NEGATIVE FEEEDBACK INTERACTION OF HO-1/iNOS IN PBMC OF ACHF PATIENTS

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Heart failure (HF) is a common clinical syndrome with frequent exacerbations requiring hospitalization. Among the various mechanisms that underlie the pathogenesis of HF, the activation of the immune system leads to a progressive and redundant release of proinflammatory cytokines responsible for a variety of deleterious effects in heart failure, such as endothelial dysfunction, apoptosis of myocytes, activation of MMPs (Matrix Metallo Proteinases) and oxidative stress, with the result of decreased inotropism and clinical syndrome such as pulmonary edema,. The condition of oxidative stress induces the expression of genes coding for the proteins inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1). Twenty-five hospitalized cardiology patients with symptomatic acute congestive HF (NYHA Class III-IV) and impaired left ventricular (LV) function (ejection fraction <35%) were included in the study. The aim of this study was to evaluate the cytokines plasma concentrations and the expression and activity of iNOS and HO-1 proteins in peripheral blood mononuclear cell (PBMC) extracted from patients in comparison to control group (IL-1α 3.8±1.7 pg/ml *vs* 7.3±0.4; IL-6 10.8±2.1 pg/ml *vs* 80.3±5.3; INF-γ 1.2±0.3 pg/ml *vs* 5.4±0.8; TNF-α 3.5±0.8 pg/ml *vs* 27.1±1.3). In ACHF; left ventricular ejection fraction (LVEF) percent was reduced. Furthermore; iNOS and HO-1 expression and cytokines plasma levels were significantly higher in patients with ACHF as compared to controls group. Moreover the enzyme activity presents an opposite trend compared to that obtained in the analysis of the transcript and proteins. Our studies suggest a negative feedback interaction between iNOS and HO-1 important in the physiopathology of heart failure that could be considered a good candidate as a future therapeutic target for the development of new drugs.

The important role of inflammation in HF has been recognized. Among the various mechanisms that underlie the pathogenesis of CHF, the activation of the immune system leads to a progressive and redundant release of pro-inflammatory cytokines such as TNF-α and interleukin (IL)-6, responsible for tissue damage, loss of capillaries and disease progression (1, 2).

Acute Congestive heart failure (ACHF) is a syndrome characterized by the inability of the heart to fill with or eject blood due to structural or functional cardiac disorders (3, 4).

Key words: heart failure, iNOS, cytokines, heme-oxygenase 1

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The difficult management of HF is due to several conditions such as the older patient population, the episodes of acute decompensation, the complexities of the required lifestyle changes, medication regimen, laboratory monitoring and interactions with co-morbidity (5).

However a concept still in the evolutionary stage is the pivotal role of the inflammatory pathway inducing the state of failure; the production of cytokines, in high concentration in the plasma, is often associated with severe symptoms of congestive heart failure (CHF); this state also presents high plasma levels of tumour necrosis factor (TNF-α) (1). Cytokines are implicated in a variety of deleterious effects in heart failure, such as endothelial dysfunction, decreased inotropism, apoptosis of myocytes, pulmonary edema, activation of MMPs (Matrix Metallo Proteinases) and oxidative stress (6, 7).

A fundamental process subsequent to the release of such cytokines is the activation of regulatory molecules such as Nuclear Factor-KappaB (NF-Kb), a transcription factor that leads to turning on several genes, including those which express the inducible nitric oxide synthase (iNOS) (8).

Massive production of nitric oxide (NO), should help reduce the inotropic state due to the CHF (9-11). However, there are recent experimental and clinical studies that failed to prove a causal relationship between iNOS induction and heart failure (11, 12).

NO is an important molecule involved in the regulation of various physiological and pathophysiological mechanisms of the cardiovascular system, nervous system and immune system (13, 14).

The condition of oxidative stress, in addition to inducing the expression of enzymes involved in antioxidant defence, also induces the expression of genes coding for the heat shock proteins such as heme-oxygenase 1 (HO-1) that play a key role for an anti-inflammatory, antiproliferative, antiapoptotic and antioxidant effect (15).

HO-1 was the first isoform to be isolated, subsequently a second isoform HO-2 was isolated by using untreated rat brain, testes and liver tissue, and this one was found to be costitutively expressed in many organs under normal conditions . A third isoform, HO-3, has been described in rats, but it has since become evident that it is a pseudo gene derived from the HO-2 transcript. HO-1, the inducible isoform of HO, catalyzes the rate-limiting step of heme oxidation to biliverdin, CO, and free ferrous iron (16).

Biliverdin is then rapidly converted by biliverdin reductase to bilirubin, a molecule with antioxidant properties, and free iron is sequestered by ferritin. CO also mediates other effects in vasculature via the activation of soluble guanylyl cyclase (sGC) (17, 18). However, its pathophysiological role in cardiac remodeling and chronic heart failure (HF) is unknown (18, 19).

Our objective was to evaluate the possible role of this protein in the pathogenesis of acute congestive heart failure (ACHF) and to analyze what mechanisms lead to an alteration of iNOS/NO and HO-1/CO systems, trying to identify a possible interaction between the pathways of these two enzymes.

MATERIAL AND METHODS

Study design

Between March and September 2011, all consecutive patients with diagnosis of ACHF (defined as acute and progressive resting dyspnea associated with clinical signs of pulmonary or peripheral congestion requiring hospitalization and treatment with an intravenous diuretic) admitted in Intensive Cardiology Unit of San Camillo De Lellis Hospital (Manfredonia, Italy) were invited to participate in this study. The experimental protocol was approved by the local Ethics Committee.

After being informed about the nature and purpose of the study all patients gave their voluntary consent. Eligible subjects were 18 to 75 years of age, with left ventricular ejection fraction (LVEF) ≤35%. Subjects were excluded if they had had acute cardiac decompensation within the previous 7 days, need for coronary revascularization, acute coronary syndrome, significant primary valvular diseases, or significant hepatic or renal dysfunction. Significant hepatic dysfunction was defined as serum aminotransferase levels above 2x the upper limit of normal.

Patient population

Twenty-five patients with ACHF (LVEF <35%) were enrolled in the study. Medical and surgical history, physical condition, and medical treatment were recorded (20).

After inclusion, a heparinised blood sample was drawn from an indwelling arterial line for determination

of parameters for this study. Subsequently a blood sample was drawn after one day of therapy. Simultaneously, laboratory parameters indicating renal function (creatinine, urea) and hepatic function - aspartate aminotransferase (AST), alanine aminotransferase (ALT) and complete haematocytometer exam - were determined (Table II).

Echocardiographic-doppler evaluation

Echocardiography was performed using an ultrasound system (ACUSON SequioiaC512 Siemens Medical Solutions USA, Inc) with a 3.7 MHz transducer. LVEF was evaluated from apical four- and two- chamber views, using the Simpson's biplane method. Each representative value was obtained from the average of three measurements according to the American Society of Echocardiography criteria. The valvular assessment included the evaluation of the function of the mitral, aortic and tricuspid valves. Color-Doppler echocardiography was performed after optimizing gain and Nyquist limit, and standard continuous and pulsed-wave Doppler recordings were acquired. Stenotic and regurgitant valve diseases were evaluated according to semi-quantitative and quantitative methods recommended by the American Society of Echocardiography (20, 21). Tricuspid regurgitation was visualized from the apical 4-chamber view. The PAPs was estimated from the peak tricuspid regurgitation jet, using the simplified Bernoulli equation (PAPs=4 x v2 + right atrial pressure), where "v" is the peak velocity of the tricuspid regurgitation jet (m/s), and the right atrial pressure is estimated from the diameter and breath-induced variability of the inferior venacava. Data were stored for further off-line analysis.

Isolation of human peripheral adherent mononuclear cells

Primary blood mononuclear cells (PBMC) were isolated as previously described (12). The cell viability, determined by trypan blue exclusion, was >99%. The media and sera were of very low LPS content, as determined by testing the chromogenic assay using Limulus amebocyte lysate (Whittaker Bioproducts, Walkersville, MD).

PCR analysis for iNOS and HO-1

Semi-quantitative reverse-transcribed polymerase chain reaction (RT-PCR) was used to determine mRNA levels of the inducible Nitric Oxide Synthase. The analysis was performed as previously described (22).

The following primer pairs were used: sense 5'-CGT AAA GAC CTC TAT GCC AA-3' and antisense 5'-AGC CAT GCC AAA TGT CTC AT-3' for iNOS, sense 5'-CAG GCA GAG AAT GCT GAG TTC-3' and antisense 5'- GCT TCA CAT AGC GCT GCA-3' for HO-1 primers, 18 S primers, 0.15 mM of both sense 5'-TAC GGA GCA GCA AAT CCA C-3' and antisense 5'-GAT CAA AGG ACT GCA GCC TG-3', 2U Termophilus Acquaticus (Taq) DNA polymerase (Celbio, Milan, Italy), and water to a final volume of 50 µl. These samples were overlaid with mineral oil and subjected to 35 cycles at 95°C for 60 s, 60°C for 60 s, and to one cycle at 72°C for 7 min for iNOS and 40 cycles at 95°C for 60 s, 58°C for 60 s, and to one cycle at 72°C for 7 min for HO-1. PCR products were run on 2% agarose gel electrophoresis and photographed after ethidium bromide staining under UV light. Bands on the gel were scanned using a computerized densitometric system (Bio-Rad Gel Doc 1000, Milan, Italy).

Western blot analysis for Nf-Kb, iNOS and HO-1

Total protein extracts were prepared by treating cells with lysis buffer (RIPA). Nuclear extracts were prepared as previously described (23). Proteins were quantified using the Bradford method. Western blot analysis was performed as described previously using the following primary antibodies: anti--human NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA),and anti-human HO-1 (Stressgen, Ann Arbor, MI, USA).

Nitrocellulose was then washed in TBS, and incubated with secondary antibody HRP-conjugated (dilution 1:10000, Pierce) for 1 h, washed again, and developed. The nitrocellulose was scanned using a computerized densitometric system (Bio-Rad Gel Doc 1000, Milan, Italy). Protein levels were normalized to the housekeeping protein β-actin (A5441; Sigma-Aldrich) to adjust for variability of protein loading and expressed as a percentage of the vehicle control.

Cytokines Elisa

Quantitative measurement of human cytokines were assayed using specific ELISA development systems to detect IL-1α, IL-6, INF-γ and TNF-α (Searchlight®, Aushon Biosystems, MA, USA) as described previously (24).

Citrulline synthesis (nitric oxide activity)

The measure of the conversion of L-arginine to L-citrulline is a standard assay method currently used for quantitative NOS activity. The assay was performed as described previously (25).

Briefly, 10 µl of radioactive arginine, L-(2, 3, 4, 5-3H) Arginine Monohydrochloride 64 Ci/mM, 1µCi/µl (Amersham, Arlington Heigths, Illinois, USA), 50 µl NADPH 10 mM, 50 µl CaCl2 6 mM, (Calbiochem, CA USA) were added to each cell homogenate sample and incubated for 30 min at room temperature. After incubation, the reactions were stopped with 400 µl of stop-buffer (50 mM HEPES, pH 5.5, 5 mM EDTA) and the equilibrate resin was added into each sample. The equilibrated resin bound

unreacted arginine. After centrifugation, the radioactivity corresponding to L-(3H)-citrulline was measured with liquid scintillation spectrometry. Calcium was omitted from these incubations to favour the determination of the calcium-independent iNOS isoform (18).

Assay for HO-1 activity

HO activity assay was performed as described previously (26). Briefly, microsomes from harvested cells were added to a reaction mixture containing 0.8 mM NADPH (Sigma), 2 mM glucose 6-phosphate (Sigma), 0.2U glucose-6-phosphate dehydrogenase (Sigma), 2 mg rat liver cytosol prepared from a 105 000 3 g supernatant fraction as a source of biliverdin reductase, potassium phosphate buffer (100 mM, pH 7.4) and 20 mM hemin (Sigma). The reaction was conducted at 37°C in the dark for 1 h and then placed on ice for 2 min to terminate the reaction.

Bilirubin was determined by calculation from the difference in absorbance between 464 and 530 nm (extinction coefficient, $40mM^{-1}$ cm^{-1} for bilirubin). HO activity was expressed as picomoles of bilirubin formed per mg of microsomal protein per h. The total protein content of confluent cells was determined using a Bio-Rad DC protein assay (Bio-Rad, Herts, UK) by comparison with a standard curve obtained with bovine serum albumin.

Statistical analysis

All qualitative variables were summarized as frequency and percentage and all quantitative variables as mean and standard deviation (SD). The results were reported separately for the ACHF group and the control group. Statistical significance of differences between groups for qualitative variables were assessed using the chi-squared test or Fisher's Exact Test, when appropriate. Non-parametric Mann-Whitney U test was applied for assessing the comparison of the quantitative variables between the two groups (ACHF group and control group).

RESULTS

The demographic, clinical and echocardiographic characteristics

Of the 33 patients hospitalized, 25 fulfilled the diagnostic criteria for ACHF. The other 8 subjects were considered as control group. Baseline characteristics of the study population are given in Table I. Of these patients (mean age 62±5 years) 12 patients were males, 13 female, 14 patients with III NYHA functional class and 11 patients with IV NYHA functional class. Means+SD of laboratory data (hepatic and renal functions, hemocytometer examination) for control group and patients are summarized in Table II. A significant variation was observed in the two groups in the following parameters: AST, ALT, HGB L, red cells, white cells, haematocrit and LVEF.

iNOS and HO-1 mRNA expression

The results (fig. 1) show that in peripheral blood mononuclear cell (PBMC) from HF patients both transcripts analyzed, iNOS and HO-1, were significantly higher than controls. Indeed, it has become increasingly clear that impaired regulation of the systems synthesizing these two enzymes upon induction by pathologic stress, namely inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1), constitutes one of the pathogenic mechanisms in cardiovascular disease. With growing interest and advancing research endeavors, many excellent reviews on iNOS/NO and HO-1/CO have become available in recent years. Yet, because these two fields have evolved independently of each other, studies generally have investigated the role of one pathway or the other in the control of biological activities, with little emphasis on the possible interactions between these two closely related systems.

Expression of proteins NFkB, iNOS and HO-1

Western-blot analysis was performed to analyze the expression in PBMC of the nuclear transcription factor NFkB, and proteins iNOS and HO-1. In addition to the control of the immune response, NF-kB regulates the expression of proteins involved in the pathogenesis of the inflammatory process such as cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), a multitude of proinflammatory cytokines and proteins responsible for adhesion and migration of monocytes/ macrophages through the vessel wall such as ICAM-1, VCAM-1 and E-selectin (10, 27). In addition, cytokines that are stimulated by NF-kB, such as tumor necrosis factor α (TNFα) and IL-1β, are also strong inducers of NF-kB, establishing a positive adjustment mechanism that can amplify the inflammatory response and establish a chronic inflammatory process. Because of its role in inflammation, proliferation and cell survival, NF-kB is considered a therapeutic target in various pathological conditions, particularly

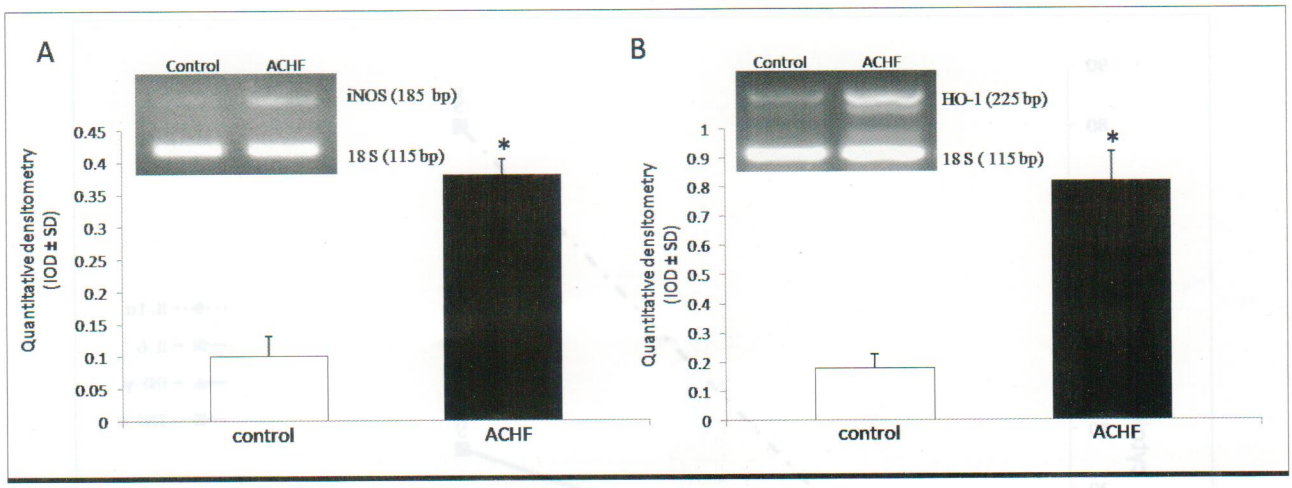

Fig. 1. *Representative image of RT-PCR experiments. mRNA level of iNOS (**A**) and HO-1 (**B**) in PBMC extracted from healthy controls (n=8) and from patients with ACHF (n=25). Both the representative images and histograms show that in heart failure patients there is a strong up-regulation of iNOS and HO-1 transcript. The data represent relative density normalized to 18S. Values are given as mean±SD (*p<0.05 respect to control).*

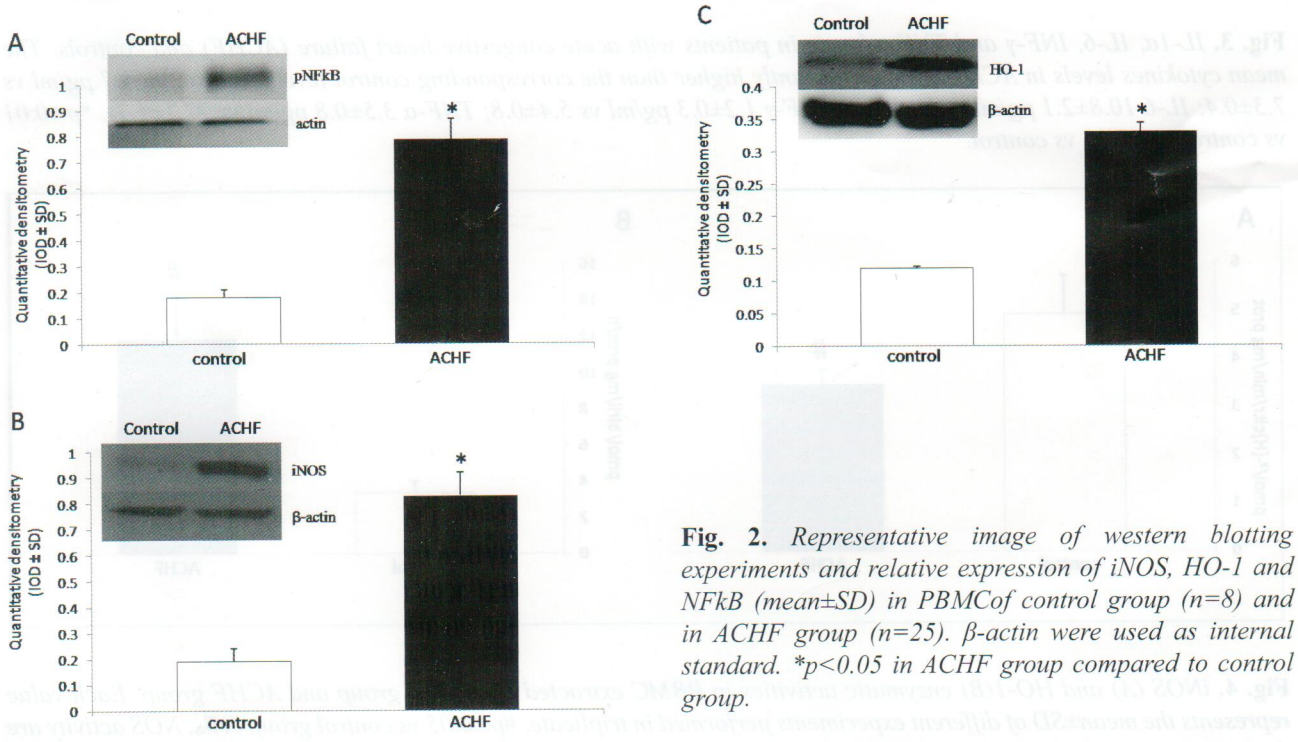

Fig. 2. *Representative image of western blotting experiments and relative expression of iNOS, HO-1 and NFkB (mean±SD) in PBMCof control group (n=8) and in ACHF group (n=25). β-actin were used as internal standard. *p<0.05 in ACHF group compared to control group.*

in degenerative diseases of the circulatory system (10). As shown in fig. 2, an increased expression of both iNOS (fig. 2B) and HO-1 (fig. 2C) is evident in patients compared to controls. The role of iNOS in heart failure pathogenesis is contentious, and biological activity of NO generated by iNOS is altered during human heart failure (14). The functional consequences of altered NOS expression and NO bioactivity in the failing human heart are only just beginning to be explored. Interestingly, HO-1 is known to promote angiogenesis (28). Although HO-1 has been acknowledged as a cardioprotective

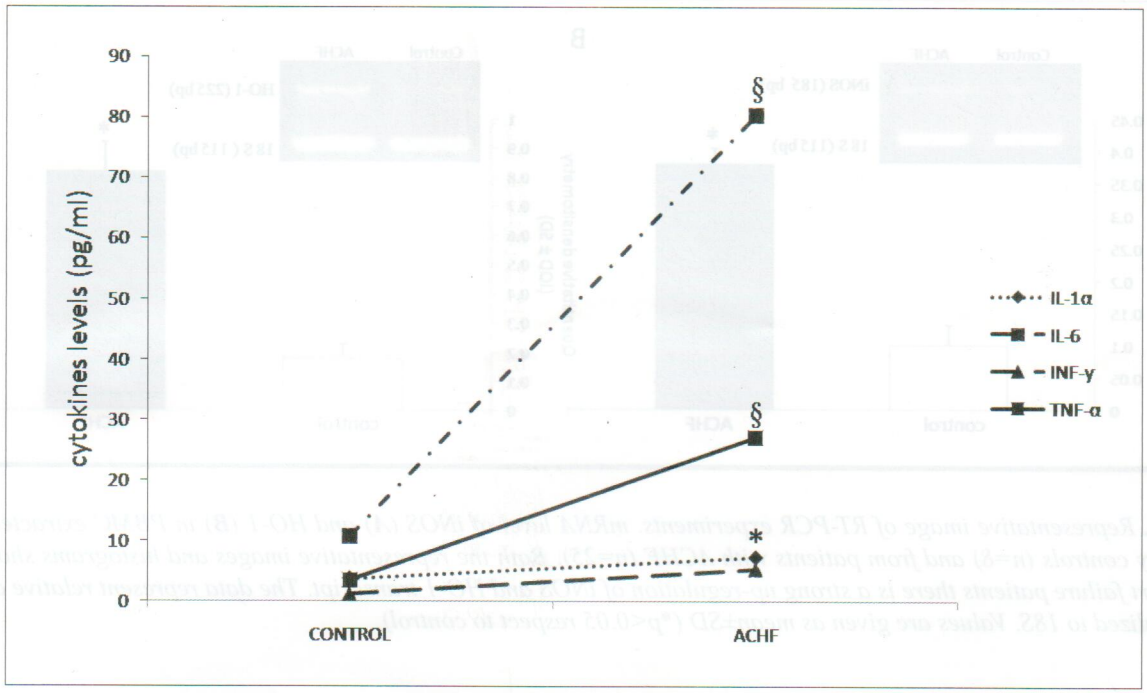

Fig. 3. *IL-1α, IL-6, INF-γ and TNF-α levels in patients with acute congestive heart failure (ACHF) and controls. The mean cytokines levels in ACHF were significantly higher than the corresponding control levels (IL-1α 3.8±1.7 pg/ml vs 7.3±0.4; IL-6 10.8±2.1 pg/ml vs 80.3±5.3; INF-γ 1.2±0.3 pg/ml vs 5.4±0.8; TNF-α 3.5±0.8 pg/ml vs 27.1±1.3). *p<0.01 vs control, §p<0.05 vs control.*

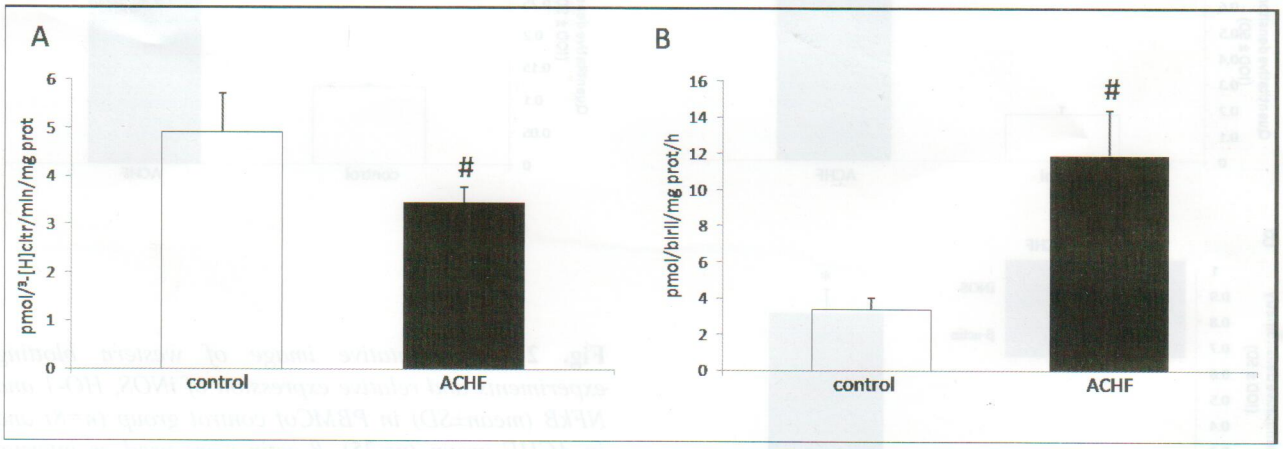

Fig. 4. *iNOS (A) and HO-1(B) enzymatic activities in PBMC extracted by control group and ACHF group. Each value represents the mean±SD of different experiments performed in triplicate. #p<0.05 vs control group cells. NOS activity are expressed in pmol/β-(H)citr/min/mg prot and HO-1 activity are reported in pmol/biril/mg prot/min.*

protein in various cardiovascular disease models, the cardio-protective mechanism of HO-1 is still incompletely understood (17). In addition, the role of HO-1, especially in cardiac regeneration, is not known.

Cytokines Elisa

Just recently it has been highlighted that heart

Table I. *Demographic details of the population studied.*

Patient characteristics	ACHF	Control
Age (mean±SD)	62±5	59±3
Sex (M/F)	12/13	5/3
BMI (Kg/m^2)	28.71±2.39	29.58±1.38
NYHA functional class III	14	-
NYHA functional class IV	11	-

Table II. *Laboratory data.*

	Control	ACHF	p-value
Creatinine (mg/dL)	1.23±0.05	1.33±0.08	ns
Urea (mg/dL)	43±2	45±4	ns
AST	21±1	25±2	p<0.05
ALT	19±1	26±1.5	p<0.01
HGB L (g/dL)	12.6±1.01	13±0.3	p<0.05
Red cells ($10^6/\mu l$)	4.6±0.2	4.1±0.2	p<0.05
White cells	7.3±0.4	8.5±0.5	p<0.01
Haematocrit	41.08±2.59	33.91±1.51	p<0.01
LVEF (%)	57.3±2.6	33.4±1.7	p<0.01

AST=Aspartate aminotransferase; ALT=Alanine aminotransferase; HGB=Hemoglobin; LVEF=Left Ventricular Ejection Fraction.

failure is no longer considered a disease only due to a dysfunction of cardiac activity, but rather a clinical syndrome characterized by an altered multiorgan function and homeostatic human systems, including the neuro-hormonal, renal-excretory system, liver function, musculoskeletal system, and also the immune-competent system (5, 6). The link between heart failure and inflammation has been highlighted in the 1990 study by Levine and colleagues, who first reported high levels of TNF in patients with heart failure (28). Our results support the bibliographic data, which show that IL-1α, IL-6, IFN-g and TNF-α are elevated in PBMC of patients with ACHF compared to controls (fig. 3). This increase may cause a transient depression of the contractile function of the heart muscle through the induction of iNOS expression as shown in fig. 2B and a decrease of Ca^{2+} release from the sarcoplasmic reticulum, acting on the ryanodine receptor.

Activity of iNOS and HO-1 proteins

Citrulline synthesis in PBMC extracts from both controls and from ACHF patients is respectively of 4.91±1.2 pmol/min/mg and 3.46±0.74 pmol/min/mg (Fig. 4). The enzyme activity presents an opposite trend compared to that obtained in the analysis of the transcript and proteins. Several experimental studies have shown that low (submicromolar) doses of NO exert small positive inotropic effects, which may serve to enhance basal cardiac function (9). Different research groups have provided evidence of the ability of CO to relax vascular tone in the heart similar to that of NO (16, 30). This discovery is of significance because the HO-1/CO system is believed to constitute a novel cardiac defense mechanism protecting cells and tissues when they are exposed to different stress stimuli (19, 31). HO-1 and the subsequent metabolites of heme catabolism appear to play vital roles in regulating important biological

responses, including inflammation, oxidative stress cell survival and proliferation (15, 17). Depending of the cell type and the nature of stimuli, HO-1 induction may be mediated by different signaling pathways. Since we found a reduced activity of iNOS, we also evaluated whether the inducible isoform of HO-1 presented the same trend.

The increase in HO-1 increases the catabolism of heme which represents an important substrate for the activation of some heme proteins such as iNOS. Fig. 4 shows increased synthesis of bilirubin, 11.92±2.46 pmol/mg protein/h in PBMC of ACHF patients compared to 3.39±0.61 pmol/mg protein/h in the PBMC of controls. The enzyme activity is therefore related to the expression of mRNA and protein of HO-1. It demonstrates that HO-1 serves as a potent scavenging agent against oxidative stress. The iNOS, to catalyze the conversion of L-arginine into L-citrulline and nitric oxide, requires the heme cofactor. We hypothesize that the increase in HO-1 metabolism and the consequent reduction of heme availability does not cause an activation of iNOS. In fact, the elimination of free-heme limit the assembly of new and functional proteins iNOS active, since its activity requires the incorporation of two molecules of heme.

In addition to the impoverishment of free-heme, the decrease in the enzymatic activity of iNOS, may be explained by increased CO which in turn binds to its heme moiety inactivating the enzyme.

The inactivation of iNOS could represent a compensatory mechanism natural system of HO-1 in response to heart failure (32, 33).

The endpoints of this feedback could be: decreased conversion of NO to reduce oxidative stress and increased production of CO to perform signaling functions equivalent to those NO.

Since nitric oxide synthesized by iNOS has a high affinity for the superoxide radical, the reduction of its activity would avoid the formation of nitrogen radicals which are very harmful and similar to peroxynitrites.

DISCUSSION

Cardiovascular diseases (atherosclerosis, stroke, ischemia, diabetes and myocardial infarction) are the leading causes of death in Western countries. Most of the diseases that affect the vascular system are caused by endothelial cell dysfunction that is accompanied by an altered expression of adhesion molecules, the rise in the production of chemotactic cytokines, pro-inflammatory cytokines, growth factors, metalloproteases, as well as by an altered proliferation and/or cell death (5, 6).

Currently, the understanding of molecular and cellular events underlying the alterations of the vascular wall has led us to consider these changes as a result of a chronic inflammatory condition that involves the interaction between soluble factors, monocytes, endothelial cells (EC) and smooth muscle cells (VSMCs) (34). Although the exact mechanism of systemic inflammation is unknown, a growing body of data shows that inflammation plays a role in both the development and the progression of HF, and influences not only myocardial function but that of other organs, thus actively participating in the full manifestation of the complex syndrome of HF (1). HO-1and iNOS are involved in the control of several important cellular functions, including cell growth, inflammation and apoptosis (35, 36). HO-1 derived CO activates the sGC/cGMP pathway, which in turn leads to smooth muscle relaxation, inhibition of smooth muscle proliferation and inhibition of platelet aggregation (37). The specific induction of the HO-1 pathway in certain disease states may represent a fine expedient to protect against injury and to restore cellular homeostasis.

Among the inflammatory mediators, reactive oxygen species, nitric oxide (NO) in particular, initiates a wide range of toxic oxidative reactions causing tissue injury (38).

Our studies demonstrate a negative feedback interaction between iNOS and HO-1 important in the physiopathology of heart failure and could be considered a good candidate as a future therapeutic target for the development of new drugs.

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