[Frontiers in Bioscience 14, 2230-2237, January 1, 2009]

Role of oxidative and nitrosative stress biomarkers in chronic heart failure

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1. ABSTRACT

In this review, we present recent insights on chronic heart failure (CHF) and the potential role of tumor necrosis factor (TNF)-alpha, interleukins, myeloperoxidase (MPO), and nitrosative stress in the progression of this disease process. Reactive oxygen species (ROS) are produced as a consequence of aerobic metabolism. Under physiologic conditions, their unfavourable effect in causing oxidative damage is counteracted by antioxidants. An imbalance in favour of oxidants leads to oxidative stress, and contributes to myocyte apoptosis, direct negative inotropic effects, and reduced bioavailability of nitric oxide (NO). Together, these effects lead to impaired vasodilatation of the coronary, pulmonary and peripheral vascular beds. In patients with moderate to severe forms of CHF, TNF-alpha leads to the formation of nitrotyrosine and consumption of nitric oxide by virtue of activation of myeloperoxidase. Further studies are required to better elucidate the complex interaction of oxidative stress, endothelial dysfunction and inflammatory activation in CHF. Such insights would likely lead to development of better strategies for the assessment of the disease severity by monitoring of new bio-humoral indices and better treatment approaches.

2. NO/REDOX IMBALANCE, ENDOTHELIAL DYSFUNCTION AND MPO ACTIVITY

The role of oxygen and oxygen-dependent processes in the physiology of human heart is complex. Oxygen is a major determinant of gene expression in heart muscle. Oxygen is involved in the generation of NO, a signalling molecule, fundamental to the establishment of vessel tone and cardiac contractility. Oxygen also plays a central role in the complex series of reactions which lead to the generation of reactive oxygen species (ROS, Reactive Oxygen Species). ROS is formed in the heart and other tissues in response to stimulation by cytokines and growth factors. Angiotensin II, PDGF and TNF-alpha, for example, can induce, in vascular smooth muscle cells and other cell types including cardiomyocytes, H_2O_2 and $O_2^{\cdot-}$ via NAD(P)H oxidases (1). In the failing myocardium of patients with ischemic or dilated cardiomyopathy, NAD(P)H oxidase-derived ROS are up-regulated, and level of TNF-alpha and platelet-derived NAD(P)H oxidase activity in the plasma are increased (2). ROS induce irreversible cellular damage and death through oxidation of cellular constituents (such as proteins critical for excitation-contraction coupling), by diminishing bioactivity of NO, by oxidation of proteins (direct competition), or by influencing

NO binding (allosteric modulation). Moreover, interactions between H_2O_2 and peroxidases, especially myeloperoxidase, lead to formation of oxidizing and nitrosating species that are particularly atherogenic (3). Despite these negative effects, ROS do have important signalling properties within the vessel wall (3). H_2O_2, in particular, is a relatively stable and uncharged molecule that can easily diffuse between cells. Hydrogen peroxide is an endogenous hyperpolarizing factor in vessels, that inhibits phosphatases, activates guanylate cyclase, stimulates NO production and alters gene expression.

Oxidative stress (OxS) is defined as an imbalance that favours oxidant effects. Such an imbalance can lead to cell damage. Most biological systems are characterized by a complex system of reduction-oxidation reactions which should, under normal conditions, remain in equilibrium. Tipping this balance causes adaptive responses that may get overcompensated by OxS. Paradoxically, such an imbalance manifests, by an increased redox environment. Reductive stress is closely linked to oxidative stress: a typical example being the overproduction of reducing equivalents such as NAD(P)H, that can lead to increased redox cycling of substances, followed by repetitive rounds of oxidation/reduction, and an increased generation of superoxide anion radical ($O_2^{-\cdot}$) and secondary oxidants. This hypoxia-like metabolic imbalance has been implicated in generation of ROS.

The concept of *nitrosative stress* (NtS) originated from a growing appreciation of the interplay between ROS and Reactive Nitrogen Species (RNS). NtS can be described as an increase in S-nitrosated compounds combined with a concomitant decrease of intracellular thiols, which may be associated with numerous biological responses important to vascular pathophysiology.

2.1. Oxidative/Nitrosative stress: the role of radicals

Any species which contains unpaired electrons and is capable of independent existence is defined as a *free radical*. Free radicals differ in their reactivity based on their structure and the molecules they encounter. The reaction between two radicals leads to the formation of a fast covalent bond by the joining of their unpaired electrons, resulting in a non radical compound. An example relevant to the vessel wall is the very fast reaction of $O_2^{-\cdot}$ with $^{\cdot}NO$ which leads to the formation of peroxynitrite:

$$O_2^{-\cdot} + {}^{\cdot}NO = ONOO^- \text{ (peroxynitrite)}$$

The reaction of peroxynitrite with CO_2 as a preferential biological target leads to metastable species, that can promote nitration (addition of a nitro group $-NO_2$), nitrosation (addition of -NO, nitrogen monoxide group), and oxidation. Both in the presence or absence of CO_2, the redox pathway of $ONOO^-$ is, at least in part, mediated by nitrogen dioxide ($^{\cdot}NO_2$), a strong oxidizing radical that can initiate and promote lipid (including LDL) peroxidation and formation of nitrotyrosine. It should be noted that $^{\cdot}NO_2$ can also derive from other sources, such as by enzymatic reactions (4).

Non radical species differ from radical species by the lack of unpaired electrons. These species can oxidize other molecules, with different reactivity. H_2O_2 is probably the most abundant non radical species with a weak oxidant activity that can directly oxidize thiol (-SH) groups, and can react with some heme proteins. While radical oxidants commonly initiate lipid peroxidation, non radical oxidants like ONOOH (peroxynitrous acid) and HOCl (hypochlorous acid) react *preferentially* with proteins (cysteine and methionine residues, followed by tyrosine, tryptophan, phenylalanine and lysine residues). The schematic representation of reactions that lead to nitrosative stress are illustrated in Figure 1.

3. REDOX MECHANISMS IN BLOOD VESSELS: SOURCES OF ROS

The NADPH oxidases represent a major source of ROS in vascular cells. They catalyze single-electron reductions of O_2 using NAD(P)H as electron sources. The vascular enzymes, similar to the NAD(P)H oxidase in macrophages, contain Nox family subunits, such as Nox1, Nox4, $p22^{phox}$ and $gp91^{phox}$. The Nox proteins represent the catalytic subunits of these enzymes and vary in respect to their mode of activation and require a cofactor for activation. A variety of pathological stimuli activate NADPH oxidases in vascular smooth muscle and endothelial cells, e.g. stretch, endothelin-1, angiotensin II, thrombin, and catecholamines (3). There is a crosstalk between NAD(P)H oxidase and XO: NAD(P)H oxidase activity maintains endothelial XO levels and participates in the conversion of XDH to XO (5). Both XO and NAD(P)H oxidases are inhibited by NO, and provide a mechanism through which NOS activity regulates superoxide production and thereby maintains $O_2^{-\cdot}$/NO homeostasis.

3.1. Xanthine oxido-reductase

Xanthine oxido-reductase is a flavoprotein, present in high a concentration in endothelial cells of capillaries and sinusoids. XO exists in two forms: xanthine dehydrogenase (XDH, which is predominant species) and xanthine oxidase (XO). XDH uses NAD^+ to receive electrons from hypoxanthine and xanthine, yielding NADH and uric acid. XO can generate $O_2^{-\cdot}$ and H_2O_2 using oxygen as an electron acceptor from these same substrates. XDH can be converted into the oxidase form by non-reversible proteolytic attack or by reversible oxidation of thiol groups. The ratio of XO to XDH is critical in determining the amount of ROS produced by these enzymes. Conversion of XDH to XO may be stimulated by inflammatory cytokines (such as TNF-alpha), and by oxidation of critical cysteine residues by oxidants, such as peroxynitrite (6). The vascular activity of XO correlates inversely with endothelial function in patients with chronic heart failure or atherosclerosis (7).

3.2 NOS enzymes

NOS are a family of enzymes (iNOS, eNOS and nNOS) that catalyze the oxidation of L-arginine to L-citrulline and $^{\cdot}NO$, a potent vasodilator. $^{\cdot}NO$ may react with metal complexes, and oxygen (autoxidation in hydrophobic environments) to form $^{\cdot}NO_2$ and N_2O_3, or with $O_2^{-\cdot}$ (rapid reaction leading to $ONOO^-$) to form RNS.

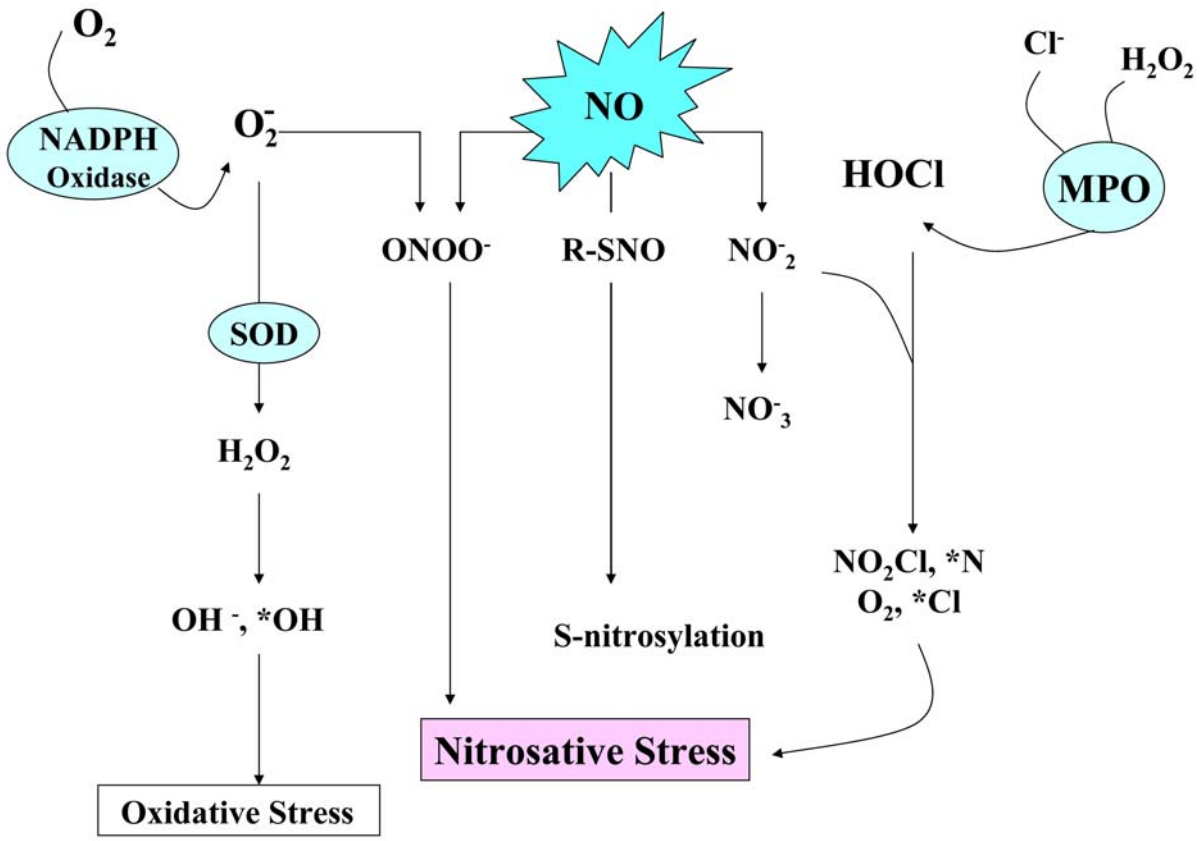

Figure 1. Overview of generation of reactive nitrogen species. XO system, mitochondrial respiration chain and NADPH oxidase are main sources of superoxide anion, that can be converted into hydrogen peroxide, spontaneously or under the influence of SODs. The interaction of inflammatory molecules with oxidative stress and excessive NO presence may induce the production of strong oxidizing RNS. Furthermore, nitration can be due to activation of myeloperoxidase by H2O2, with production of NO2-. HOCl, as a product of MPO-catalyzed Cl- oxidation, can also oxidize NO2- to nitrating intermediates. NOS: nitric oxide synthase, MPO: myeloperoxidase, SOD: superoxide dismutase, HOCl: hypochlorous acid.

eNOS and iNOS are the most relevant NOS isoforms in the vasculature. eNOS is derived from endothelial cells, certain brain cells and peripheral neurons. eNOS is calcium dependent, and can lead to the synthesis of small amounts of NO on demand through activation of a number of molecules such as acetylcholine, bradykinin, thrombin, ADP, and serotonin. Interestingly, eNOS can be up-regulated by physical exercise. Its molecular targets are heme proteins, soluble guanylate cyclase, and thiols. iNOS is functionally calcium independent, and is expressed in macrophages, neutrophils, mast cells, endothelial cells and vascular smooth muscle cells. iNOS can continuously lead to the synthesis of a large amount of NO. It must be noted that a fully induced macrophage can release an amount per minute that is thousands of times higher than that released from NOS in endothelial cells. This may explain the role of NO as a lethal molecule in activated immune cells.

Under specific circumstances, in the absence of cofactors such as tetrahydrobiopterin (BH4) or L-arginine, eNOS switches from a coupled state (that generates NO) to an uncoupled state (to generate $O_2^{-\cdot}$). The increase of $O_2^{-\cdot}$ production in the vasculature alters endothelium-dependent vascular relaxation through interaction with NO. Moreover, the resultant peroxynitrite oxidize BH4 which leads to a deficiency of BH4, with the pathogenic uncoupling of NOS (Figure 2).

3.3. Mitochondrial damage

Mitochondria provide energy to the cells through oxidative phosphorylation, a process by which electrons are transferred from NADH or FADH2 (generated through the Krebs cycle) to molecular oxygen, to form ATP. Between 1-4% of the oxygen reacting with the respiratory chain is incompletely reduced to O2-.. and then H2O2. This small amount of O2-. is scavenged by manganese SOD (MnSOD/SOD2). When mitochondrial oxidative phosphorylation occurs under pathologic conditions, the electron transport chain may become uncoupled, leading to increased O2-. production. Mitochondrial enzyme activity can be altered by a number of different stimuli including hyperglycemia, cyclic strain, leptin, or cigarette smoking. Excessive exposure of endothelial or vascular smooth muscle cells to exogenous peroxynirite or H2O2 leads to the damage of mitochondrial DNA.

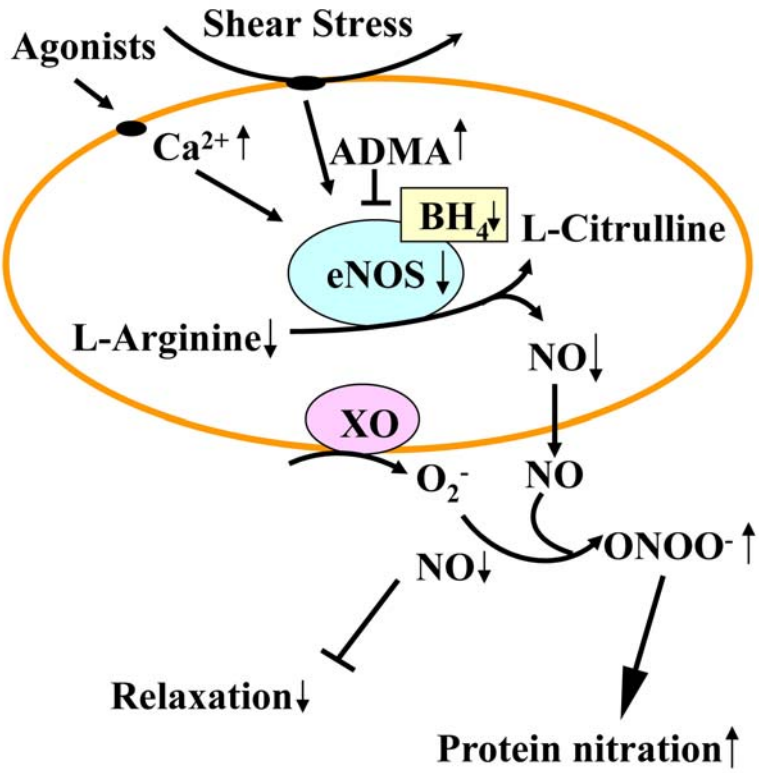

Figure 2. Schematic representation of NO generation by conversion of L-Arginine to L-citrullin in endothelial cells, mediated by eNOS in the presence of BH4 (tetrahydrobiopterin). In patients with CHF, NO production is low due to low bioavailability of L-arginine and blunted activity of eNOS. NO reacts with oxygen radicals and leads to the formation of ONOO- (peroxynitrite).

3.4. Hemoglobin (SNO synthase and heme-oxidase)

Hemoglobin is the largest reservoir of O_2 and NO in the human. O_2 is carried by hemes, and NO by hemes and cysteine thiols (S-nitrosohemoglobin, SNO-Hb). The O_2/NO-binding functions of hemoglobin (Hb) are mostly governed by an equilibrium between deoxy (T) and oxy (R) structures. The allosteric change from R to T structure lowers the affinity of hemes for O_2 and promotes the transfer of NO groups from SNO-Hb to acceptor thiols (8). Red blood cells, thereby, provide a NO mediated vasodilator activity that increases blood flow (and hence O_2 delivery). Rebinding of O_2 to Hb in the lungs regenerates SNO-Hb by promoting intramolecular NO transfer from heme to thiol. Hemoglobin also possesses an intrinsic heme-oxidase activity, which leads to production of superoxide. The release of superoxide by Hb, with heme oxidation, is favoured in the T structure. Thus, sustained or excessive desaturation of hemoglobin, characteristic of chronic heart failure (CHF), would increase ROS production. Nonetheless, when O_2 saturation is low, NO may migrate from the beta chains (which dispense NO bioactivity) to the alpha chains, which are impaired in their ability to support NO production. It was recently reported that CHF is characterized by accumulation of heme-NO (compared to controls) and venous desaturation (9). NO/redox disequilibrium *in situ* may impair red blood cell-related vasodilation and tissue ischemia.

4. ENDOTHELIAL DYSFUNCTION AND CHF

The endothelial lining is strategically located to separate the vascular wall from the circulating blood and blood components. Endothelial cells regulate blood vessel tone by the release of nitric oxide (NO) in response to stimulation by agonists such as acetylcholine or bradykinin or by mechanical stimuli such as changes in blood flow velocity or endothelial shear stress. This interaction leads to the relaxation of the vascular smooth muscle. eNOS is constitutively expressed in endothelial cells, endocardial cells and possibly cardiomyocytes. nNOS is present at nerve endings. iNOS is expressed in response to inflammation, particularly in end stage heart failure, and generates large amounts of NO independently of agonist stimulation or calcium level (10).

Some systematic studies in animals with pacing-induced cardiomyopathy indicate that heart failure is associated with normal or even increased NO production in the early phase of the disease, while a reduction of NO occurs only during cardiac decompensation (11). Moreover, during cardiac decompensation there is a reduced production of nitrate and nitrite across the heart. As a result of these changes, there is an increase in myocardial oxygen consumption, with a shift in substrate use from fatty acids to glucose (12).

As endothelial dysfunction is an early event in the clinical spectrum of CHF, altered endothelial signalling might contribute to the progression of left ventricular dysfunction by causing increased afterload and central effects such as myocardial ischemia (13). If the reduction in eNOS enzymes contributes to the development of cardiac decompensation, then strategies designed to restore eNOS activity and NO production should be effective in the treatment of CHF.

ACE inhibitors can increase local bradykinin levels or prolong the half life of bradykinin by blocking kinin metabolism; the blockade of the breakdown of kinins leads to stimulation of NO production through the interaction between the B2 kinin receptor and eNOS. Also statins can contribute to maintain NO production by phosphorylation of eNOS, a process that has been demonstrated to increase eNOS activity (10). The supplementation with L-arginine (the substrate of eNOS for the production of NO) can also reverse endothelial dysfunction; numerous factors may account for this improvement, including a direct process of scavenging of free radicals, that are increased in CHF (10). Free radicals may play a key role in the pathogenesis and progression of the disease. ROS may account for impaired endothelium-dependent vasodilation in response to agonists or flow, by scavenging endothelium-released NO. The balance between radical producing and scavenging mechanisms (that are reduced in CHF) is crucial to the maintenance of normal endothelial function.

5. BIOMARKERS OF OXIDATIVE STRESS AND INFLAMMATION

5.1. Myeloperoxidase

Myeloperoxidase (MPO), which is indepensable to innate host defense, is the most abundant component of azurophilic granules of leukocytes, secreted upon leukocyte activation. MPO is found predominantly in neutrophils, monocytes and some subtypes of tissue macrophages. MPO is a member of the heme peroxidase superfamily, which amplifies the oxidative potential of its co-substrate hydrogen peroxide, forming potent oxidants capable of chlorinating and nitrating phenolic compounds (14). The H_2O_2 substrate is derived from a number of sources *in vivo*, including leukocyte NA(P)H oxidases, XO, uncoupled NOS and various Nox isoenzymes. MPO has the peculiar ability to generate hypochlorous acid (HOCl), that reacts with electron-rich moieties in a large number of biomolecules (14):

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_20$$

MPO, which is the only human enzyme known to generate HOCl, and chlorinated molecules, is considered a specific marker of oxidation reactions. The myeloperoxidase/H_2O_2/Cl^- system which leads to the formation of HOCl also oxidizes nitrite to the non-radical oxidant nitryl chloride (NO_2Cl) and the radical $^{\cdot}NO_2$, both of which promote nitration and convert tyrosine into 3-nitrotyrosine. The formation of 3-nitrotyrosine is strictly dependent on the availability of $^{\cdot}NO_2$.

MPO seems to play a direct role in the development of endothelial dysfunction through limitation of bioavailability of NO. MPO-generated oxidants inhibit the activity of NOS. Chlorination of arginine following reaction with HOCl reduces availability of its substrate and directly inhibits enzymatic activity of NOS, and reduces the formation of NO metabolites by endothelial cells *in vitro*. RNS generated by MPO can also uncouple NOS. The final effect of reduction of NO-dependent relaxation in arterial conduits likely derives from combination of both direct catalytic consumption, radical-radical scavenging effects and modulation of NOS activity through oxidation of its substrates and cofactors (14).

MPO binds to endothelial cells and is subsequently transcytosed to the subendothelial space, where it lies together with nitrotyrosine. This suggests that MPO is anatomically positioned between endothelial cells and their smooth muscle cells target to intercept NO and to limit its bioavailability.

Levels of MPO correlate with the abnormal left ventricular remodelling (15). However, there are only limited data regarding the distribution of MPO in patients with CHF. Elevated level of MPO has been reported in a small population of (n=102) patients with CHF as a marker for the assessment of severity of the disease without a reference as being an active contributor for the progression of disease.

5.2. Interleukins

Cytokines have detrimental effects on the cardiovascular system and many peripheral organs. These mediators can effectively contribute to the progression of left ventricular dysfunction and to the development of a chronic heart failure syndrome (so-called *cytokine hypothesis*). There are common pathways that lead to the secretion of the acute phase proteins. Among these are complement apoproteins, fibrinogen, cytokines (particularly IL-6 and TNF alpha), opsonins and pentraxins (like C-reactive protein). Such an inflammatory cascade, is highly energy-dependent and increase hepatic protein synthesis at the expense of peripheral muscles. In the long term, this mechanism can be detrimental due to progressive tissue catabolism and wasting, leading to cachexia typical of the advanced phase of CHF. TNF alpha acts on hypothalamic nuclei and causes anorexia; by virtue of modulating leptin, corticotrophin-relaxing hormone and serotonin, and produces a number of changes in lipid metabolism (i.e. increased levels of FFAs, triglycerides and VLDL released from periphery, and decreased activity of lipoprotein lipase), and gastrointestinal functions. TNF-alpha is directly pro-apoptotic, and can induce left ventricular dysfunction with subsequent CHF progression in healthy animals (116-7). TNF-alpha is consistently elevated in advanced CHF and is an independent marker of an adverse prognosis (17). In advanced CHF, there is a chronic stimulation of TNF alpha by various mechanisms, that includes local expression of this cytokine due to mechanical overload and to pathologic absorption of endotoxins and bacterial translocation at the bowel wall

level, due to mesenteric venous congestion (18). CRP is a pentraxin that tends to increase with age in healthy subjects, presumably reflecting the increasing incidence of subclinical pathologies (19). In most diseases, CRP reflects an ongoing inflammatory and/or tissue damage far more accurately than do other laboratory parameters of the acute-phase response, such as plasma viscosity or the erythrocyte sedimentation rate. The acute-phase CRP values show no diurnal variation, are unaffected by eating, and are only impaired by liver failure. CRP concentration although being useful, is only a non specific biochemical marker of inflammation. Assessment of serum level of CRP contributes to the screening of organic disease, monitoring of the response to treatment of inflammation and infection, and to the detection of intercurrent infections in immunocompromised patients (19).

In recent years a complex cytokine system has been identified, which is comprised of an increasing number of components with about 25 functionally-related chemokines. All these mediators are pleiotropic (the same molecule has multiple and sometimes even opposite effects) and somewhat redundant (the same action can be played by different molecules). Chemokines are divided into pro-inflammatory and anti-inflammatory groups. Although, the pro-inflammatory chemokines are activated in the course of CHF syndrome, the level of anti-inflammatory chemokines does not significantly get altered in the different phases of the disease (17).

6. BNP AND NT-pro-BNP

Determination of the level of B-type natriuretic peptide (BNP) and its precursor N-terminal pro-B-type natriuretic peptide (pro-BNP) can be used with a great sensitivity in the assessment of left ventricular dysfunction. However, specificity and positive predictive values of such assays are relatively low in community screening. Other clinical applications include diagnosis of acute heart failure, risk stratification in both acute and established HF and in acute coronary syndromes, and in prediction of cardiovascular risk in the general population (20). In a recent work (21), a multimarker approach was used in a community screening for the diagnosis of heart failure. CRP and MPO, together with pro-BNP have incremental value in the detection of left ventricular dysfunction with a specificity and positive predictive value, which are higher than those determined by the use of a single marker.

7. NITROSATIVE STRESS AND ITS RELATIONSHIP WITH SYSTEMIC INFLAMMATION IN PATIENTS WITH CHF

In patients with CHF, increased oxidative and nitrosative stress and production of ROS have been linked to endothelial dysfunction and deterioration of heart function (22-24). ROS which are produced by a variety of enzymes (25-29), induce the expression of inflammatory cytokines (30). Conversely, cytokines, particularly TNF-alpha, promote the production of ROS (30-32). These findings underline the presence of a cross-talk between inflammatory response and mechanisms that lead to an increased oxidative and nitrosative stress (30-33).

We recently evaluated systemic levels of oxidative and nitrosative stress biomarkers, systemic inflammation and pro-Brain Natriuretic Peptide (pBNP) in plasma and sera of 80 patients with CHF. The severity of the disease was based on the guideline of New York Heart Association (NYHA) ranging from class I to III. Among the patients, 9 had NYHA class I, 34 had class II and 23 had class III disease. The control group included 14 healthy volunteers, matched for age and sex, without any documented signs or symptoms of heart failure, or history of left ventricular dysfunction. The presence of circulating NT correlated significantly with the circulating markers of systemic inflammation TNF-alpha ($r=0.32$, $p<0.01$), CRP ($r=0.47$, $p<0.0002$), MPO ($r=0.37$, $p<0.003$), Also nitrite (NO_2^-) levels correlated with the markers of systemic inflammation TNF-alpha ($r=0.30$, $p<0.01$), CRP ($r=0.45$, $p<0.0004$), MPO ($r=0.34$, $p<0.006$). Total NO correlated significantly with TNF-alpha ($r=0.46$, $p<0.0002$), CRP ($r=0.52$, $p<0.0001$), MPO ($r=0.28$, $p<0.02$), NT ($r=0.25$, $p<0.04$), and IL-6 ($r=0.47$, $p<0.0002$). MPO, potentially involved in the NO consumption, correlated significantly with TNF-alpha ($r=0.43$, $p<0.0006$), CRP ($r=0.49$, $p<0.0001$), and IL-6 ($r=0.52$, $p<0.0001$). A sub-analysis of NT and MPO levels in NYHA III clearly showed a better correlation in this subgroup of patients with more advanced functional impairment ($r=0.59$, $p<0.005$, NYHA III) compared to less compromised patients ($r=0.37$, $p<0.003$ NYHA I and NYHA II). These preliminary data show increased serum and plasma levels of nitrotyrosine, nitric oxide, nitrite and myeloperoxidase in patients with moderate to severe chronic heart failure. In addition to these changes, in patients with severe form of CHF, there is also an increased level of TNF-alpha and proBNP. Although "R" values are low, the level of nitrotyrosine and total NO correlate with the levels of TNF-alpha, CRP, MPO and proBNP. Together, these findings show a correlation between the severity of the CHF and level of nitrosative stress.

There is close relationship between the serum/plasma level of TNF-alpha, and those of NT and MPO in patients who suffer from CHF of increasing severity (34-37). Such a relationship signifies of possible stimulatory action of TNF alpha, via MPO activation, in inducing consumption of NO and formation of NT in patients who have moderate to severe form of CHF. Other components of systemic inflammation such as IL-1, IL-6 and their receptors, are also increased in patients with CHF patients. The findings reported here were initially presented at the Annual Congress of the European Society of Cardiology (38).

8. ACKNOWLEDGEMENTS

This work was supported by Fondazione S. Maugeri, IRCCS, Ricerca corrente.

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Abbreviations: MPO: myeloperoxidase; NO: nitric oxide; ROS: reactive oxygen species; XO: xanthine oxidase; XDH: xanthine dehydrogenase; SOD: superoxide dismutase; NOS: nitric oxide synthase; NYHA: new york heart association; TNF: tumor necrosis factor; BNP: brain natriuretic peptide; CHF: chronic heart failure; NT: nitrotyrosine; IHD: ischemic CHF; ICM: idiopathic CHF; LVEF: left ventricular ejection fraction; HOCl: hypochlorus acid

Key Words: Nitrosative stress, Oxidative Stress, Endothelial Dysfunction, Review

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