Low Serum Ferroxidase I Activity is Associated with Mortality in Heart Failure and Related to Both Peroxynitrite-Induced Cysteine Oxidation and Tyrosine Nitration of Ceruloplasmin

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ABSTRACT

Rationale: Ceruloplasmin (Cp) antioxidant function is mainly related to its Ferroxidase I (FeOxI) activity, which influences iron-dependent oxidative and nitrosative radical species generation. Peroxynitrite (ONOO\textsuperscript{-}), whose production is increased in heart failure (HF), can affect Cp antioxidant function through aminoacid modification.

Objective: We investigated the relationship between FeOxI and Cp tyrosine and cysteine modification and explored in a cohort of HF patients the potential clinical relevance of serum FeOxI.

Methods and Results: In chronic HF patients (n=96, 76±9 years, NYHA class 2.9±0.8), and age-matched controls (n=35,CTR) serum FeOxI, FeOxII, Cp, nitrotyrosine-bound Cp, BNP, norepinephrine, and hsCRP were measured and the patients were followed-up for 24 months. Cp, BNP, norepinephrine and hsCRP were increased in HF vs CTR. FeOxI was decreased in HF (-20%) and inversely related to nitrotyrosine-bound Cp (r= - 0.305, P=0.003). In HF, FeOxI lower tertile had a mortality rate doubled compared to middle-higher tertiles. FeOxI emerged as a mortality predictor (HR 2.95, CI 1.29-6.75, P=0.011) after adjustment for age, sex, hypertension, smoking, sodium level, eGFR and hsCRP. In experimental settings, ONOO\textsuperscript{-} incubation of serum samples and isolated purified Cp reduced FeOxI activity while increasing Cp tyrosine nitration and cysteine thiol oxidation. Reduced glutathione prevented ONOO\textsuperscript{-}induced FeOxI drop, tyrosine nitration and cysteine oxidation; flavonoid(-)-epicatechin, which prevented Cp tyrosine nitration but not cysteine oxidation, partially impeded ONOO\textsuperscript{-}induced FeOxI drop.

Conclusions: Reduced activity of serum FeOxI is associated with Cp nitration and reduced survival in HF patients. Both Cp tyrosine nitration and cysteine thiol oxidation may be operant in vivo in ONOO\textsuperscript{-}induced FeOxI activity inhibition.

Keywords: Heart failure, Ferroxidase I activity, ceruloplasmin, nitrosative stress, oxidative stress, mortality, peroxynitrite

Nonstandard Abbreviations and Acronyms:
Cp ceruloplasmin
EF ejection fraction
eGFR estimated glomerular filtration rate
EPI (-)-epicatechin
FeOx ferroxidase
GSH reduced glutathione
HF heart failure
IAF iodoacetamidofluorescein
MAD malondialdehyde
ONOO\textsuperscript{-} peroxynitrite
RDW red cell distribution width
TNF-\textalpha tumor necrosis factor-\textalpha
INTRODUCTION

The progression of heart failure (HF) is associated with aberrant oxygen- and nitric oxide-derived free radical production in either quantity or spatial cellular localization (1,2). Ferrous iron(II) is a catalyst in the formation of oxygen- and nitric oxide-derived free radicals and its oxidation to ferric iron(III) generates through Fenton’s reaction the propagation of oxidative and nitrosative stress (3). The ability to oxidize ferrous iron(II) ions to less toxic ferric (III) ions and to inhibit iron-dependent free radical and oxidants generation (4) is indicated as ferroxidase (FeOx) activity (5).

Ceruloplasmin (Cp), an alpha2-glycoprotein mainly synthesized by hepatocytes, whose functions include the transport of serum copper (10), the acute phase inflammation reactant, is also involved in iron metabolism through its FeOx activity (5). In fact, Cp represents the main contributor of FeOx activity in human plasma and is called FeOxI, which differentiates from FeOxII because of its almost complete inhibition by azide (4,6).

It has been recently reported that high Cp circulating levels have an independent prognostic value in stable patients undergoing elective diagnostic cardiac catheterization procedure (7) and are associated with the incidence of HF and mortality in the Atherosclerosis Risk in Communities study population (8). In another prospective study it was reported a relationship between serum Cp levels and the subsequent development of myocardial infarction in the elderly general population (9).

In settings of experimental and human HF, increased peroxynitrite (ONOO\textsuperscript{–}) generation, from nitric oxide and superoxide or from hemoperoxidases (10), leads to extensive tyrosine protein nitration (3,11,12). Tyrosine nitration may affect protein structure with a loss, a gain, or no effect on function (13). Cysteine can also be modified by ONOO\textsuperscript{–} and in particular sulfhydryl oxidation can lead to misfolding of proteins and remarkably influence their function (14,15).

Cp has been identified among nitrated proteins in patients with acute respiratory distress syndrome and in hemodialysis patients (13,16). It is reported that Cp tyrosine nitration affects protein function, resulting in a reduction of FeOx activity (13). In the HF population no data are available on the relationship between Cp, its tyrosine nitration, and on FeOxI activity and even less on the clinical relevance of Cp-mediated FeOxI activity. In addition, no information in clinical or experimental settings are available on an involvement of other amino acid changes such as cysteine thiol oxidation in ONOO\textsuperscript{–} - induced FeOxI activity drop of Cp. Based on these premises, we undertook a prospective study on a cohort of stable chronic elderly HF patients to investigate the prognostic value of Cp-mediated FeOxI activity and to evaluate the relationship of Cp-mediated FeOxI activity and its tyrosine nitration. We also explored in experimental settings the role of tyrosine nitration and cysteine thiol oxidation in ONOO\textsuperscript{–} - mediated Cp FeOxI activity inhibition.

METHODS

Study cohort and follow-up of patients.
Ninety six consecutive stable chronic HF patients referred to the heart failure outpatient Clinic of the Cardiorenal Research Unit of the Department of Clinical and Experimental Medicine of the University Hospital of Parma were prospectively studied for 24 months. The diagnosis of HF was based on symptoms and clinical signs according to guidelines issued by the European Society of Cardiology (17) and by the American College of Cardiology (18). The patients had no clinical nor laboratory signs of acute infection, rheumatoid or other autoimmune diseases, primary cachectic states (cancer, thyroid disease, severe liver disease, severe chronic lung disease) neuromuscular disorders, myocardial infarction...
within the previous 20 weeks, diabetes mellitus, or severe chronic renal failure (serum creatinine level > 2.0 mg/dl, >177 μmol/L). All patients were clinically stable and on constant therapy at least 8 weeks prior to entering the study. The study was approved by the University of Parma Ethics Committee, complied with the Declaration of Helsinki, and all participants provided written informed consent.

Thirty five age-matched healthy subjects were recruited as controls (CTR) from healthy subjects reporting for a periodical check-up at the cardiovascular prevention Clinic of the same Department. On study entry, a complete medical history, a physical examination, basal laboratory tests (total blood count including red cell distribution width (RDW), serum creatinine, electrolytes, lipid profile), plasma neurohormonal and inflammatory markers determination, an electrocardiogram and an echocardiogram were obtained from all patients. Estimated glomerular filtration rate (eGFR) was calculated from the four-component Model of Disease in Renal Disease (MDRD) equation incorporating age, race, sex and serum creatinine level: estimated eGFR=186 * (serum creatinine [in milligrams per deciliter])−1.154 * (age [in years])−0.203. For women the product of the equation was multiplied by a correction factor of 0.742 (19). All patients were seen at least 4 times per year in our HF outpatient clinic or as per their specific clinical need. The primary endpoint was all-cause mortality: this information was obtained by their general physician and by relatives. Morbidity data expressed as time to first hospital admission, number of hospitalizations for heart failure and cumulative length of hospital stay during the follow-up period were also recorded.

Venous blood sampling procedure and biochemical assays.

Venous samples were obtained after at least 30 minutes of supine rest from an indwelling catheter and collected in polypropylene tubes containing an EDTA (ethylene diamine tetraacetic acid) buffer (1.5 mg/ml), except for BNP where a mix of protease inhibitors (phenylmethylsulfonyl fluoride, trypsin inhibitor, and aprotinin 500 units/ml) was added and for serum copper where EDTA was excluded. Fresh serum samples were used to measure FeOxI and FeOxII activity, whereas multiple aliquots of plasma samples were stored at -80°C until assay time for norepinephrine, BNP, free malondialdehyde (MAD), total nitrated proteins, Cp, nitrotyrosine-bound Cp and total copper. All laboratory measurements were performed without any freeze-thaw cycles of the samples and by investigators blind to the clinical data. FeOxI and FeOxII activity was measured by ferrous ion as substrate (Fe(II); ferrous ammonium) according to the method of Erel (20). Briefly, serum was incubated with Fe(II) in 0.45 mol/l acetate buffer (pH 5.8), and the remaining nonoxidized ferrous ions formed a colored complex with the 3-(2-pyridyl)-5,6-bis(2-[5-furylsulfonic acid])-1,2,4-triazine chromogen. The difference in the ferrous ion concentration before and after the reaction corresponded to the amount of Fe(II) oxidized to Fe(III) and directly expressed the total FeOx activity of the serum. For FeOxII, the same procedure was applied using serum previously mixed with sodium azide, which instantly and completely inhibited FeOxI. FeOxI activity was calculated from the difference between total and FeOxII activities. Serum concentrations of copper were analyzed by use of atomic absorption spectrophotometry. Intra and inter-assay values for coefficient of variation were 4.1 and 4.6 %, respectively.

Norepinephrine, BNP, free MAD and high sensitivity C-reactive protein (hsCRP) were determined as previously described (21,22). Serum Cp was evaluated using an immunonephelometry kit (N antiserum against human ceruloplasmin, Dade Behring, Marburg, Germany) according to the manufacturer’s instruction. Total nitrated proteins levels were assessed using a sandwich ELISA assay kit (Oxis Research International Inc. Foster City, CA USA), and nitrotyrosine-bound Cp was measured by the same ELISA Kit after Cp immunoprecipitation. The intra-assay and inter-assay coefficients of variation were 4% and 14% and the analytical sensitivity was 2 nmol/L.

Peroxynitrite synthesis and ex vivo experiments on Cp-related ferroxidase activity.

In order to evaluate the effects of ONOO⁻ (0.15 mmol/L) and decomposed ONOO⁻ addition on Cp-related FeOxI activity and its relationship with Cp nitration, separate ex vivo experiments were performed in a subset of CTR subjects (n=18). Serum samples (350 μl of fresh serum) from these subjects had FeOxI and
II activities measured before and after addition of ONOO' and decomposed ONOO' prepared as previously described (23). The samples were incubated for 15 min at 37°C with ONOO' or with decomposed ONOO' (0.15 mmol/L) in phosphate buffer (K2HPO4 0.1 mol/L, 650 μL pH 7.40): these analyses were done in triplicate. The concentration of ONOO' used in the present study is of pathophysiological relevance (3).

Separate in vitro experiments (n=10), using lyophilized Cp (Sigma-Aldrich Chemical Co. St Louis, Missouri, USA) dissolved at 50 μg/4.5 units in K2HPO4 0.1 mol/L, pH 7.40 have been performed and FeOxI and II activities were measured before and after addition of ONOO' (0.15 mmol/L in phosphate buffer) and decomposed ONOO' for 15 min at 37°C. FeOx activities were also measured in presence of reduced glutathione (GSH, 850 μM, Acros Organic, Thermo-Fisher Scientific, USA) or (-)-epicatechin, (EPI, 50 μM, Sigma-Aldrich Chemical Co. St Louis, Missouri, USA), a flavanol known for its ability to protect against nitration but not from oxidation induced by ONOO' (24). GSH and EPI at these concentrations, in previous in vitro experiments, induced similar reductions in nitration of free tyrosine after ONOO' exposure. Standard solutions of (-)-epicatechin were prepared in dimethyl sulfoxide (DMSO) and stored at –20°C: after dilution, the final concentration of DMSO was 0.01%.

Cp Immunoprecipitation and immunoblotting for nitrotyrosine and cystein thiol oxidation.
For immunoprecipitation we used a polyclonal anti-human anti-Cp antibody (ABIN680108 antibodies-online GmbH, Aachen, Germany) that was cross-linked to Dynabeads protein A (Dynal Biotech, Oslo, Norway). Immunoprecipitated Cp was then tested for nitrotyrosine with immunoblotting and enzyme-linked immunosorbent assays (ELISA assay Kit, Oxis Research International Inc. Foster City, CA USA) as previously described (25). Nitrotyrosine-bound Cp was evaluated in serum from HF patients and CTR but also in ex vivo experiments on CTR serum (n=18) after incubation of ONOO' or decomposed ONOO' and in in vitro experiments (n=10) after ONOO' or decomposed ONOO' incubation of isolated purified Cp in a phosphate buffer bath. In addition to nitrotyrosine, the evaluation of cysteine thiols oxidation after ONOO' or decomposed ONOO' incubation was performed on isolated purified Cp in a phosphate buffer bath. To detect cysteine thiol status we labeled untreated and ONOO' or decomposed ONOO'-treated Cp free thiol groups with iodoacetamidofluorescein (IAF). IAF-labeled thiol groups were detected using 1 mg/ml mouse anti-fluorescein-Oregon Green (Molecular Probes) as previously described (26). For detailed experimental methods on Cp immunoprecipitation and immunoblotting on Cp nitrotyrosine and cystein thiol oxidation detection, please see the Online Data Supplement.

Data analysis.
Values are presented as mean ± SD. Comparisons of the baseline characteristic variables among the tertiles of FeOxI activity were made with 1-way analysis of variance model followed by the Bonferroni post hoc test. Univariate and multivariate analysis using Cox regression techniques were performed to examine the relationship between variables and mortality after 2-years follow-up period. Variables accepted as being of prognostic value in HF were included in the Cox model (age, gender, hypertension, smoking status, plasma sodium level, eGFR, hs-CRP, BNP). Four models of analysis including different covariates are reported in Table 3. Statistical comparisons were performed by Kaplan–Meier survival curves to analyse the cumulative event rate linked to tertiles of FeOxI activity (tertile 1 FeOxI < 336 UI/L; tertile 2 336<FeOxI>432 UI/L; tertile 3 FeOxI >432 UI/L). Data on time to first hospitalization, number of hospitalizations for heart failure and cumulative length of hospital stay are given as median and interquartile range (IQR). All statistical analyses were performed using SPSS for Windows 18.0 (SPSS Inc). Relations between parameters including FeOxI, Cp, nitrotyrosine-bound Cp, total nitrated protein, hsCRP, TNF-α, BNP, MAD, RDW were analyzed by linear regression analysis using Pearson correlation coefficients. The D'Agostino-Pearson normality test was passed for all parameters, except hsCRP that was log transformed to create a normal distribution. P ≤ .05 was considered statistically significant.
RESULTS

Ninety six HF patients met the inclusion criteria and agreed to participate to the study (47 female and 49 male). Their mean age was 76±9 years and their mean New York Heart Association (NYHA) functional class was 2.9±0.8 (class II/III/IV: 38/31/27). The baseline features are outlined in Table 1 and clinical parameters were compared to age-matched CTR subjects (n=35). HF patients had a lower ejection fraction (EF, 42±11%) and eGFR (46±16 ml/min) compared to CTR group. Setting at 45%, the cut-off for EF, 57 (59%) HF patients had a reduced EF (34±8%) and 39 (41%) had a preserved EF (53±5%). HF cause was ischemic in origin in about 81% of the patients, and 61% of them suffered from hypertension. Eighty one percent of the patients were treated with either an ACE-inhibitor or an angiotensin-II-receptor-1 antagonist, 66% with beta-adrenergic blocker, 34% with aldosterone receptor antagonist, 87% with diuretics and 30% with digoxin. HF patients showed significant higher plasma levels of norepinephrine, hsCRP, free MAD, Cp, nitrotyrosine-bound Cp, and total nitrated proteins as compared to CTR subjects (Table 1). Total serum copper levels were higher in HF patients compared to CTR (21.63 ± 6.77 vs 16.45 ± 4.87 umol/L, p<0.01).

FeOxI activity was significantly reduced in HF vs CTR patients (-20%; p<0.01, Table 1), whereas no difference in Fe OxII was observed. When the comparison of FeOxI activity was done dividing the patients on the basis of their NYHA class, a significant reduction of FeOxI was observed: NYHA class IV patients compared to class II (P<0.05) and to CTR subjects (P<0.01) as well as NYHA class III patients compared to CTR subjects (P<0.01) had a lower FeOxI activity (Figure 1A). FeOxI activity was significantly reduced in HF with reduced EF vs those with preserved EF (354 ± 21 UI/L vs 426 ± 19 UI/L; p=0.014).

In Table 2, the baseline characteristics of HF patients are depicted separately for each tertile of FeOxI activity. No differences were observed among tertiles with regards to age, body mass index, systolic blood pressure, heart rate, haemoglobin, eGFR, hsCRP, free MAD, ferritin and total nitrated proteins (Table 2) and total copper levels.

There were no significant differences in the medical therapy among tertiles of patients. Tertile 1 (FeOxI activity ≤ 336 UI/L) had lower diastolic blood pressure, lower EF, higher BNP and nitrated Cp compared to the tertile 3 (FeOxI activity ≥ 432 UI/L). No significant differences were found in plasma norepinephrine levels among FeOx tertiles even if a trend was observed toward higher levels in tertiles 1 and 2 versus tertile 3 (p=0.063) (Table 2). Serum Cp was higher in tertile I as compared to tertile II but not different from tertile III (Table 2). Follow-up was complete in all patients. After 24 months of follow-up, 40 patients (41%) of the HF group died: 20 patients (64%) died in the tertile 1, 11 (33%) in the tertile 2 and 9 (28%) in the tertile 3 of FeOxI activity. As shown in Table 3, multivariate Cox regression analysis revealed that FeOxI activity was an independent predictive factor for death occurrence (HR 2.95, CI 1.29-6.75, P=0.011) even after adjustment of the model for clinical variables including age, sex, hypertension, smoking habit, plasma sodium level, eGFR and hsCRP. BNP addition to the model induced the loss of significance of predictive power of FeOxI activity (HR 2.34, CI 0.99-5.54, P=0.053) (Table 3). The Kaplan-Meier survival curves depicting cumulative death rate shows a significantly worse outcome for HF patients in the lowest tertile of FeOxI activity (tertile 1 FeOx ≤ 336 UI/L) compared with those included in the middle (tertile 2: 336<FeOx<432 UI/L) and the higher ones (tertile 3: FeOx≥432 UI/L) (Figure 2).

In addition to mortality outcomes, data on hospitalization rates were collected. Median time to first hospitalization was shorter (less than half) in FeOxI activity tertile 1 vs tertile 2 and 3 (71 days IQR 44-121 for tertile 1 vs 195 days IQR 83-368 in tertile 2 and 262 days IQR 141-493 in the third tertile respectively, P<0.01). Cumulative length of hospital stay was higher in FeOxI tertile 1 vs tertile 3.
(median 32 days IQR 18-56 for tertile 1 vs 26 days IQR 16-33 in tertile 3, p=0.025) but not significant vs tertile 2 (tertile 2 median 23 days IQR 12-45, p=0.152). No significant differences among FeOxI tertiles were found on number of hospitalizations in 24 months (median 3 IQR 2-4 for tertile 1, median 2 IQR 1-3 for tertile 2, median 2 IQR 1-3 for tertile 3, p=0.467).

In HF patients, no correlation was found between serum FeOxI activity and Cp (r = 0.016, p = 0.872, Figure 1B) whereas a significant inverse linear relationship was observed between FeOxI and nitrotyrosine-bound Cp (r = - 0.305, p = 0.003, Figure 1C), total nitrated protein (r = -0.289, P = 0.007), free MAD (r = -0.246, P =0.015), hsCRP (r = -0.245, p = 0.017), TNF-α (r = -0.288, p = 0.009), BNP (r = -0.427, p < 0.001), and RDW (r = -0.327, p < 0.001).

When HF patients were dichotomized on the basis of EF, those with preserved EF had significantly lower Cp levels (2265 ± 419 vs 2523 ± 563 nmol/L, p=0.017) as well as hsCRP (2.20 ± 5.71 vs 6.61 ± 8.09 mg/dl, p=0.004) as compared to HF patients with reduced EF, and a trend in reduction was found for nitrotyrosine-bound Cp (9.27 ± 7.53 vs 13.31 ± 10.13 nmol/L, p=0.068). Mean FeOxI activity in HF with reduced EF was lower than in those with preserved EF (355 ± 144 UI/L vs 426 ± 129 UI/L, P=0.015). At the end of follow-up, 27 patients out of 57 (47%) died in the group with reduced EF compared with 13 out of 39 (33%) of those with preserved EF. Median time to first hospitalization was shorter in HF with reduced EF (105 days (interquartile range) 56-239) as compared to those with preserved EF (271 days IQR 105-482, p<0.01). Median number of hospitalizations during follow-up period were higher in HF with reduced EF (median 3 IQR 2-4) vs those with preserved EF (median 2 IQR 1-3, p=0.004). Median values of cumulative number of days of hospital stay during follow-up period were longer in HF with reduced EF (31 days IQR 18-52) vs those with preserved EF (19 days IQR 11-29, p<0.01). A negative relationship was observed in the HF patients with reduced EF between FeOxI and hsCRP (r = -0.325, p = 0.013, n=57) but not in those with preserved EF (r = 0.206, p = 0.201, n=39). Both preserved and reduced EF HF patient groups showed a negative relationship between FeOxI and nitrotyrosine-bound Cp (preserved EF r = - 0.337, p = 0.035, n=39 and reduced EF r = -0.256, p = 0.054, n=57).

Peroxy nitrite effect on FeOx I activity, Cp tyrosine nitration and cysteine oxidation.

After immunoprecipitation, Cp from plasma of HF and CTR patients was analyzed by Western blot, and two bands at about Mr 140,000 were observed (Figure 3A). Figure 3B shows typical results of immunoblotting assays of eluates from HF and CTR subjects tested against anti-nitrotyrosine antibody after Cp immunoprecipitation. Enzyme-linked immunosorbent assays showed that HF patients had higher plasma nitrotyrosine-bound Cp levels (+203%, P<0.01) compared with CTR (Table 1).

In ex vivo experiments, the effects of ONOO− on FeOxI and II activities was evaluated in plasma from a subset of CTR patients (n=18 out of 35): ONOO− addition caused a significant drop in FeOxI activity (from 448±111 to 238±68 UI/L, - 47%, Figure 4A, P<0.01) with a concomitant increase in nitrotyrosine-bound Cp (from 5.92±2.06 to 21.98±10.49 nM, +371%, Figure 4B, P<0.01). Decomposed ONOO− addition did not induce any changes in FeOxI activity neither in nitrotyrosine-bound Cp levels (Figure 4A and 4B).

The effects of ONOO− and decomposed ONOO− were also verified on purified and lyophilized Cp dissolved in phosphate buffer. The results showed that addition of ONOO− induced a 77 % reduction in FeOxI activity (Figure 5A) associated with an increase in nitrotyrosine-bound Cp (3.3 times the basal values, P<0.001, Figure 5B). We also investigated the effects of ONOO− on cysteine thiol groups of Cp. ONOO− treatment of Cp decreased IAF labeling of isolated Cp (-64%, P<0.001), thereby indicating an increased cysteine thiol oxidation after ONOO− incubation (Figure 5C). GSH at a concentration of 850 μM completely prevented ONOO−induced FeOxI drop, tyrosine nitration and cysteine thiol modification.
(Figure 5 A,B,C). In the presence of EPI, ONOO− induced FeOxI activity drop was partially prevented (Figure 5A). Cp tyrosine nitration was completely prevented (Figure 5B) whereas IAF labeling was still decreased indicating no effects of EPI on cysteine thiol oxidation after ONOO− incubation (Figure 5C). When DMSO, the vehicle of EPI was tested at the dilution used in our experiments, no changes in FeOxI activity, in tyrosine nitration nor in IAF labeling was observed after ONOO− incubation (Figure 5A,B,C). The result of the immunoblotting assay for IAF labeling of cystein thiol of isolated and lyophilized Cp dissolved in phosphate buffer before and after ONOO− incubation in presence and absence of GSH, EPI is depicted in Online Figure I.

DISCUSSION

Three main results arise from this study on a cohort of chronic HF patients and on in vitro experimental observations. First, we confirm the increase of circulating nitrated proteins in HF patients compared to CTR subjects, as a result of increased ONOO− generation. Second, we found an elevation of nitrotyrosine-bound Cp levels in advanced HF patients was associated with a lower serum FeOxI activity with a close inverse relationship. In support of their close link, we have demonstrated in ex vivo and in vitro experiments, that ONOO− induces Cp tyrosine nitration and cysteine thiol oxidation, and these amino acid residue changes result in a significant reduction of Cp-related FeOxI activity.

Third, in our cohort of moderate-to severe HF patients, the reduced FeOxI activity was associated with a significant increase in 2-year mortality. To our knowledge, this is the first study reporting an independent prognostic value for FeOxI activity in HF patients even after adjustment for clinical and biochemical variables.

All these findings deserve specific comments. First, the evidence for protein nitration in vivo is abundant and solid in both physiological and pathological conditions (11,12,25). In particular, in both experimental and human HF, nitrotyrosine protein accumulation reflects a disruption in the balance between oxygen and nitric oxide-derived oxidant formation and antioxidant defence mechanisms (2,11). In the last decade, several experimental studies in HF(1,10,12), but only few in humans (14,27), suggested a potential pathogenetic link between progressive deterioration of heart function and increased protein nitration, although they rarely identified which protein was nitrated. The results of our study fully agree with those reported by others showing that severely diseased HF patients express the highest levels of plasma nitrated proteins. We go further by indicating that Cp in HF patients is identified as one of the plasma nitrated protein. Protein nitrotyrosine formation has been claimed as a “footprint” for ONOO− generation, but only recently specific nitrated proteins have been identified as having a direct pathogenic role in heart function deterioration (11-13,27-29). Originally, nitration was thought to be the result of only ONOO− generation but alternative mechanisms of nitration have been shown to take place in vivo, involving the generation of the NO2− radical by myeloperoxidase and eosinophil peroxidase in the presence of hydrogen peroxide (3,10,28). Tyrosine nitration is strongly enhanced by the presence of transition metals, in particular iron and copper, due to the formation of secondary radicals at the metal center reacting to NO2− (30). Therefore, serum FeOxI activity that regulates the balance between toxic ferrous iron and its nontoxic ferric form represents a fundamental mechanism of protection from iron-mediated free radical injury. Serum Cp acts as an antioxidant in vivo by binding copper and preventing free copper ions from catalyzing oxidative damage and in particular by its FeOxI activity (4,6).We found a progressive decline of serum FeOxI activity with increasing severity of the patients’ HF along with a parallel increase of nitrotyrosine-bound Cp. Nitrotyrosine-bound Cp was identified by immunoprecipitation, immunoblot and ELISA assays; however we did not investigate which tyrosine residue (Cp has 6% of tyrosine residues in its sequence of 1046 amino acids) underwent a nitration process and which has the most important role in FeOxI activity reduction. Hence, we used experimental
settings to investigate the effects of ONOO' on Cp-related FeOx activity and tyrosine and cysteine residue modifications.

Our observations provide direct evidence that ONOO' is a potent inhibitor of Cp-related FeOxI activity. Decomposed ONOO' had null effect on FeOxI activity. We also demonstrated that ONOO' effects are related not only to modified tyrosine but also involves cysteine residues. This is the first report showing an involvement of both cysteine thiol oxidation and tyrosine nitration in the regulation of Cp-related FeOxI activity and that GSH, which inhibits both ONOO'-induced Cp tyrosine nitration and cysteine thiol oxidation, completely prevent FeOxI activity drop. In the presence of EPI, a flavonoid found in cocoa, grapes and green tea, which impedes tyrosine nitration but not cysteine thiol oxidation, a partial drop of FeOxI activity was observed after ONOO' incubation, indicating that both cysteine and tyrosine are necessary and contribute to full FeOxI activity of Cp. Unfortunately we did not investigate which residues in the Cp sequence are modified nor the mechanisms by which the interaction of cysteine and tyrosine residues are important in FeOxI activity. It should be noted from the results obtained in in vitro and ex vivo experiments and in clinical samples that a clear and definite causal link cannot be drawn between ONOO'-induced reduction of FeOxI activity and Cp cysteine thiol oxidation and tyrosine nitration in HF patients. Our observations may suggest that these mechanisms of post-translational modification of Cp may be also operant in vivo.

In the present study, we observed that in a cohort of elderly stable chronic moderate to severe HF patients a lower serum FeOxI activity is associated with higher incidence of all-cause mortality in a 2-year follow up period. This observation has never been reported before and is consistent with the concept that as HF deteriorates, declining FeOxI activity is related to increased nitrotyrosine-bound Cp and could contribute to worse outcomes in HF patients. Interestingly, the predictive mortality performance of FeOxI activity was maintained after the model was adjusted for the patients’ clinical characteristics and several biochemical predictors of outcomes in HF including plasma sodium level, eGFR and hsCRP. But when BNP, one of the most powerful biomarker in HF patients, was included in the model the prognostic performance of FeOxI activity was strongly reduced and become not significant (p=0.053). From our results we cannot argue if high BNP and low FeOxI are only reflecting the severity of HF clinical situation of patients or if they are directly pathogenetically linked.

In our study, we were unable to observe, as recently reported recently in the literature, a strong independent prognostic value of high Cp circulating levels in stable patients undergoing elective coronarography and in a group of patients without HF or cardiovascular disease taken from the Atherosclerosis Risk in Communities Study (7,8). As a matter of fact, our findings show that Cp and FeOxI were unrelated in pooled preserved and reduced EF HF patients. It is quite unclear why Cp had no predictive value in our HF patients, the majority (81 %) of whom had an ischemic origin. However, the population enrolled in our study was significantly older (mean age 76 years old), included patients with advanced stages of heart failure (almost 60% with mean ejection fraction of 34%) compared to the younger patients in the two larger studies by Tang et al. and Dadu et al. (mean age 62 years-old) (7,8) and at least in the Atherosclerosis Risk in Communities Study in subjects without heart failure and cardiovascular disease at enrolment (8). Only FeOxI was found to predict the mortality risk and a close inverse relationship was found with nitrotyrosine-bound Cp in both HF patients with preserved and reduced EF and with hsCRP in HF patients with reduced EF. In addition, the analysis of hospitalization rate of our patient’s population, showed that time to first admission was much shorter in patients with low FeOxI activity whereas no difference in number of hospital admission during the follow-up period was observed among FeOxI tertiles. It should be noted that the number of hospitalizations recorded in our population of HF patients is small and there was insufficient power to detect a difference among tertiles. Overall, our findings imply that a reduced anti-oxidative capacity as evidenced by decreased Cp-related FeOxI activity plays a role in increasing the mortality risk in moderate to severe HF elderly patients. Low FeOxI activity can in part affect the progression of left ventricular dysfunction as found in our study, but
others mechanisms are involved and are probably more important. In fact, our data shows that left ventricular EF was similar in the 2 tertiles with low FeOxI activity, thus suggesting the possibility that FeOxI activity may be more closely related to peripheral derangements and vascular alterations. In this regard, unfortunately, we did not measure vascular parameters in our patients such as endothelial function, peak reactive hyperemia, or aerobic capacity that could clearly influence the prognosis of HF patients.

Our study has some other limitations. First the results obtained from a single centre require a validation in large external cohorts of patients. Second, the clinical criteria of enrolment were quite restrictive: in fact diabetic patients, a large portion in elderly HF population, were excluded. However, while the sample size is limited, a large number of deaths occurred during the follow-up period in the elderly HF patients in our study, which made it possible to perform an analysis of FeOxI as an independent mortality predictor.

In conclusion, our findings provide evidence that in elderly chronic HF patients, lower FeOxI activity related to higher Cp nitration, is associated with increased mortality risk. Both Cp tyrosine nitration and cysteine thiol oxidation that are responsible in the in vitro setting of ONOO-induced FeOxI activity inhibition, may be the mechanisms also operant in vivo in HF patients. However, further studies are required to deeper scrutinize these experimental and clinical research areas related to the effects of ONOO- on cysteine and tyrosine residues in Cp and to evaluate the clinical significance of these results, and to establish whether preservation or restoration of FeOxI activity in HF could become a target for future treatment.

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The authors thank Maria Giovanna Fedeli, for assistance in biomarkers analysis, and the staff nurse of the out patients Clinic of Clinica Medica e Nefrologia.

DISCLOSURES
None.
REFERENCES


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11


FIGURES LEGENDS

**Figure 1.** A: Boxplots of serum Ferroxidase I activities in Controls (n=35) and Heart Failure patients (NYHA class II n=38 , III n=31 , IV n=27). One way ANOVA (P < 0.001) showed a significant difference among the groups (Class IV and III vs Controls, † P<0.01; Class IV vs Class II, * P < 0.05). B-C: Scatterplots of serum Ferroxidase I Activity against Ceruloplasmin (1B) and Nitrotyrosine-bound Ceruloplasmin (1C) in HF patients. r = Pearson correlation coefficient.

**Figure 2.** Relationship between serum Ferroxidase I Activity and mortality in 2-years follow-up of stable chronic heart failure patients. Kaplan-Meier analysis shows the comparison of mortality according to serum Ferroxidase I Activity tertiles groups (tertile 1 FeOx ≤ 336 UI/L, tertile 2 336 < FeOx > 432 UI/L, tertile 3 FeOx ≥ 432 UI/L).

**Figure 3.** Representative immunoblotting of immunoprecipitated Ceruloplasmin (Cp) incubated with polyclonal anti-Cp antibody (A) and with anti-nitrotyrosine antibody (B). A. MM: molecular wt markers. Lane 1: blank; lanes 2-5 heart failure (HF) patients; lane 6 control (CTR). B. HF and CTR lanes.

**Figure 4.** Serum Ferroxidase I Activity (A) and Nitrotyrosine-bound Ceruloplasmin (B) before and after exposure to peroxynitrite (ONOO⁻) and decomposed ONOO⁻ in Controls (n=18), * P < 0.05, † P < 0.01.

**Figure 5.** Boxplots of Ferroxidase I activity (A), Nitrotyrosine-bound Ceruloplasmin (Cp) (B), and iodoacetamidofluorescein (IAF) labeling of cysteine thiol groups of purified Cp dissolved in phosphate buffer (C) before and after exposure to peroxynitrite (ONOO⁻) in presence of GSH, Epicatechin (EPI) and DMSO (vehicle of EPI). * P < 0.05, † P < 0.01.
Table 1. Clinical characteristics of heart failure patients and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 35)</th>
<th>Heart Failure (n = 96)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>76 ± 10</td>
<td>76 ± 9</td>
</tr>
<tr>
<td>Gender, male (%)</td>
<td>16 (45)</td>
<td>49 (51)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.9 ± 4.4</td>
<td>24.3 ± 3.5</td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td>---</td>
<td>78 (81)</td>
</tr>
<tr>
<td>Hypertensive heart disease</td>
<td>---</td>
<td>48 (50)</td>
</tr>
<tr>
<td>Idiopathic heart disease</td>
<td>---</td>
<td>4 (4)</td>
</tr>
<tr>
<td>NYHA class II, III, IV</td>
<td>---</td>
<td>38, 31, 27</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>63 ± 6</td>
<td>42 ± 11 †</td>
</tr>
<tr>
<td>Ejection fraction &lt;45%</td>
<td></td>
<td>57 (59)</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>9 (26)</td>
<td>58 (61)</td>
</tr>
<tr>
<td>Hypercholesterolemia (%)</td>
<td>13 (37)</td>
<td>39 (41)</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>9 (26)</td>
<td>29 (30)</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>13.3 ± 1.3</td>
<td>12.9 ± 1.2</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>12.5 ± 0.6</td>
<td>13.6 ± 1.0 †</td>
</tr>
<tr>
<td>eGFR (ml/min)</td>
<td>62.7 ± 18.7</td>
<td>46.0 ± 16.1 †</td>
</tr>
<tr>
<td>Na (mEq/l)</td>
<td>140 ± 4</td>
<td>139 ± 5</td>
</tr>
<tr>
<td>hsCRP (mg/dl)</td>
<td>1.19 ± 1.16</td>
<td>4.81 ± 7.52 *</td>
</tr>
<tr>
<td>Norepinephrine (pg/ml)</td>
<td>229 ± 86</td>
<td>406 ± 198 †</td>
</tr>
<tr>
<td>Free Malondialdehyde (umol/L)</td>
<td>0.29 ± 0.11</td>
<td>0.40 ± 0.13 †</td>
</tr>
<tr>
<td>Ceruloplasmin (nmol/L)a</td>
<td>2118 ± 478</td>
<td>2419 ± 523 *</td>
</tr>
<tr>
<td>Ferritin (mg/dl)</td>
<td>130.2 ± 86.4</td>
<td>134.3 ± 90.0</td>
</tr>
<tr>
<td>Ferroxidase I Activity (UI/L)</td>
<td>478 ± 133</td>
<td>384 ± 141 †</td>
</tr>
<tr>
<td>Ferroxidase II Activity (UI/L)</td>
<td>12.7 ± 5.1</td>
<td>14.0 ± 4.4</td>
</tr>
<tr>
<td>Total Nitrated Proteins (nmol/L)a</td>
<td>262 ± 74</td>
<td>381 ± 94 †</td>
</tr>
<tr>
<td>Nitrotyrosine-bound Ceruloplasmin (nmol/L)a</td>
<td>5.85 ± 2.01</td>
<td>11.89 ± 9.29 †</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD; numbers in parentheses are percentages. RDW: Red cell distribution width; eGFR: estimated Glomerular Filtration Rate; BNP: B type natriuretic peptide; hsCRP: high sensitivity C reactive protein; * P < 0.05, † P < 0.01. a Data in control group are obtained in 27 out of 35 patients.
Table 2. Characteristics of heart failure patients divided for Ferroxidase I activity tertiles.

<table>
<thead>
<tr>
<th></th>
<th>Tertile 1 (n=31) FeOx&lt;336 UI/l</th>
<th>Tertile 2 (n=33) 336&gt;FeOx&lt;432UI/l</th>
<th>Tertile 3 (n=32) FeOx&gt;432 UI/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>77 ± 9</td>
<td>76 ± 8</td>
<td>75 ± 10</td>
</tr>
<tr>
<td>Gender, male</td>
<td>21</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.9 ± 2.8</td>
<td>23.4 ± 3.8</td>
<td>25.3 ± 3.8</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>127 ± 24</td>
<td>128 ± 22</td>
<td>136 ± 17</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>70 ± 15</td>
<td>72 ± 13</td>
<td>80 ± 14 *</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>72 ± 13</td>
<td>72 ± 11</td>
<td>71 ± 13</td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td>24 (77)</td>
<td>27 (82)</td>
<td>23 (72)</td>
</tr>
<tr>
<td>Hypertensive heart disease</td>
<td>9 (29)</td>
<td>10 (30)</td>
<td>19 (59)</td>
</tr>
<tr>
<td>Idiopathic heart disease</td>
<td>3 (10)</td>
<td>0 (0)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>NYHA class (II, III, IV)</td>
<td>6, 12, 13</td>
<td>13, 8, 12</td>
<td>18, 11, 3</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>39 ± 13</td>
<td>40 ± 12</td>
<td>47 ± 9 * †</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>13.1 ± 1.1</td>
<td>12.8 ± 1.4</td>
<td>13.1 ± 1.2</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>14.1 ± 1.1</td>
<td>13.2 ± 0.7 *</td>
<td>13.2 ± 0.9 *</td>
</tr>
<tr>
<td>eGFR (ml/min)</td>
<td>44.1 ± 12.5</td>
<td>43.5 ± 14.0</td>
<td>50.4 ± 20.3</td>
</tr>
<tr>
<td>hsCRP (mg/dl)</td>
<td>5.39 ± 7.94</td>
<td>6.10 ± 7.61</td>
<td>2.90 ± 6.81</td>
</tr>
<tr>
<td>Tumor Necrosis Factor-α (pg/ml)</td>
<td>5.25 ± 1.59</td>
<td>4.50 ± 2.38</td>
<td>3.70 ± 1.61 *</td>
</tr>
<tr>
<td>Norepinephrine (pg/ml)</td>
<td>445 ± 191</td>
<td>433 ± 247</td>
<td>339 ± 122</td>
</tr>
<tr>
<td>BNP (pg/ml)</td>
<td>282.8 ± 179.3</td>
<td>175.8 ± 146.1 *</td>
<td>113.5 ± 78.8 *</td>
</tr>
<tr>
<td>Free Malondialdehyde (umol/L)</td>
<td>0.42 ± 0.14</td>
<td>0.42 ± 0.13</td>
<td>0.35 ± 0.11</td>
</tr>
<tr>
<td>Ceruloplasmin (nmol/L)</td>
<td>2545 ± 568 †</td>
<td>2233 ± 468</td>
<td>2485 ± 493</td>
</tr>
<tr>
<td>Ferritin (mg/dl)</td>
<td>120.5 ± 79.3</td>
<td>154.3 ± 110.9</td>
<td>132.2 ± 68.6</td>
</tr>
<tr>
<td>Ferroxidase II Activity (UI/L)</td>
<td>13.7 ± 2.9</td>
<td>14.1 ± 5.4</td>
<td>14.5 ± 4.6</td>
</tr>
<tr>
<td>Total Nitrated Proteins (nmol/L)</td>
<td>399.02 ± 111.86</td>
<td>390.22 ± 83.16</td>
<td>354.26 ± 83.67</td>
</tr>
<tr>
<td>Nitrotyrosine-bound Ceruloplasmin (nmol/L)</td>
<td>16.05 ± 11.80</td>
<td>10.55 ± 7.76 *</td>
<td>9.24 ± 6.40 *</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD; numbers in parentheses are percentages. RDW: Red cell distribution width; eGFR estimated glomerular filtration rate; BNP: B type natriuretic peptide; hsCRP: high sensitivity C reactive Protein * indicates P less than 0.05 vs Tertile 1, † indicates P less than 0.05 vs Tertile 2.
Table 3. Unadjusted and adjusted Cox proportional hazard models for mortality according to tertiles of Ferroxidase I activity.

<table>
<thead>
<tr>
<th></th>
<th>Upper FeOxI tertile 3rd (FeOx&gt;432 UI/l)</th>
<th>Middle FeOxI tertile 2nd (336&gt;FeOx&lt;432UI/l)</th>
<th>Lower FeOxI tertile 1st (FeOx&lt;336 UI/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 32</td>
<td>N = 33</td>
<td>N = 31</td>
</tr>
<tr>
<td>Events at 24 months, n (%)</td>
<td>9 (28)</td>
<td>11 (33)</td>
<td>20 (64)</td>
</tr>
<tr>
<td>Models</td>
<td>HR (95%CI) - P value</td>
<td>HR (95%CI) - P value</td>
<td>HR (95%CI) – P value</td>
</tr>
<tr>
<td>Unadjusted</td>
<td>1.0</td>
<td>1.27 (0.53-3.07) – 0.592</td>
<td>3.65 (1.66-8.04) – &lt;0.001</td>
</tr>
<tr>
<td>Adjusted for clinical variables (a)</td>
<td>1.0</td>
<td>0.89 (0.35-2.27) – 0.805</td>
<td>3.23 (1.37-7.64) - 0.009</td>
</tr>
<tr>
<td>Adjusted for clinical variables (a) and hsCRP</td>
<td>1.0</td>
<td>0.87 (0.34-2.21) – 0.764</td>
<td>2.95 (1.28-6.75) – 0.011</td>
</tr>
<tr>
<td>Adjusted for clinical variables (a) and BNP</td>
<td>1.0</td>
<td>0.81 (0.31-2.10) – 0.661</td>
<td>2.34 (0.99-5.54) – 0.053</td>
</tr>
</tbody>
</table>

(a) The model adjusted for clinical variables includes age, sex, hypertension, smoking habit, plasma sodium level and estimated glomerular filtration rate; hsCRP (High-sensitivity C- Reactive Protein); BNP (B type natriuretic peptide); TNF- (Tumor Necrosis Factor- ); EF (Ejection Fraction).
Novelty and Significance

What Is Known?

- By regulating the balance between the toxic ferrous and the non-toxic ferric form of iron, serum ferroxidase (FeOx) protects against oxygen and nitrogen free radical injury.

- In human plasma, serum Ceruloplasmin (Cp), the major copper transporter, has antioxidant properties via its FeOx I activity.

- Advanced heart failure (HF) is associated with increased generation of aberrant oxygen free radicals and nitric oxide-derived peroxynitrite (ONOO').

What New Information Does This Article Contribute?

- Low serum FeOxI activity is associated with increased severity of heart failure and has important prognostic implications.

- Increased tyrosine nitration of Cp is closely related to the drop in FeOx I activity in heart failure patients.

- Cysteine thiol oxidation and tyrosine nitration of serum Cp may be operant in vivo in ONOO-induced FeOxI activity inhibition in HF patients.

The FeOxI and FeOxII activities of serum ferroxidase regulate the balance between the toxic ferrous iron and nontoxic ferric form of iron, and protect against iron-mediated oxygen and nitrogen-derived free radical injury. Ceruloplasmin (Cp), an alpha2-glycoprotein that transports serum copper also has antioxidant properties through its FeOx I activity. Increased peroxynitrite (ONOO') generation, as observed in heart failure (HF), can affect protein folding and influence protein function through tyrosine nitration and cysteine sulfhydryl oxidation. However, in the HF population the relationship between Cp, ONOO' generation, tyrosine nitration, and FeOxI activity has not been established and the clinical relevance of Cp-mediated FeOxI activity remains unclear. We found that increasing severity of HF in patients is associated with a drop in serum FeOxI activity is inversely related to Cp tyrosine nitration. In experimental studies, both cysteine thiol oxidation and tyrosine nitration of serum Cp were identified to be responsible for ex vivo and in vitro decrease in FeOx I activity. Thus, low FeOx I activity appears to have important prognostic implications in patients with chronic HF and that it may be an important target in HF treatment.
Figure 1

A

Ferroxidase 1 Activity, UI/l

Controls  Class II  Class III  Class IV

†

B

Ceruloplasmin, nM

r=0.016, p=0.872, n=96

Ferroxidase 1 Activity (UI/l)

C

Nitrotyrosine-bound Cp, nM

r=-0.305, p=0.003, n=96

Ferroxidase 1 Activity (UI/l)
Figure 2

[Graph showing survival over follow-up period with different FeOx activity levels.]
Figure 4

A

Ferroxidase 1 Activity (UI/l)

B

Nitrotyrosine, nmol

before ONOO⁻

after ONOO⁻

before ONOO⁻

after decomposed ONOO⁻
Figure 5

A

Ferroxidase 1 Activity, UI

B

Nitrotyrosine-bound Cp, nM

C

IAF labelling Cp, %

before ONOO⁻

after ONOO⁻

after ONOO⁻ + GSH

after ONOO⁻ + EPI

after ONOO⁻ + DMSO
Low Serum Ferroxidase I Activity is Associated with Mortality in Heart Failure and Related to Both Peroxynitrite-Induced Cysteine Oxidation and Tyrosine Nitration of Ceruloplasmin
Aderville Cabassi, Simone Maurizio Binno, Stefano Tedeschi, Valerie Ruzicka, Simona Dancelli, Rossana Rocco, Vanni Vicini, Pietro Coghi, Giuseppe Regolisti, Alberto Montanari, Enrico Fiaccadori, Paolo Govoni, Massimo F Piepoli and Jacques de Champlain

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SUPPLEMENTAL MATERIAL

Low serum ferroxidase I activity is associated with mortality in heart failure and related to both peroxynitrite-induced cysteine oxidation and tyrosine nitration of ceruloplasmin.

Cabassi et al Ferroxidase I activity and heart failure

Methods

Cp Immunoprecipitation

For immunoprecipitation we used a polyclonal anti-human anti-Cp antibody (ABIN680108 antibodies-online GmbH, Aachen, Germany) that was cross-linked to Dynabeads protein A (Dynal Biotech, Oslo, Norway). Lyophilized rabbit anti-Cp antibody were reconstituted in purified distilled water to obtain a stock solution of 1µg/ul. After washing, 50 µl of Dynabeads (1.5 mg) were resuspended after magnetic separation (Dynal MPC) in 0.1 M Na₂HPO₄ pH 8.0, and transferred to a polypropylene test tube. The solution was incubated with rotation (Dynal MX1-Mixer) for 20 minutes at room temperature with 200 µl of phosphate buffer saline (pH 7.4) containing 2.5 µg of antibody. The supernatant was then magnetically removed and the beads-Ab complex resuspended with 200 µl phosphate buffer saline (pH 7.4) with 0.02% Tween 20 and washed by gently pipetting. Two hundred and fifty µl of diluted serum from the patients (1:20 with 0.1 M Na₂HPO₄ pH 8.0) were incubate with tilting and rotation for 45 minutes at room temperature. Test tubes were then placed on the magnet for 3 min to collect the beads beads-Ab complex on the tube wall. After washing 3 times (1 ml phosphate buffer saline, pH 7.4), immune complexes were released after incubation by tilting and rotation for 10 min at 25°C with 0.1 M glycine-HCl (pH 2.7) buffer. The tube was then placed on the magnet and the supernatant containing eluted antibody and Cp transferred to a clean tube. Eluted Cp solution was adjusted by adding 1 M Tris (Sigma T1503), to obtain a pH of 7.4. The protein concentration of the supernatant was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA). Appropriate control tests were established (one out of ten serum patient samples) by adding Cp to the serum at a known concentration and verifying its recovery. The eluted Cp was then tested for
Immunoblotting for nitrotyrosine and cystein thiol oxidation detection

Eluates from Cp immunoprecipitation were subjected to standard SDS-PAGE and Western blotting techniques using 5% for stacking and 8-12% polyacrylamide separating gel and transferred to polyvinylidene difluoride membranes (Immun-Blot PVDF Membrane, Bio-Rad Laboratories, Hercules, California, USA) at 4 mA/cm² for 45 min as previously described (1). In brief, equal amounts of protein concentrations from each sample were loaded into the gel and run for 45 min at 200 V. A 12-μl volume of Rainbow molecular marker standards was used for molecular weight markers. Gel running buffer consisted of 1× Tris-glycine-SDS buffer. The membrane was then incubated with polyclonal anti-nitrotyrosine (1:2500; 06-284, Upstate, Millipore), and the signal amplified with an Opti-4CN Detection Kit (goat anti-rabbit HRP-conjugated secondary antibody, 1:10,000 Bio-Rad Laboratories, Hercules, California, USA). Nitrotyrosine-bound Cp was evaluated in serum from HF patients and CTR but also in ex vivo experiments on CTR serum (n=18) after incubation of ONOO⁻ or decomposed ONOO⁻ and in in vitro experiments (n=10) after ONOO⁻ or decomposed ONOO⁻ incubation of isolated purified Cp in a phosphate buffer bath.

In addition to nitrotyrosine, the evaluation of cysteine thiol oxidation after ONOO⁻ or decomposed ONOO⁻ incubation was performed on isolated purified Cp in a phosphate buffer bath. To detect cysteine thiol status we labeled untreated and ONOO⁻ or decomposed ONOO⁻-treated Cp free thiol groups with iodoacetamido-4,5-fluorescein (IAF). Lyophilized Cp (50 μg/4.5 units, Sigma-Aldrich Chemical Co. St Louis, Missouri, USA) was dissolved in 350 μl of 0.2 M Tris·HCl (pH 7.40) and incubated for 10 min at 37°C in the presence or absence of ONOO⁻ and decomposed ONOO⁻ at a dose of 0.15 mmol/L. Samples were incubated with 0.5% SDS for 15 min at room temperature and then with 500 μM iodoacetamido-4,5-fluorescein for 30 min in the dark at room temperature. Cysteine (5 mM) was added to quench any nonreacted iodoacetamido-4,5-fluorescein. IAF-labeled thiol groups were detected using 1 mg/ml mouse anti-4,5-fluorescein-Oregon Green (Molecular Probes) diluted 1:4,000 into 1× Tris Buffer Saline with 0.05% Tween 20. Membranes were incubated with the appropriate primary antibody for 1 h at room temperature and then washed five times in 1× Tris Buffer Saline with 0.05% Tween 20.
Tween 20. Membranes were incubated again for 1 h with goat anti-mouse HRP-conjugated secondary antibody (31430, Pierce, ThermoFisher Scientific, Rockford, Illinois, USA) diluted 1:25,000 into 1× TBS with 0.05% Tween 20 and washed five times, for 5 min each, in fresh 1× TBS with 0.05% Tween 20 as previously described (2).

The result of the immunoblotting assay for IAF labeling of cystein thiol of isolated and lyophilized Cp dissolved in phosphate buffer before and after ONOO⁻ incubation in presence and absence of GSH, EPI is depicted in Online Figure I.

**Online Figure I**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>ONOO⁻, 150 μM</td>
<td>+</td>
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<tr>
<td>Dec. ONOO⁻, 150 μM</td>
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<tr>
<td>GSH, 850 μM</td>
<td>-</td>
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<td>+</td>
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<tr>
<td>EPI, 50 μM</td>
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<td>DMSO, 0.01%</td>
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</table>

Online Figure I: Representative immunoblotting assay for IAF labeling of cystein thiol of isolated and lyophilized Cp before and after ONOO⁻ incubation in presence and absence of GSH, EPI. Peroxynitrite (ONOO⁻)-induced decrease in iodoacetamidofluorescein labeling implies that peroxynitrite modifies cysteine thiol groups. Purified and lyophilized Cp was treated with 0,15 mM of ONOO⁻ (lane 1 alone, lane 3 in presence of GSH, lane 5 in presence of EPI, lane 7 in presence of DMSO) or with decomposed (Dec. ONOO⁻ (lane 2 alone, lane 4 in presence of GSH) or not treated (lane 6).
References
