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Oxidized proteins in plasma of patients with heart failure: Role in endothelial damage

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Abstract

Background: Oxidative stress is increased in the failing heart, and this might contribute to the pathogenesis of myocardial remodelling and heart failure (HF).
Aim: To identify the oxidized proteins in plasma of chronic HF patients and to evaluate their possible role in endothelial damage.
Methods: Plasma levels of oxidized proteins were measured by immunoassay and by analysis in albumin and immunoglobulin depleted plasma using a proteomic approach, in 40 HF patients and in 20 age-matched normal subjects. Analysis of the effects of proteins oxidized *in vitro* on human endothelial cell (EC) viability was also performed.
Results: Plasma levels of oxidized proteins were significantly higher in HF patients than in controls ($p<0.01$). We identified two proteins, α-1-antitrypsin and fibrinogen, which underwent oxidation. Oxidation of α-1-antitrypsin resulted in loss of its protease inhibitor activity, thus leading to EC death in the presence of elastase. Fibrinogen, when oxidized, became otherwise cytotoxic and induced apoptosis in EC.
Conclusions: This study shows that plasma levels of oxidized proteins are increased in HF, and permitted the identification of two proteins, namely α-1-antitrypsin and fibrinogen, which underwent oxidation. *In vitro* results highlighted the potential role of oxidized proteins in the EC damage that occurs in HF.

Keywords: α-1-antitrypsin; Apoptosis; Endothelium; Fibrinogen; Heart failure

1. Introduction

Increasing experimental evidence supports the concept that oxidative stress is increased in the failing heart and contributes to the pathogenesis of myocardial remodelling which leads to heart failure (HF) [1]. Indeed, markers of oxidative stress are elevated in chronic HF patients and have been correlated with myocardial dysfunction and overall severity of HF [1]. Furthermore, it has been reported that plasma antioxidative enzyme activity is decreased in patients with HF [2]. During the last decade, considerable research effort has been directed at the identification of changes in oxidative stress and in antioxidative enzymes as one of the possible mechanisms underlying the development of HF [3]. Among these, an increase in lipid peroxidation markers and encroachment of antioxidant reserves in HF, have been reported in humans [4]. Indeed, products of free radical reactions, i.e. plasma lipid peroxides and expired breath pentane levels, have also been found to be elevated [5]. Proteins are major targets for reactive oxygen species (ROS) because of their abundance in biological systems and because they are primarily responsible for most functional processes within cells. It has been estimated that proteins can scavenge the majority (50–75%) of ROS [6]. Oxidative damage to proteins is induced either directly by ROS

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or indirectly by reaction of secondary by-products of oxidative stress and can occur via different mechanisms, leading to peptide backbone cleavage, cross-linking, and/or modification of the side chain of virtually every amino acid [6]. Most protein damage is irreparable, and oxidative changes of protein structure can have a wide range of downstream functional consequences, such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity [6]. It is now recognised that oxidation of proteins plays an essential role in the pathogenesis of an important number of degenerative diseases [7]. Compared to control samples, proteins are more oxidized in tissues from animals and patients suffering from Alzheimer's disease, rheumatoid arthritis, atherosclerosis or amyothrophic lateral sclerosis [6]. The most widely studied oxidative stress-induced modification of proteins is the formation of carbonyl groups on lysine, proline, arginine and threonine residues. The amount of carbonyls is considered a marker of oxidative stress and it is used to quantify the level of oxidative damage in polypeptide chains [6]. Moreover, the analysis of protein carbonyl groups may have some advantages in comparison with other methods: as their formation is considered a common product of protein oxidative reactions, and they are produced early, relatively stable and induced by almost all types of ROS [8]. This study is designed to: (a) analyze the presence of carbonylated proteins in plasma of HF patients; (b) identify by a proteomic approach the specific targets of carbonylation; (c) analyze the alteration of protein function in *in vitro* cultured endothelial cells.

2. Materials and methods

2.1. Patients and sample collection

The study population consisted of 40 patients with severe HF. All patients were in a stable clinical condition, none had elevated liver enzymes or suffered from liver disease, osteoporosis, multiple myeloma, osteolytic metastases, rheumatoid arthritis, or cancer. Patients were receiving standard medication including ACE-inhibitors, ß-blockers, digitalis, and diuretics. No patient was on systemic glucocorticoid treatment. We grouped our patients according to HF aetiology. Twenty patients had ischaemic heart disease [New York Heart Association (NYHA) class III, $n=13$; class IV, $n=7$)] and 20 patients had idiopathic dilated cardiomyopathy (normal coronary angiography, NYHA class III, $n=11$; class IV, $n=9$). All patients belong to a cohort of HF subjects regularly followed at our heart failure clinic.

The control group consisted of 20 healthy subjects comparable for gender, smoking habit, and age.

Informed consent was obtained from all subjects and the study protocol was approved by the Ethics Committee of Centro Cardiologico Monzino. The investigation conforms to the principles outlined in the Declaration of Helsinki (Br Med J 1964;II:177).

Heart size was measured by echocardiography. Venous blood collected from the antecubital vein was transferred into chilled sterile tubes containing heparin (68IU, Sigma)), centrifuged at 3000 rpm for 10 min at 4 °C, immediately frozen, and stored at −80 °C.

2.2. Quantification of protein carbonyl groups

Protein carbonyl measurements were performed by Zentech PC test ELISA (Biocell). Briefly, samples were reacted with 2,4-dinitrophenylhydrazine (DNPH) before probing with an antibody against protein-conjugated DNPH. Absorbances were read with a 450 nm filter. Standard curve of oxidized albumin and samples were assayed in triplicate and intra- and inter-assay coefficients of variation were below 2.1 and 3.2%, respectively.

2.3. Elastase activity assay

Human active elastase (HNE) was measured by Innozyme activity assay (Calbiochem). Briefly, activity of captured HNE is detected with the fluorogenic substrate MeOSuc-Ala-Ala-Pro-Val AMC. Released free AMC is measured fluorometrically at excitation 360 nm and emission 450 nm. Lower detection limit is 0.0625 ng/mL.

2.4. Sample preparation and 2-DE for the detection of protein carbonyls

In order to eliminate two of the most abundant plasma proteins, albumin and immunoglobulins, samples were pretreated with ProteoPrep Blue Albumin Depletion Kit (Sigma). 300 μg of plasma proteins, measured by Bradford method, were then diluted with a buffer yielding final concentrations of 7 mol/L urea, 2 mol/L thiourea, 0.2% SDS, 4% CHAPS, 2% v/v carrier ampholytes pH 3–10, 20 mmol/L Tris, 55 mmol/L dithiothreitol, and bromophenol blue. IPG ready strips, 11 cm, pH 3–10 non linear gradient or 4–7 linear gradient (Biorad), were actively rehydrated at 50 V for 24 h. Samples were loaded at the cathode and focused for a total of 20 kV h. Following isoelectric focusing, the oxidized proteins were derivatized by the "in-strip DNPH derivatization" method of Conrad et al. [9]. Briefly, the IPG strips were incubated in 2 N HCl with 10 mmol/L DNPH, washed with 2 mol/L Tris/30% glycerol and equilibrated first with a solution containing 50 mmol/L Tris–HCl, 6 mol/L urea, 30% v/v glycerol, 2% SDS and 2% dithiothreitol, and then with the same buffer containing 4.5% iodoacetamide instead of dithiothreitol. The second dimension was run on 7%–17% polyacrylamide gradient gels. Every sample was run in duplicate, one gel *per* sample was stained with colloidal blue, for total protein content analysis, and the other was electroblotted to polyvinylidene fluoride (PVDF) membrane for DNP immunostaining, for carbonylated protein evaluation. This procedure was performed in triplicate for each sample. Gels were stained with colloidal blue for total protein content analysis, and electroblotted to PVDF for DNP immunostaining.

Before immunodetection, the membranes were stained in 0.2% w/v Ponceau S in 3% w/v trichloroacetic acid and

Table 1
Clinical characteristic of patients and control subjects

Clinical variables	Control	Ischaemic HF	Idiopathic HF
	(n=20)	(n=20)	(n=20)
Age, years*	64.2±8.9	68.8±6.6	64.1±9.9
Men, n (%)	19 (95)	20 (100)	17 (85)
Smoking, n (%)	2 (10)	6 (30)	3 (15)
TC (mg/dL)*	190±77	179±65	209±45
LDL-C (mg/dL)*	136±36	113±55	127±45
HDL-C (mg/dL)*	48±7.7	43±11	48±12
TG (mg/dL)*	129±53	122±44	137±56
Hypertension, n (%)	–	5 (25)	10 (50)
Diabetes, n (%)	–	7 (35)	8 (40)
Obesity, n (%)	–	2 (10)	5 (26)
LVEDV, mL*	–	224.2±58.1	188.3±54.2
LVEF, %*	–	28.2±7.8	34.6±11.7
LVEDD, mm*	–	68.5±9.3	64.5±9.2
Diuretics	–	19	20
Anti-aldosterone drugs	–	17	15
Beta-blockers	–	13	15
ACE-inhibitors	–	16	12
AT1-blockers	–	4	6
Amiodarone	–	9	9
Anticoagulant	–	12	7
Ca^{++} channel blockers	–	1	1
Nitrates	–	4	1
Aspirin	–	5	3
+ inotropic drugs	–	3	4

*Mean±SD; LVEDV, left ventricular end-diastolic volume; LVEDD, left ventricular end-diastolic diameter; TC, total cholesterol; TG, triglycerides, AT = angiotensin receptor blockers, + = positive.

the spot position marked in order to facilitate computer-assisted matching on the colloidal blue stained gel. Immunodetection was performed as previously described [9], using biotinylated anti-DNP antibody (Molecular Probes). The immunoreactive spots were detected using avidin horseradish peroxidase-conjugated (Biorad) and a chemiluminescence detection system (Amersham). All images were scanned with a GS-800 densitometer (Biorad).

Immunoreactive proteins were identified by mass spectrometry after excision of the matching spot on the superimposed gel stained with colloidal blue.

2.5. Mass spectrometry analysis

Proteins selected for analysis were in-gel reduced, alkylated, and digested with trypsin as previously described [10]. Mass spectrometry analysis was performed by Voyager DE-STR MALDI-TOF spectrometer (Applied Biosystem). The instrument was operated in the positive ion reflectron mode at 20 kV accelerating voltage with time-lag focusing. Spectra obtained from each digested protein were internally calibrated using trypsin autolysis products and processed with Data Explorer v 4.0. Monoisotopic masses of tryptic peptides were searched against the protein databases, Swiss Prot and NCBInr, using the Protein Prospector MS-Fit software v 4.0.5 (http://prospector.ucsf.edu). Search parameters allowed oxidation of methionine, carbamidomethylation of cysteine, one missed cleavage, and 50 ppm mass accuracy. Where necessary, additional searches were performed using the programs Mascot (http://www.matrixscience.com/) or ProFound (http://prowl.rockefeller.edu).

2.6. Cell cultures experiments

Human umbilical vein endothelial cells (HUVEC) were obtained as previously described [11]. Lactate dehydrogenase (LDH), an index of cell cytotoxicity, was measured by an enzymatic assay (Roche). Oxidation of α-1-antitrypsin was achieved by incubating the purified protein (1 mg/mL) for 16 h in the presence of 1 mmol/L H_2O_2. Oxidized fibrinogen was prepared by incubation with H_2O_2 and $FeSO_4$ (1 mmol/L each). Following these procedures, both α-1-antitrypsin and fibrinogen showed a two-fold increase in carbonyls group content, evaluated by immunoassay and Western blotting using biotinylated anti-DNP antibody, in comparison with native proteins. Native and oxidized proteins (0.25 mg/mL) were then added to the cell culture medium.

2.7. TUNEL assay

DNA fragmentation of apoptotic cells was assessed using the terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) assay (Roche). TUNEL test was performed on HUVEC cultured on glass coverslips and fixed for 10 min in 4% paraformaldehyde. Incorporated fluorescein was detected by antifluorescein antibody Fab fragments, conjugated with horseradish peroxidase, according to the manufacturer's instructions. After substrate reaction, stained cells were analyzed under a light microscope.

2.8. Statistical analysis

Numerical data are given as mean±SD or as median and interquartile range, as specified. Clinical characteristics were compared among the three groups by analysis of variance (ANOVA). Non parametric test (Wilcoxon Mann–Whitney) was also performed to compare controls and patients. Multivariable analysis, adjusting for HF risk factors (diabetes,

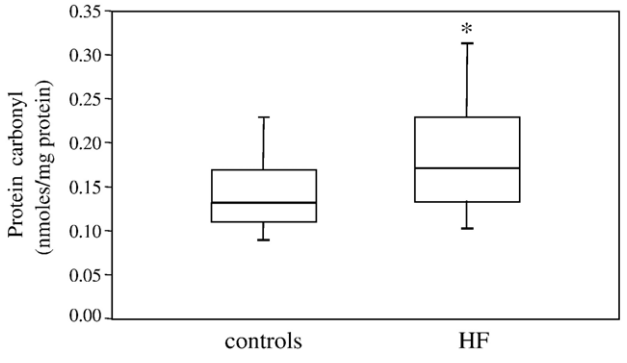

Fig. 1. Protein carbonyl measurement by ELISA in plasma of HF patients (n=40) and control subjects (n=20). Boxes represent 25th and 75th percentiles; line within boxes, median. *p<0.01 vs. control subjects.

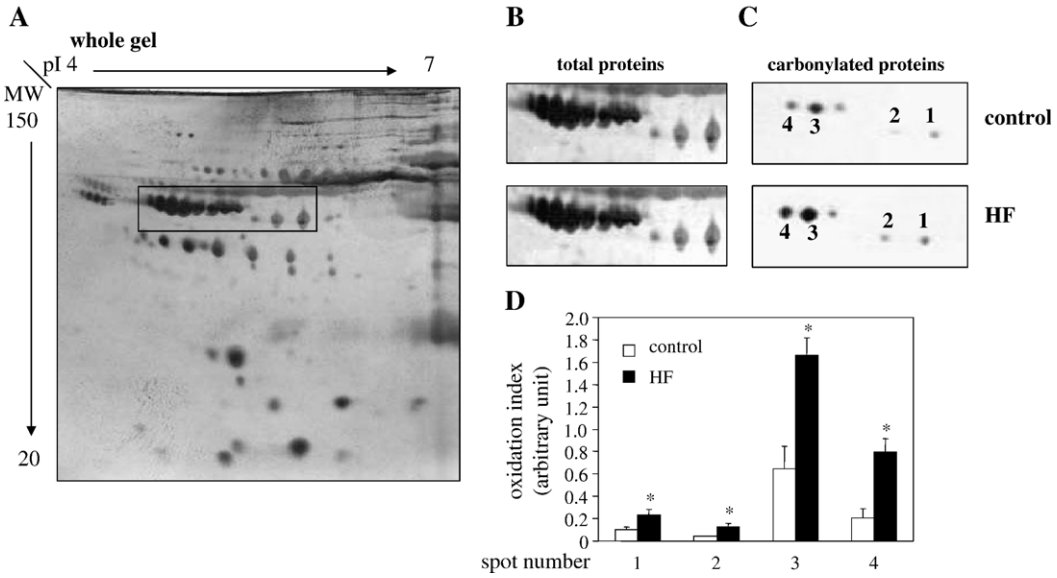

Fig. 2. Representative analysis of carbonylated plasma proteins by 2-DE. Panel A: Whole 2-DE gel stained with Colloidal blue to visualized all proteins. Panel B: Expanded views of the "area of magnification" of total proteins isolated from control subjects (upper panel) and HF patients (lower panel) stained with Colloidal Blue. Panel C: Expanded views of the immunostained "area of magnification" of DNP-derivatized proteins isolated from control subjects (upper panel) and HF patients (lower panel). Panel D: Histograms of oxidation index, ratio between spot immunointensity divided by intensity of protein stain with Colloidal Blue (mean ± SD, *$p<0.05$ vs controls). Images are representative of experiments performed on 10 control and 10 HF samples selected as described in the Results section.

hypertension and obesity) was performed using covariance analysis (ANCOVA). To evaluate gel reproducibility and to improve the reliability of the qualitative and quantitative changes in protein expression by electrophoresis, we analyzed each sample in triplicate. PDQuest software (v 7.3.1, Biorad) was used for spot detection, spot quantification, gel matching, and normalization for total spot volume in the gel. The cut-off level for a differentially expressed protein was defined as at least a two-fold increase or decrease in spot intensity. Statistically significant differences between groups for each protein were computed by Student's *t*-test. The level of significance was set at $p<0.05$. All statistical analyses were performed using SAS version 8 (SAS Institute, Cary, NC, USA).

3. Results

The clinical characteristics of patients and control subjects are summarized in Table 1. Gender, age, and smoking habit were well balanced in the three groups. There were no differences in plasma levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol or triglycerides (TG) between patients and control subjects. The levels of carbonylated proteins were significantly higher in plasma of both groups of patients than in controls ($p<0.01$ using Wilcoxon Mann–Whitney analysis, Fig. 1). However, carbonyl levels did not differ significantly ($p=0.34$) between ischaemic and idiopathic HF patients (0.21 ± 0.1, 0.17 ± 0.06, 0.14 ± 0.04, mean ± SD for ischaemic, idiopathic, and controls, respectively). HF risk factors did not significantly influence, *per se*, our results; indeed, after adjusting for diabetes, hypertension and obesity by ANCOVA, plasma levels of carbonylated proteins remained significantly higher in HF patients than in control subjects ($F=4.79$, $p<0.05$). For the identification of carbonylated proteins, this study exploited the high resolving power of 2-DE, combined with the high specificity of immunoblotting. The immunoblotting analysis with anti-DNP antibody was performed on 10 samples from HF patients and 10 from healthy controls. The samples were selected according to the ELISA results, choosing patients with a protein carbonyl content close to the average value [12]. Different isoforms of two proteins were carbonylated to a greater extent in the HF plasma samples than in the control samples after DNP immunostaining (Fig. 2, and Table 2). Using an annotated 2-DE image for human plasma from the Expasy (Expert Protein

Table 2
Identification of protein showing differences in protein carbonylation

Protein name	Accession code[a]	MW kDa/pI	Sequence coverage %/no. peptides	MOWSE score[b]	Spot no.
Fibrinogen γ chain precursor	FIBG_HUMAN	50/5.6	50/19	6.4 e+8	1
Fibrinogen γ chain precursor	FIBG_HUMAN	50/5.5	53/20	2.5 e+9	2
α-1-antitrypsin	A1AT_HUMAN	55/5.15	41/12	1.78 e+8	3
α-1-antitrypsin	A1AT_HUMAN	55/5.10	35/8	5.6 e+7	4

[a] Accession code in Swiss-Prot.
[b] MOWSE Score from MS-Fit.

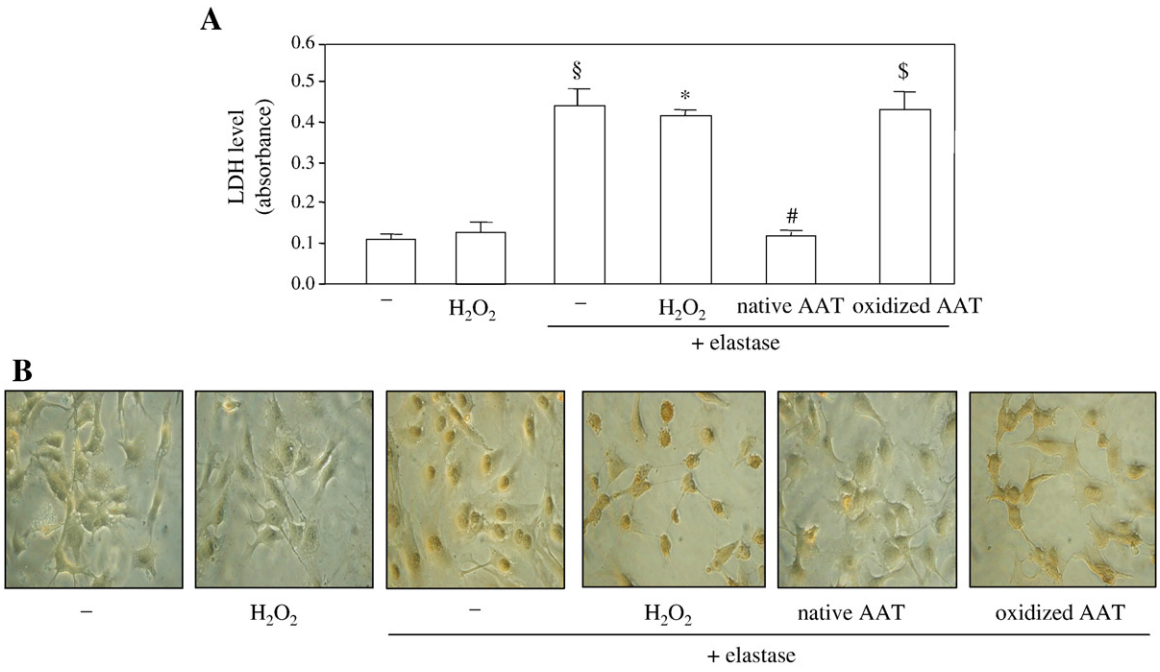

Fig. 3. Effect of oxidized α-1-antitrypsin on endothelial cell viability. Panel A: LDH released by HUVEC incubated for 2 h with elastase (0.05 μg/mL) alone, in the presence of H_2O_2, of native α-1-antitrypsin (0.25 mg/mL) or of oxidized α-1-antitrypsin (0.25 mg/mL). Control cells were incubated with the same concentration of H_2O_2 used in oxidized α-1-antitrypsin-treated cells. −, untreated cells. Values are the means ± SD of 4 individual experiments performed in duplicate. §$p < 0.01$ vs untreated cells; *$p < 0.01$ vs H_2O_2-treated cells; #$p < 0.01$ vs. elastase treated cells; $$p < 0.01$ vs. native α-1-antitrypsin plus elastase treated cells. Panel B: TUNEL assay of HUVEC incubated as described in panel A. The experiment is representative of 3 independent experiments. TUNEL positive cells are stained (brown) by the TUNEL treatment. AAT: α-1-antitrypsin.

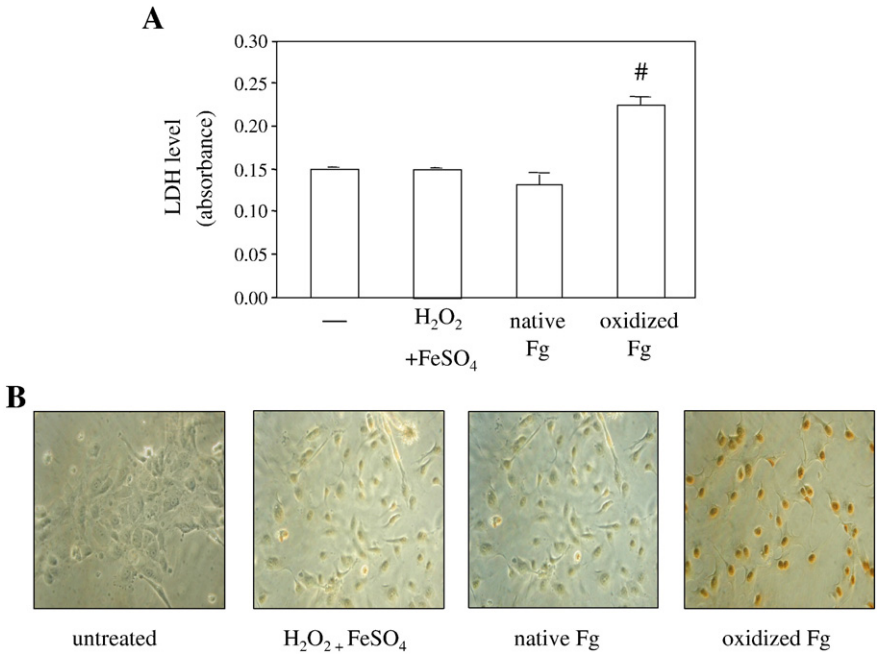

Fig. 4. Effect of oxidized fibrinogen on endothelial cell viability. Panel A: LDH released by HUVEC incubated with native or oxidized fibrinogen (both 0.25 mg/mL) for 7 h. Control cells were incubated with the same concentration of H_2O_2 and $FeSO_4$ used in oxidized fibrinogen-treated cells. −, untreated cells. Values are the means ± SD of 4 individual experiments performed in duplicate. #$p < 0.01$ vs. native fibrinogen-treated cells; Panel B: TUNEL assay of HUVEC incubated as described in panel A. The experiment is representative of 3 independent experiments. TUNEL positive cells are stained (brown) by the TUNEL treatment. Fg: fibrinogen.

Analysis System) proteomics server of the Swiss Institute of Bioinformatics, carbonylated proteins were identified by their pI and molecular weight on the gels. Moreover, these carbonylated protein spots were excised from the 2-DE gel, digested with trypsin, and analyzed by MALDI-TOF. Spots were positively identified as fibrinogen γ chain and α-1-antitrypsin (Table 2).

In order to evaluate the altered functionality of carbonylated α-1-antitrypsin and fibrinogen, native proteins were oxidized *in vitro* with a resulting two-fold increase in carbonyl group content, an extent similar to that observed in HF plasma. Native and oxidized α-1-antitrypsin were then added to HUVEC in the presence or absence of neutrophil elastase. Control cells, incubated with the same concentration of H_2O_2 used in oxidized α-1-antitrypsin-treated cells, did not show cytotoxicity. In our experimental conditions, elastase strongly induced the appearance of an apoptotic phenotype to the same extent either alone or in the presence of H_2O_2. Native α-1-antitrypsin completely prevented elastase-induced apoptosis. Oxidized α-1-antitrypsin did not affect cell viability, *per se*, but drastically impaired its ability to counteract elastase activity, as demonstrated by increased levels of LDH (Fig. 3, panel A) and by the appearance of endothelial cell apoptosis (Fig. 3, panel B). Moreover, levels of elastase activity were higher in HF patients in comparison with healthy subjects (0.162±0.02 ng/mL and 0.200±0.04, for control and HF patients, respectively, $p<0.05$). Furthermore, cells were treated with native and oxidized fibrinogen. Results show that fibrinogen, only when oxidized, became cytotoxic and induced apoptosis in endothelial cells (Fig. 4). Cells, incubated with the same concentration of H_2O_2 and $FeSO_4$ used in oxidized fibrinogen-treated cells, did not show cell toxicity (Fig. 4).

4. Discussion

This study shows that oxidative stress-induced modification of proteins is increased in the plasma of HF patients, using the proteomic approach allowed the identification of two proteins which undergo oxidation, α-1-antitrypsin and fibrinogen. Oxidation of α-1-antitrypsin resulted in loss of its protease inhibitor activity, thus leading to endothelial cell death in the presence of elastase. *Vice versa* oxidation of fibrinogen generated cytotoxic effects and induced apoptosis in endothelial cells. Therefore, the physiological cell experiments performed with the identified carbonylated proteins support the hypothesis that oxidized biomolecules are not only surrogate markers for oxidative stress, but might also have a potential pathological role.

Heart failure patients were grouped according to aetiology. Indeed, the presence of ischaemic heart disease could *per se* influence the protein carbonyl content. In our study, we found no difference between HF groups thus suggesting that the oxidative process, which is enhanced in HF, might occur independently from HF aetiology. However, since we studied patients with severe chronic HF, it is still possible that protein carbonyl content could be different in the early stages of HF, either in terms of the total value and/or in relation to the aetiology.

The amount of protein carbonyls is considered a marker of severe oxidative stress and is used to quantify the level of oxidative damage in polypeptide chains [13]. This approach may have some advantages in comparison with the analysis of lipid peroxidation products because the formation of protein-bound carbonyl groups seems to be a common phenomenon of protein oxidation and because of the relatively early formation and relative stability of oxidized proteins. It is known that cells degrade oxidized proteins within hours if not days, whereas lipid peroxidation products are detoxified within minutes [14]. Interestingly, protein carbonyl groups form early, and circulate for longer periods in the blood of patients compared with other parameters of oxidative stress [15]. Finally, the chemical stability of protein carbonyls makes them suitable targets for laboratory measurement [16]. Many different physiological and environmental processes can promote the generation of ROS, including a number of free radicals, various non radical oxygen derivatives, and highly reactive lipid- or carbohydrate-derived carbonyl compounds. In addition, carbonyl groups are produced only in the presence of severe oxidative stress. Detection of elevated levels of protein carbonyls is a sign of both oxidative stress and protein dysfunction [6]. Carbonyls, however, are not an index of all oxidative modifications of proteins, such as the conversion of tyrosine residues to 3-chlorotyrosine, 3-nitrotyrosine, dityrosine or methionine oxidation [6]. We cannot therefore exclude that other types of modifications might occur in heart failure patients.

Level of oxidized protein reflects the balance between the rate of protein oxidation and the rate of oxidized protein degradation. This balance is a complex function of numerous factors that lead to the generation of both ROS, and of multiple factors that determine the concentrations and/or activities of the proteases that degrade oxidatively damaged protein. Furthermore, oxidized forms of some proteins are not only resistant to proteolysis but, can inhibit the ability of proteases to degrade the oxidized forms of other proteins [17]. The vicious circle of decreased proteolysis and accumulation of increasing amounts of oxidatively damaged proteins continues until the protein aggregates cause metabolic dysfunction or the initiation of apoptotic or necrotic events.

Moreover, oxidative damage often leads to loss of specific protein function [7]. The relationship between protein oxidation, protein dysfunction, and diseases remain largely unclear; however, it is known that oxidative modifications of enzymes and structural proteins plays a significant role in the pathophysiology of diseases such as Alzheimer's disease [18] and atherosclerosis [19].

One of the main findings of the present study is that α-1-antitrypsin and fibrinogen undergo oxidation in HF patients. α-1-antitrypsin, one of the major serine proteinase inhibitors in human plasma, inhibits overexpressed proteinases during inflammation [20]. Proteinase activity is tightly regulated by these inhibitors under normal physiological conditions, but this equilibrium may be impaired in some pathological conditions,

where oxidative inactivation of the inhibitor may occur [21]. Indeed, oxidation of α-1-antitrypsin results in loss of its anti-neutrophil elastase activity and uncontrolled degradation of connective tissues in rheumatoid arthritis and pulmonary emphysema [15]. Also, its oxidized form activates primary human monocytes, thus contributing to the inflammatory process [22]. We show here that oxidized α-1-antitrypsin did not affect cell viability, *per se*, but drastically impairs its ability to counteract elastase activity, thus leading to endothelial cell death in the presence of elastase. Oxidized fibrinogen, on the other hand, induced cell death.

Fibrinogen is essential for fibrin formation under the influence of thrombin and forms the basic plug for plasma coagulation and platelet aggregation via glycoprotein IIb/IIIa receptors [23]. Furthermore, fibrinogen increases the binding of platelets, endothelial cells, and leukocytes to each other, which, in turn, causes leukocyte and platelet activation and release of intracellular mediators from these cells [24]. Oxidative modification of fibrinogen chains may alter its normal function which may result in increased activation of plasminogen thereby contributing to fibrinolysis and proteolysis in areas of inflammation [25]. Furthermore, fibrinogen oxidation increases the release of proinflammatory IL-8 from endothelial cells [26].

The different effects of oxidized fibrinogen and α-1-antitrypsin on cell viability might be ascribed to their main physiological role. It is conceivable that the effects of fibrinogen are mainly directed at the cell, whereas α-1-antitrypsin acts primarily inactivating circulating elastase. However, we cannot completely exclude that oxidized α-1-antitrypsin, in other experimental conditions, might have other cell-mediated functions.

In the present study, we show that oxidized fibrinogen and α-1-antitrypsin may contribute to endothelial dysfunction, which it is known to occur in HF [27]. Indeed, serum of HF patients induces endothelial cell apoptosis *in vitro* [28]. The precise serum factor(s) responsible for the induction of apoptosis in endothelial cells, however, remains to be determined. It has previously been shown that several factors, whose levels are increased in HF [3,29,30], such as tumour necrosis factor-α (TNFα), ROS, and angiotensin II, all represent potent activators of the caspase cascade, leading to endothelial apoptosis. Our data highlights the role of oxidized proteins, whose biological function is altered, in endothelial damage.

One important study limitation is that we cannot define whether the *in vitro* concentrations of oxidized fibrinogen and α-1-antitrypsin are relevant to the *in vivo* conditions. Indeed we found the first evidence of an effect on cell viability starting at a concentration of oxidized fibrinogen and α-1-antitrypsin of 0.25 mg/mL which is less than 10% of the *in vivo* concentrations. Because the percentage of *in vivo* oxidized fibrinogen and α-1-antitrypsin is unknown, our results are likely in the physiological range but definite proof of this statement is needed.

Another limit of the study is that in order to go deeper into the plasma proteome and to study carbonylation of less abundant proteins, it is mandatory to remove the most abundant plasma proteins, e.g. albumin and immunoglobulins, although these proteins might represent a target of ROS-mediated carbonylation.

Nevertheless, data herein reported indicate that oxidized proteins are increased in the plasma of HF patients, and, that oxidation of α-1-antitrypsin and fibrinogen leads to protein dysfunction; the first losing its protective inhibitory activity against elastase, and the second becoming cytotoxic, two events which, in turn, may contribute to endothelial damage associated with heart failure.

On the other hand, we could hypothesize that abundant proteins (such as fibrinogen and α-1-antitrypsin) might act as an important physiological antioxidant, protecting other molecules from free radical mediated oxidation. However, if fibrinogen acts as a sacrificial antioxidant, it should lead to disturbances of its clotting activity. Indeed, in *in vitro* studies oxidized fibrinogen loses its coagulation abilities. Moreover, inefficient removal and degradation of oxidatively modified fibrinogen forming intravascular fibrin-like deposits may lead to endothelial damage and initiation of atherogenesis [31].

In conclusion, proteomic studies allow a much broader investigation of the oxidative stress response than conventional biochemical methods. In conjunction with the development of dedicated biochemical tools (e.g. antibodies against tyrosine hydroxylation, methionine or tryptophan oxidation), and development of mass spectrometry based quantitative techniques, it will be possible to study both expression variations and oxidative modifications of unpredictable proteins using the wide-screen proteomics toolbox.

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