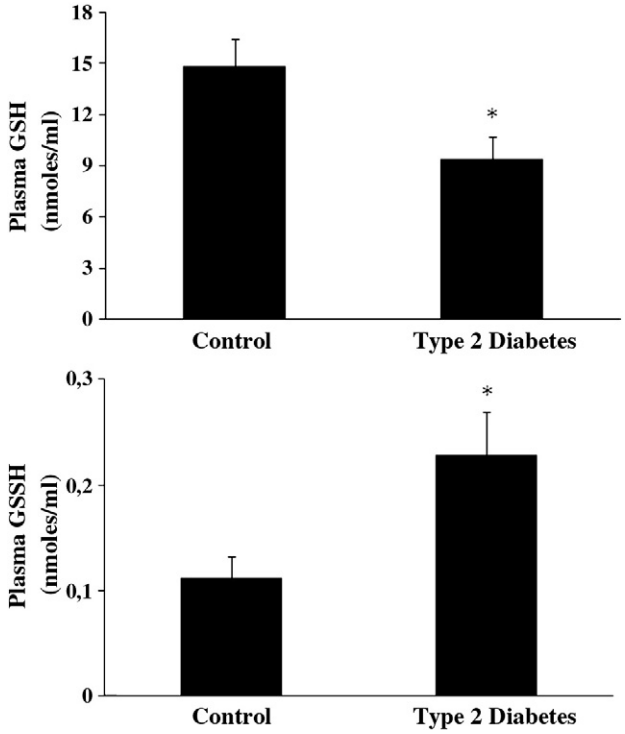

Fig. 1. Plasma levels of reduced (GSH) and oxidized (GSSG) glutathione in type 2 Diabetic Patients and Controls. Plasma GSH and GSSG contents were assayed as described in Methods. Data are expressed as mean ± SEM of 15 patients per group. *$P<0.05$ *vs* controls.

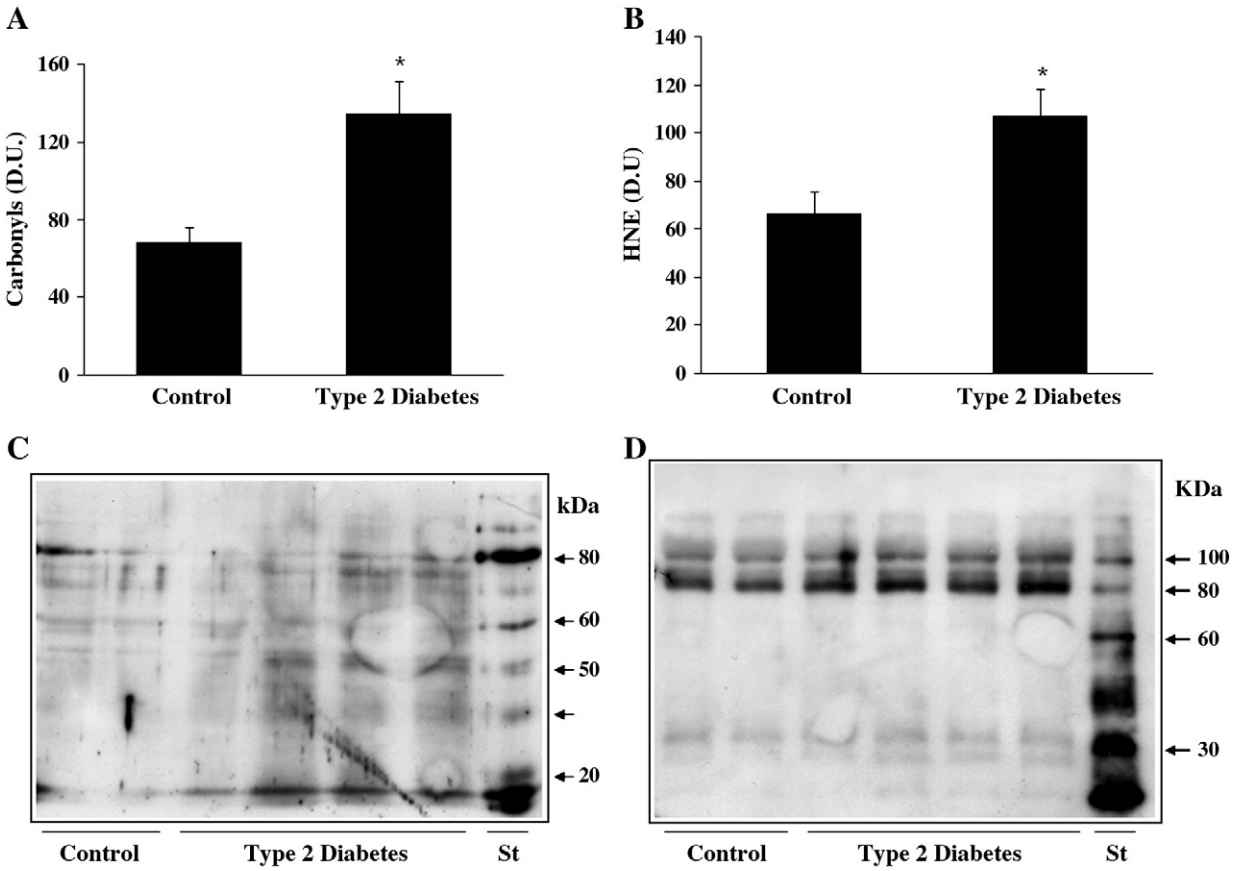

Fig. 2. Protein carbonyls and 4-hydroxy-2-nonenals levels in plasma from type 2 diabetic patients. Plasma samples from type 2 diabetic patients and age-matched controls were assayed for protein carbonyls (DNPH) (Fig. 2A) and 4-hydroxy-2-nonenals (HNE) (Fig. 2B) by western blot as described in Methods. Representative immunoblots are shown (Fig. 2C) and (Fig. 2D). The bar graph shows the densitometric evaluation and values are expressed as mean ± SEM of 15 patients per group.

step was to evaluate the effects of observed systemic pro-oxidant conditions on lipids-derived circulating F2 isoprostanes. As shown in Fig. 3B, a significant elevation ($P<0.05$) of in plasma levels of total F2-isoprostanes has been found in type 2 diabetic patients ($P<0.01$) with respect to controls.

The heat shock response contributes to establishing a cytoprotective state in a wide variety of human diseases, including inflammation, cancer, aging and neurodegenerative disorders. The vitagene family is composed of the heat shock proteins HO-1 (also called Hsp32), Hsp70, by the thioredoxin system and by sirtuin proteins [41,42]. We therefore evaluated the expression levels of cellular stress response proteins in plasma and lymphocytes in control and type 2 diabetes patients. Western blot analysis of plasma probed for thioredoxin (Trx) antioxidant protein expression is reported in Fig. 4. Trx expression was significantly ($p<0.05$) decreased in the plasma of diabetic patients, compared to controls.

Heme oxygenase-1 (HO-1), also referred to as Hsp32, belongs to the Hsp family and protects brain cells from oxidative stress by degrading toxic heme into free iron, carbon monoxide and biliverdin [29,43,44]. This latter is then reduced by biliverdin reductase (BVR) into bilirubin (BR), a linear tetrapyrrole with antioxidant properties; very recently, BR has been shown to effectively counteract also nitrosative stress, due to its ability to bind and inactivate NO and RNS [43,45,46]. We evaluated the expression of inducible HO-1 isoform in lymphocytes of type 2 diabetes patients and in controls. As shown in Fig. 5, HO-1 protein expression was higher in samples from diabetes patients compared to controls.

The 70 kDa family of stress proteins is one of the most extensively studied cytoprotective system which includes the constitutive heat shock cognate isoform (Hsc70), and the inducible isoform, Hsp70 (also referred to as Hsp72). Here we evaluated the inducible isoform and its expression is reported in Fig. 6A, which shows a significant ($p<0.05$) increase in type 2 diabetes lymphocytes with respect to healthy control subjects. A representative immunoblot is also shown (Fig. 6B).

Analysis of lymphocytes in type 2 diabetes patients, compared to control group, revealed also a significant ($p<0.05$) reduction of Trx expression (Fig. 7) which was associated with increased levels of the enzyme Thioredoxin reductase-1 (Fig. 8). Interestingly, we found a significant ($p<0.05$) decrease in the levels of sirtuin-1 as well as sirtuin-2 proteins in lymphocytes of diabetic patients than in control group (Fig. 9A,C and Fig. 9 B,D). As far as we are concerned this is the first evidence of changes in sirtuin-1 and sirtuin-2 expression in type 2 diabetes, although at the moment we can not exclude that this might not be a specific alteration of this progressive chronic inflammatory systemic disease.

4. Discussion

Prevalence of diabetes is constantly increasing in the United States, in Europe as well as in developing countries and the global burden associated with the disease is becoming one of the most relevant world health problem [47]. The long term exposure to chronic hyperglycaemia along with the increase in oxidative stress that characterizes the diabetic patients results in the formation and accumulation of AGEs [4,7,9,48,49]. AGEs have a wide range of chemical, cellular, and tissue effects that contribute to the development of chronic complication of diabetes [40,50]. The importance of AGEs as downstream mediators of tissue injury in diabetes has been demonstrated by animal studies in which the inhibition of the advanced

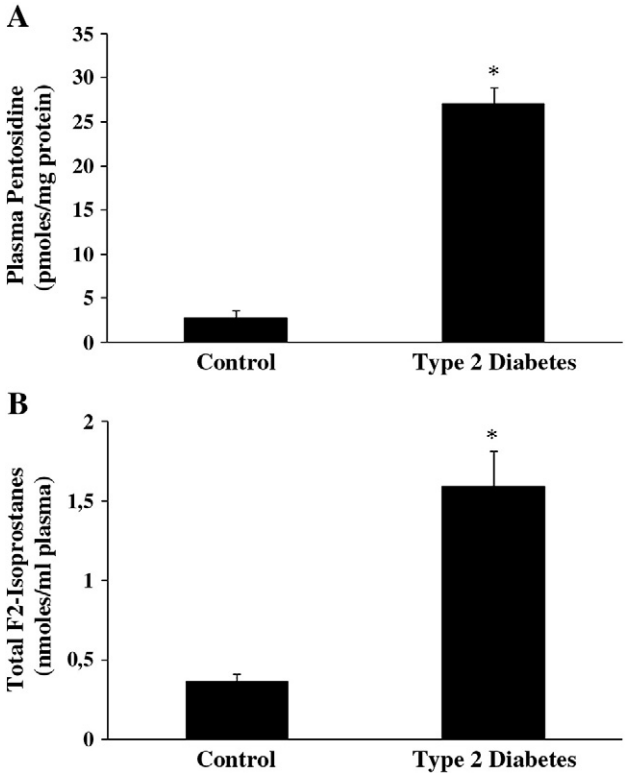

Fig. 3. Pentosidine and Total F2-isoprostanes levels in plasma from type 2 diabetic patients. Plasma samples from patients with type 2 diabetes and age-matched controls were assayed for pentosidine (Fig. 3A) and total F2-isoprostanes (Fig. 3B) as indicated in Methods. Data are expressed as mean ± SEM of 15 patients per group. *P<0.01 *vs* controls.

glycation reaction delayed the development of microvascular complications without any direct effect on the glycemic control [40]. Furthermore, AGEs-modified proteins undergo physico-chemical changes which alter charge, solubility and conformation resulting in an altered protein [40]. Finally, the effects of AGEs, together with hyperglycaemia and ROS induce growth factors and cytokines causing organ hypertrophy, as the enlarged kidneys in diabetic nephropathy and accumulation of extracellular matrix components [50]. Our results showing an elevation in plasma AGEs in patients with diabetes compared with healthy subjects are in accordance with current literature [51–53].

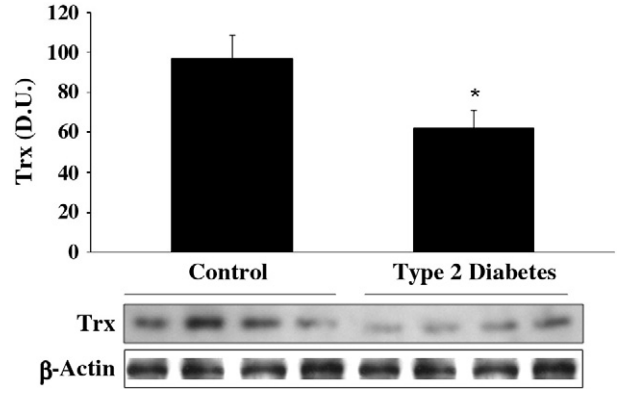

Fig. 4. Plasma levels of Thioredoxin in type 2 diabetes and controls subjects. Plasma samples from patients with nephropathy secondary to type 2 diabetes and age-matched controls were assayed for thioredoxin (Trx) by western blot as described in Methods. A representative immunoblot is shown. β-actin has been used as loading control. The bar graphs show the densitometric evaluation and values are expressed as mean ± SEM of 15 patients per group. *P<0.05 *vs* control. D.U., densitometric units.

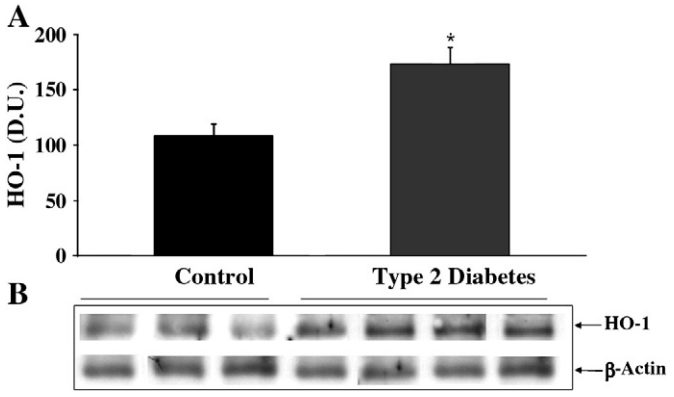

Fig. 5. Heme oxygenase-1 levels in lymphocytes from type 2 diabetic patients. Lymphocyte samples from patients with nephropathy secondary to type 2 diabetes and age-matched controls were assayed for heme oxygenase-1 (HO-1) by western blot as described in Methods. A representative immunoblot is shown. β-actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean ± SEM of independent analyses on 15 patients per group. *P<0.05 *vs* control. D.U., densitometric units.

Reactive carbonyl compounds, which are known precursors of carbonyl stress, can be generated during the AGEs-mediated free radical formation [54,55]. Our evidence of an increased levels of carbonyls both in plasma and lymphocytes from type 2 diabetic patients are in line with clinical and experimental data showing an increased generation of ROS in diabetes [5,56,57]. 4-hydroxy-2-nonenal (HNE) are formed from arachidonic acid or other unsaturated fatty acids following free radical attack and bind, by Michael addition, to proteins particularly at cysteine, hystidine, or lysine residues [14,37]. Thus, protein-bound HNE is a reliable index of lipid oxidative stress which can also occur in different organs under oxidative stress conditions [13,15,54,58]. Isoprostanes are derived by the free radical-catalyzed peroxidation of arachidonic acid, they are formed *in situ* from phospholipids and subsequently released in the circulation by a phospholipase and eventually excreted in urine [16,59]. Isoprostanes, in contrast to lipid hydroperoxides, are chemically stable end products of lipid peroxidation, and the measurement of their levels in plasma or urine may permit a sensitive and specific method for detection of lipid oxidative damage *in vivo* [19]. Their concentration in the urine is one of the most readily available index of lipid oxidation and, more in general, of the oxidative stress status of a patient population. The F2-isoprostane levels in type 2 diabetic patients are consitently increased [18,60,61]. In a previous study we demonstrated an increased level of F2 isoprostanes in type 2 diabetic patients with incipient diabetic nephropathy [38]. Our results show a significant increase in both plasma

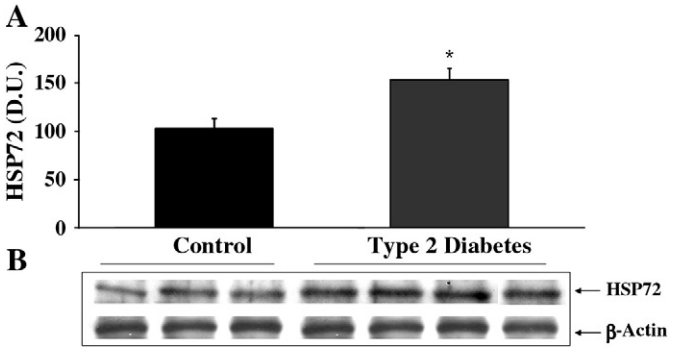

Fig. 6. Heat shock protein 70 levels in lymphocytes from type 2 diabetic patients. Lymphocyte samples from patients with nephropathy secondary to type 2 diabetes and age-matched controls were assayed for heat shock protein 70 (Hsp70) by western blot as described in Materials and Methods. A representative immunoblot is shown. β-actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean ± SEM of independent analyses on 15 patients per group. *P<0.05 *vs* control. D.U., densitometric units.

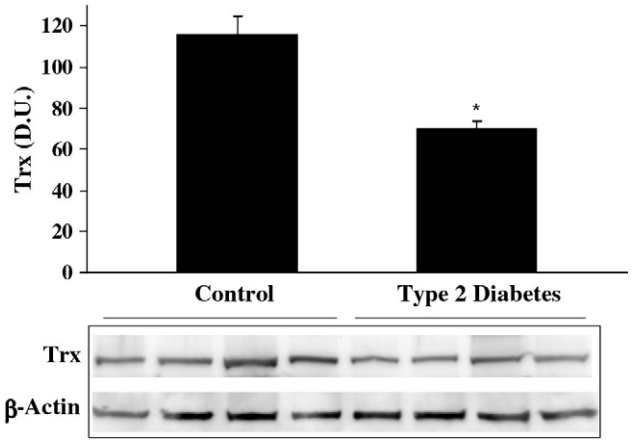

Fig. 7. Thioredoxin levels in lymphocytes from diabetic nephropathic patients. Lymphocyte samples from patients with nephropathy secondary to type 2 diabetes and age-matched controls were assayed for thioredoxin (Trx) by western blot as described in Methods. A representative immunoblot is shown. β-actin has been used as loading control. The bar graphs show the densitometric evaluation and values are expressed as mean ± SEM of independent analyses on 15 patients per group. *P < 0.05 *vs* control. D.U., densitometric units.

and urinary levels of F2-isoprostanes and provide further evidence about a condition of systemic rather than local pro-oxidant status. In recent years, it was also shown that isoprostane directly affects platelet aggregation and may therefore be implicated in macrovascular complication of diabetes [24].

Eucariotic cells have developed various pathways to counteract oxidative stress-related damage. Among these stress, induced proteins, chaperones are essential to help the correct folding and maintenance of the proper conformation of other proteins and to promote cell survival after a large variety of environmental stresses. Therefore, normal chaperone function plays a pivotal role in the endogenous response of several tissues to an increased cellular stress, whereas altered chaperone function has been associated with the development of several diseases [27,62]. To this regard, the expression of HO-1, Hsp70, as well as of TrxR-1 in peripheral lymphocytes of patients with type 2 diabetes is significantly increased. Consistent to this, we show that type 2 diabetes is associated with significant perturbation of systemic redox state, as revealed by a significant decrease in both reduced glutathione and thioredoxin protein. Hsp70 induction has been proved to be an efficient system helping the recovery from a

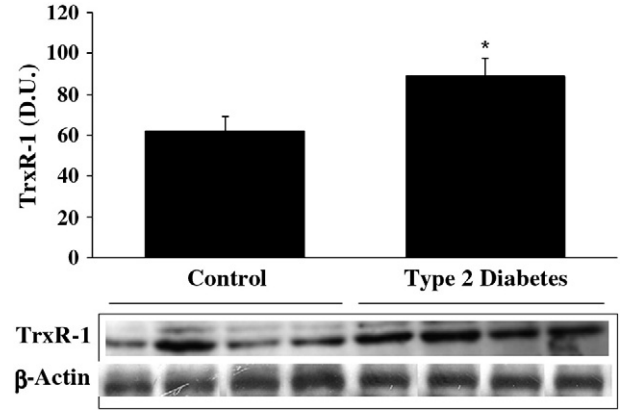

Fig. 8. Thioredoxin reductase-1 levels in lymphocytes from diabetic nephropathic patients. Lymphocyte samples from patients with nephropathy secondary to type 2 diabetes and age-matched controls were assayed for thioredoxin reductase-1 (TrxR-1) by western blot as described in Materials and Methods. A representative immunoblot is shown. β-actin has been used as loading control. The bar graphs show the densitometric evaluation and values are expressed as mean ± SEM of independent analyses on 15 patients per group. *P < 0.05 *vs* control. D.U., densitometric units.

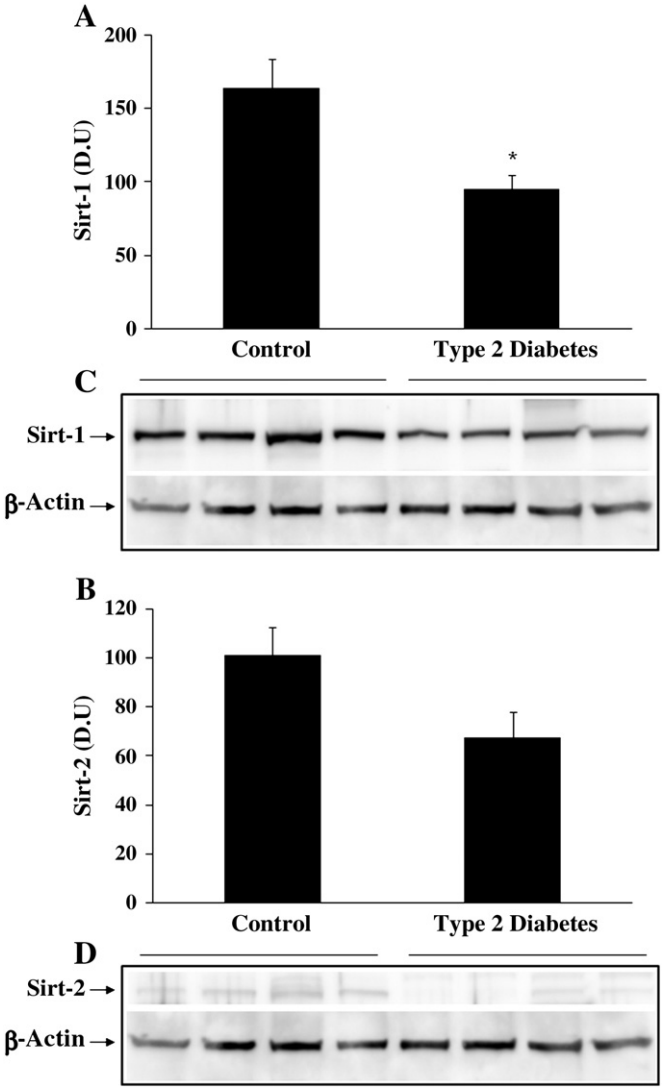

Fig. 9. Levels of sirtuin-1 (Fig. 9A) and sirtuin-2 (Fig. 9B) in lymphocytes from type 2 diabetic patients. Blood samples were assayed for sirtuins (Sirt-1, Sirt-2) by Western blot as described in Methods. Representative immunoblots are shown in C and respectively D. β-actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of independent analyses on 15 patients per group. $P \leq 0.05$ *vs* control. D.U., densitometric units.

large number of diseases, such as atherosclerotic and inflammatory disease, diabetes and neurodegenerative damage-associated pathologies [25–27,62,63]. The physiological response of HO-1 protein in human lymphocytes is also intriguing. Under oxidative stress conditions, HO-1 is one of the early genes to be induced and it exerts cytoprotective functions by inducing the metabolic pathway of prooxidant heme degradation and the production of both the vasoactive molecule carbon monoxide and biliverdin, the latter being the precursor of the powerful antioxidant and antinitrosative molecule bilirubin [27,62,64]. Thus, HO-1 increase in the lymphocytes of patients with type 2 diabetes suggests that, in response to an oxidant insult, induction of an early gene is a significant part of the antioxidant response and this is much more interesting considering the long term course of a disease such as diabetes. The thioredoxin system (thioredoxin, thioredoxin reductase, and NADPH) regulates cellular redox balance through the reversible oxidization of its redox-active cysteine residues (—Cys-Gly-Pro-Cys-) to form a disulfide bond that in turn is reduced by thioredoxin reductase and NADPH [30,31,65]. Thioredoxin plays an essential role in cell function by limiting oxidative stress directly via antioxidant effects and indirectly by protein–protein interactions

with key signaling molecules, such as the thioredoxin-interacting protein [66]. We have shown an increased TrxR protein expression in lymphocytes of diabetic patients which is in agreement with the well known effects induced by oxidative stress [67]. Our study provides evidence also that sirtuin signal is downregulated in type 2 diabetes respect to healthy control subjects. This is consistent with the recent notion that sirtuins reduce reactive oxygen species formation by modulating the acetylation of the respiratory chain and by stimulating mithocondria superoxide dismutase (SOD-2) and isocitrate dehydrogenase which generates NADPH for the glutathione pathway. Thus, changes in sirtuin expression, as observed in our study, may contribute to explain the failure in diabetic defense mechanisms against sustained oxidative stress [32]. Furthermore, given the interplay between reactive oxygen species and insulin signal transduction, it is of interest that while small physiologic amount of H2O2 mimic the action of insulin and stimulate glucose uptake, in contrast, a large body of clinical evidence shows that sustained oxidative stress correlates directly with both mean blood glucose levels, as well as glucose variability, in response to anti-diabetic therapy [68]. Consistently, it is known that glucose and lipid levels can exert a direct toxic effect on beta cell function and insulin secretion. A direct role of oxidative stress and AGE on insulin secretion has been demonstrated *in vivo*, however, few data are available *in vivo* on a potentially advantageous effects of anti oxidative therapies on beta cell function in diabetics. Data on an early positive effect anti oxidant administration on insulin secretion of patients with a family history of diabetes would suggest the need of an early intervention prior that irreversible loss of beta cell mass and function have occurred [68].

The above discussed data demonstrate the role of oxidative and glycoxidative pathways in diabetes and unravel the importance of systemic cellular stress response mechanisms, in particular vitagenes which, if stimulated, may counteract this pro-oxidant status. It is plausible to hypothesize that novel therapeutic approaches, based on combined nutritional and pharmacologic interventions, can be designed to burst cellular stress response as a mean to control and reduce oxidative stress-mediated formation of AGEs as well as to remove AGE-induced modifications, thus constituting an important component of future prophylaxis and therapy in patients with diabetes.

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