Hydroxyl Radical Generation, Levels of Tumor Necrosis Factor-Alpha, and Progression to Heart Failure After Acute Myocardial Infarction

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OBJECTIVES We used acetylsalicylic acid (ASA) as a probing agent to quantify hydroxyl radical (OH) in Controls and patients with coronary artery disease and to prospectively investigate OH production in patients with myocardial infarction (MI) complicated by heart failure (HF).

BACKGROUND Oxidative stress status (OSS) is a mechanism for transition to HF in experimental heart injury models, but evidence for its causal role in humans is still limited.

METHODS Thirty healthy subjects (Controls), 12 patients with stable angina (Group 1), and 74 patients with ST-segment elevation MI (Group 2) were enrolled. A dose of 250 mg Flectadol was given intravenously before each blood collection to determine the 2,3-dihydroxybenzoic acid/salicylic acid (DHBA/SA) ratio. We also quantified vitamin E and coenzyme Q10 to monitor antioxidant reserve, as well as tumor necrosis factor (TNF)-alpha, TNF-soluble receptors, interleukin (IL)-6, and IL-1ra to assess inflammatory status. All measurements were repeated at month 6 in Group 2.

RESULTS There were no differences between Controls and Group 1. Group 2 showed increased OH production, peaking at 24 h, whereas vitamin E and coenzyme Q10 progressively declined. Group 2 patients developing HF during hospitalization (Group 2Bii) presented with an increase of both OH production at discharge and inflammatory status, as compared with patients without HF (Group 2Ai), persisting at month 6 in post-MI patients with HF (Group 2Bii).

CONCLUSIONS We found a distinct pattern of OH generation in post-MI patients who show progression to HF. The interplay between OSS and inflammatory status should be targeted as a possible mechanism of progression to post-MI left ventricular dysfunction.

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An imbalance of oxidative stress status (OSS) has been reported to occur in acute coronary syndromes and heart failure (HF) (1). Elevation of reactive oxygen species (ROS) during myocardial ischemia occurs both at an early stage—during reperfusion (2), contributing to stunning (3,4) and reperfusion injury (5,6)—and at a late phase—days or weeks after myocardial infarction (MI) (7). Experimental studies have shown that ROS production at this later stage is convincingly associated with post-MI left ventricular (LV) remodeling and progression to HF (8–11).

An increase of lipid peroxidation markers and encroachment on antioxidant reserves in chronic HF have been widely reported in humans (12–14), but no prospective data are currently available on the association between (increased) OSS and progression to HF in post-MI patients (15).

A radical probing technique for in vivo OSS measurement, based on the ability of hydroxyl radical (OH) to attack the benzene ring of aromatic molecules, such as acetylsalicylic acid (ASA), and to produce hydroxylated compounds, such as 2,3-dihydroxybenzoic acid (DHBA), has been previously employed in humans (Fig. 1) (16,17). This method seems particularly interesting in patients with coronary artery disease, in whom ASA is indicated for primary and secondary prevention of acute cardiovascular events (18).

We aimed to prospectively investigate the pattern of OH elevation in MI patients with and without transition to HF by using a recommended intravenous dose of ASA (19) as a probe for OH production in vivo. Healthy subjects and stable patients with coronary artery disease were similarly investigated.

Endogenous antioxidant molecules—vitamin E and coenzyme Q10—and inflammatory status—tumor necrosis factor (TNF)-alpha, related soluble receptors of tumor necrosis factor-alpha (sTNFR), interleukin (IL)-6, and IL-1ra—were also monitored.

METHODS

Study population. Thirty healthy subjects and 86 patients were enrolled. Their clinical and biochemical profiles are presented in Table 1. The local Ethics Committee on Human Research approved the study protocol, and all participants gave their written, informed consent to participate in the study.

Three groups were considered: Controls comprised 30...
healthy subjects who were matched in terms of gender and age (19 men; mean age 65 ± 6 years) to the patients. None had clinical signs of acute or chronic illness or was receiving any treatment. Group 1 comprised 12 patients (8 men; mean age 68 ± 11 years) with angiographically documented coronary atherosclerotic lesions and stable angina (Canadian Cardiovascular Society classes II and III). They were treated with beta-blockers (n = 11), statins (n = 12), angiotensin-converting enzyme (ACE) inhibitors (n = 9), and calcium channel blockers (n = 5). Group 2 comprised 74 patients (49 men; mean age 67 ± 10 years) consecutively admitted for acute MI, defined as the occurrence of typical chest pain at rest lasting >20 min, accompanied by persistent ST-segment elevation of ≥1 mm in at least two standard electrocardiographic leads or ≥2 mm in at least two contiguous precordial leads. In all Group 2 patients, MI was confirmed by a rise in serum creatine kinase by twofold or more than the upper normal limit during hospitalization.

Exclusion criteria were symptoms lasting >12 h before hospitalization, history of HF before hospitalization, presence of any known neoplastic disease, diseases affecting the immune system, and ongoing infectious diseases. The infarct location was anterior in 36 patients. Fifty-five patients (74%) received accelerated tissue plasminogen activator. All Group 2 patients were receiving a standard regimen of beta-blocker, statin, and ACE inhibitor.

The occurrence of HF was defined as the presence of rest or effort dyspnea and at least one of the following: pulmonary rales at lung auscultation, evidence of pulmonary congestion on the chest X-ray, new appearance of peripheral edema, and use of diuretics.

**Blood processing. PLASMA.** Antecubital venous blood was collected in K3 EDTA-containing tubes, immediately centrifuged at 1,700 g for 15 min, and subsequently stored at −80°C. Plasma vials that would be used to measure 2,3-DHBA/SA were also frozen in liquid nitrogen before storage.

**SEBEMA.** Antecubital venous blood was collected in empty tubes and, after 45 min, centrifuged at 1,700 g for 4°C for 15 min. The serum obtained was stored at −80°C.

**OH probing in vivo.** The principle behind OH quantification is schematically shown in Figure 1 (16,17). A dose of 250 mg Flectadol was given intravenously 5 min before each blood collection. The time lag between Flectadol administration and blood collection was chosen after pilot observations in patients (n = 13) or Controls (n = 10), showing no significant changes in the 2,3-DHBA/SA ratio in the range of 3 to 20 min.

The kinetics of OH production was established in five Controls, all Group 1 patients, and 10 Group 2 patients by sampling at 6, 12, 24, 36, 48, and 72 h after symptom onset and then after 5 and 7 days and at discharge (9 ± 3 days). Based on the results obtained, the remaining 25 Controls and 64 Group 2 patients were studied at entry and at 24, 48, and 72 h after symptom onset and at discharge (10 ± 4 days).

**Extraction and quantification of 2,3-DHBA and 2,5-DHBA.** Aliquots of standard solutions or plasma samples (500 µl) were mixed with 20 µl of 5 µmol/l 3,4-DHBA (internal standard) and acidified with 25 µl of concentrated HCl (37%) in glass tubes. The samples were vortexed for 1 min and centrifuged at 2,000 g for 15 min. Ether (9%ml) was added to the supernatant. The samples were vortexed for 1 min and centrifuged at 2,000 g for 15 min. The ether phase was extracted. The extraction was repeated (with 3 ml of ether), and the ether phases were recollected. The ether phase was then dried under nitrogen steam. The dry residue was reconstituted in 500 µl of mobile phase and filtered on 0.22-µm filters (Millipore USA), and 100 µl was injected into the column (X Terra RP18—3.5 µm, 4.6 × 150-mm cartridge columns; Waters, Milford, Massachusetts). Chro-

**Figure 1. Oxidative metabolism of salicylic acid: acetylsalicylic acid (ASA) is rapidly hydrolyzed to SA by esterases and for 60% remains unmodified and can undergo hydroxyl radical (OH) attack to produce two derivate—namely, 2,3-dihydroxybenzoic acid (DHBA) and 2,5-DHBA. Enzymatic pathways through the cytochrome P-450 system can also produce the latter acid, whereas the former acid is solely formed by direct OH attack. Therefore, measurement of 2,3-DHBA or the 2,3-DHBA/SA ratio, after administration of ASA, is recognized as a sensitive and specific method to determine OH production in vivo.**
matographic separations were performed using a system consisting of a high-pressure pump (Waters Model 590, Multisolvent Delivery System) with an auto sampler (Waters Model 717 plus) and electrochemical detector (ESA Coulochem II 5200A with High Sensitivity Analytical Cell Model 5011) connected to an integrator (Millennium32, Chromatography Manager). The mobile phase consisted of 30 mmol/l each of sodium citrate and acetate at pH 3.0 (MeOH 20%) each. The flow rate was 1 ml/min, and the analytical cell potential applied is 250 mV (CH1). The detection was at 296 nm. The concentration of metabolites was determined by the ratio of respective metabolite peak areas to 2,6-DHBA (internal standard).

**Extraction and quantification of salicylic acid (SA).** Aliquots of standard solutions or plasma samples (100 μl) were mixed with 100 μl of 100 μmol/l of 2,6-DHBA (internal standard) and deproteinized by 200 μl of EtOH in 1.5-ml polypropylene conical Eppendorf micro test tubes. The samples were vortexed for 2 min and centrifuged at 1,600 g for 15 min. A total of 200 μl of supernatant was diluted with 200 μl of mobile phase. The diluted solution was then filtered on 0.22-μm filters (Millipore), and 50 μl of solution was injected into the column (NovaPak C18—60 Å, 4 μm, 3.9 × 150-mm cartridge columns made by Waters). Chromatographic separations were performed using a system consisting of a high-pressure pump (Waters Model 600E, Multisolvent Delivery System), a sample injection valve (Rhodyne Model 7725, Cotati, California) with a 50-μl sample loop, and a photodiode array detector (Waters Model 996) connected to an integrator (Millennium32, Chromatography Manager). The mobile phase consisted of 30 mmol/l each of sodium citrate and acetate at pH 3.0 (MeOH 15%). The flow rate was 0.5 ml/min, and the analytical cell potential applied was 3,026 ± 1,276 mV (CH1). The concentration of metabolites was determined by the ratio of respective metabolite peak areas to 2,6-DHBA (internal standard).

The ratio of 2,3-DHBA (μmol/l) to SA (μmol/l) is expressed as molar ratio × 1,000.

**Plasma levels of vitamin E and coenzyme Q10.** Vitamin E and coenzyme Q10 were measured by reverse-phase high-pressure liquid chromatography (HPLC) with wavelength-programmed, ultraviolet-visible absorbance detection (20). Serum triglycerides and total and high-density lipoprotein cholesterol were measured by standard enzymatic colorimetric methods. Low-density lipoprotein cholesterol was calculated with the Friedewald formula. Vitamin E levels were expressed both as an absolute figure and after adjustment for total cholesterol. Because vitamin E plasma levels, unlike coenzyme Q, are modified by diet (21), patients and controls were submitted to a standardized diet for vitamin E intake (30 IU/day) during hospitalization and for the entire period of follow-up.

**Cytokines and cytokine receptor levels.** Levels of TNF-alpha, sTNFR-I, sTNFR-II, IL-6, and IL-1ra were measured in serum from patients and controls, as previously described (22,23).

**Follow-up phase.** Group 2 patients were clinically followed after discharge, and at six months. OH probing,
Figure 2. Hydroxyl radical generation during the hospital phase. (A) Time course of 2,3-dihydroxybenzoic acid (DHBA)/salicylic acid (SA) (expressed as molar ratio × 1,000) in 10 patients with myocardial infarction (Group 2), all patients with stable angina (Group 1), and 5 Controls. (B) Time course of 2,3-DHBA/SA (expressed as molar ratio × 1,000) in the remaining 65 patients with myocardial infarction (Group 2) and 25 Controls. Sampling was performed at entry (within 12 h from symptom onset), at 24, 48, and 72 h after symptom onset, and at discharge (10 ± 4 days). The insert shows the pattern of 2,3-DHBA/SA in patients without (Group 2Ai) and with (Group 2Bi) HF during hospitalization.
vitamin E, coenzyme Q₁₀, and inflammatory status measurements were repeated.

**Chemicals.** Sodium SA, sodium citrate, sodium acetate, citric acid, 2,3-DHBA, 2,5-DHBA, 2,6-DHBA, and 3,4-DHBA were purchased from Sigma (St. Louis, Missouri), and HPLC-grade water and methanol were from Labscan Ltd. (Dublin, Ireland). All others chemicals and solvents used were of the highest purity commercially available.

**Statistical analysis.** Data are expressed as the mean value ± SD. Comparisons between two groups were performed with the Student t test or the Mann-Whitney U test for nonparametric variables. The Fisher exact test was used for categorical variables. Comparisons between more than two groups were performed by two-tailed analysis of variance, and post hoc comparisons were made using the Turkey's honest significance difference test. Correlations between variables were tested by Pearson analysis. Statistical significance was set at a level of p < 0.05 (Statistica version 6.0).

**RESULTS**

The baseline characteristics of the study population are shown in Table 1. Two patients in Group 2 (3%) died of intracranial hemorrhage and LV free wall rupture on days 2 and 3 of hospitalization, respectively; 24 (32%) presented with signs and symptoms of HF, 4 (5%) had re-infarction, and 3 (4%) showed acute pericarditis.

**Generation of ‘OH.** The kinetics of ‘OH production is shown in Figure 2A. No complication occurred in the 10 Group 2 patients enrolled in this analysis. The 2,3-DHBA/SA ratio did not differ between Controls and Group 1 patients. In Group 2 patients, 2,3-DHBA/SA was already significantly higher at entry (0.6 ± 0.1 vs. 0.16 ± 0.06 in Group 1 and 0.17 ± 0.04 in Controls, p < 0.05 for both), with a peak at 24 h (0.74 ± 0.18 vs. 0.17 ± 0.07 in Group 1 and 0.18 ± 0.06 in Controls, p < 0.001 for both), followed by a decline. Five days after the onset of symptoms, 2,3-DHBA/SA returned to normal values (0.28 ± 0.1 vs. 0.16 ± 0.08 in Group 1 and 0.15 ± 0.07 in Controls, p = 0.09 and p = 0.07, respectively).

Based on these data, in the remaining study population, we limited sampling to the timing reported in Figure 2B. The 2,3-DHBA/SA ratio was significantly elevated in Group 2 at entry (0.4 ± 0.09 vs. 0.24 ± 0.04 in Controls, p < 0.05), with a peak after 24 h (0.72 ± 0.15 vs. 0.23 ± 0.05 in Controls, p < 0.01). Contrary to the data reported in Figure 2A, increasing the sample size from 10 to 64 resulted in a significant difference in ‘OH production between Group 2 and Controls, even at discharge. In Group 2, the 2,3-DHBA/SA ratio failed to correlate with the peak levels of troponin I or creatine kinase, MB fraction and C-reactive protein.

**Plasma levels of antioxidants.** Figure 3 shows plasma levels of vitamin E (Fig. 3A) and coenzyme Q₁₀ (Fig. 3B). Levels of both these antioxidants did not change in Group 1 and Controls throughout the study. On the other hand, both antioxidant compounds showed a progressive decline in Group 2 patients. Vitamin E levels were significantly decreased 24 h after symptom onset (27 ± 5 vs. 39 ± 2.3 μmol/l in Controls, p < 0.05), with a further decline thereafter. Coenzyme Q₁₀ decreased significantly at discharge (0.9 ± 0.3 vs. 1.7 ± 0.5 μg/ml in Controls, p < 0.05).

**Role of HF.** The different patterns of ‘OH production in Group 2 patients with respect to the absence (Group 2Ai) or presence (Group 2Bi) of HF during hospitalization are shown in the insert of Figure 2B. Group 2Bi patients tended to maintain a higher degree of ‘OH production after the peak at 24 h, which became significant at discharge. In contrast, as shown in Table 2, the degree of antioxidant consumption was not different between Groups 2Ai and 2Bi, either considered as the absolute difference or percentage of decrease.

**Cytokines and cytokine receptor levels.** Cytokines and cytokine receptor levels are reported in Table 3. All tested cytokines were significantly increased in Group 2 compared
with Group 1 patients and Controls. All tested cytokines were significantly elevated in Group 2Bi versus Group 2Ai (TNF-alpha: 34 ± 16 pg/ml vs. 24 ± 7 pg/ml, p < 0.0001; sTNFR-I: 2,235 ± 1,132 pg/ml vs. 1,518 ± 702 pg/ml, p < 0.0001; sTNFR-II: 3,185 ± 1,420 pg/ml vs. 2,222 ± 914 pg/ml, p < 0.0001; IL-6: 41 ± 42 pg/ml vs. 14 ± 19 pg/ml, p < 0.0001; IL-1ra: 1,294 ± 888 pg/ml vs. 966 ± 685 pg/ml, p < 0.04, respectively). Group 2 patients with high (above median value), compared with those with low levels of TNF-alpha, sTNFR-I, and sTNFR-II, showed a significant increase in 2,3-DHBA/SA levels at discharge (Fig. 4).

**Correlations.** The percentage of vitamin E decrease during hospitalization was significantly related to that of coenzyme Q$_{10}$ decrease (r = 0.61, p < 0.05). The percentage of vitamin E decrease correlated significantly with 2,3-DHBA, 2,3-DHBA is not the product of any enzymatic reaction (in relation to its rate constant for diffusion-controlled rate constant of salicylate with concentration of the drug molecule), 2,3-DHBA is metabolized but undergoes renal clearance. Unlike 2,5-DHBA, 2,3-DHBA is not the product of any enzymatic reaction.

**DISCUSSION**

In vivo measurement of highly reactive free radicals, such as •OH, in humans is difficult. Consequently, secondary products of oxidative stress have been measured (24,25), the most common being thiobarbituric acid test, conjugated dienes, and diene-conjugated hydroperoxides. However, all these markers are considered rather unreliable, and studies on their specificity have always given conflicting results (26–29).

High oral doses of ASA (1,000 mg) have recently suggested for probing •OH in patients (17,30). The method is based on the ability of •OH to attack the benzene ring of aromatic molecules, such as ASA, and to produce stable, long-lasting hydroxylated compounds (Fig. 1) (16,17). For a radical probing technique to be reliable in vivo, it is necessary that: 1) the probe has a sufficiently high concentration (in relation to its rate constant for •OH) to be able to compete with other scavenger molecules; 2) the hydroxylated product(s) cannot be further metabolized; and 3) the product(s) hydroxylated by •OH is (are) different from possible enzyme-produced hydroxylated metabolites. It should be noted that the probing technique herein employed satisfies all three criteria owing to the very high, diffusion-controlled rate constant of salicylate with •OH (1.2 × 10$^{10}$ mol/l$^{-1}$ s$^{-1}$). 2,3-DHBA is not further metabolized but undergoes renal clearance. Unlike 2,5-DHBA, 2,3-DHBA is not the product of any enzymatic reaction.

**Table 2.** Antioxidant Consumption in Patients With Acute Myocardial Infarction (Group 2, n = 74)

<table>
<thead>
<tr>
<th>Variables</th>
<th>All</th>
<th>Group 2Ai</th>
<th>Group 2Bi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Entry Discharge</td>
<td>Entry Discharge</td>
<td>Entry Discharge</td>
</tr>
<tr>
<td>Vitamin E (µmol/l)</td>
<td>29 ± 6</td>
<td>30 ± 7</td>
<td>28 ± 6</td>
</tr>
<tr>
<td>Vitamin E/cholesterol (mmol/mol)</td>
<td>5.2 ± 1.3</td>
<td>5.0 ± 1.1</td>
<td>5.2 ± 1.2</td>
</tr>
<tr>
<td>Coenzyme Q$_{10}$ (µg/ml)</td>
<td>1.5 ± 0.5</td>
<td>1.6 ± 0.7</td>
<td>1.5 ± 0.4</td>
</tr>
</tbody>
</table>

*p < 0.05 discharge versus entry. Data are presented as the mean value ± SD. Vitamin E adjusted for total cholesterol and coenzyme Q$_{10}$ values significantly decreased at discharge with respect to hospital admission levels. The degree of antioxidant consumption was not different between patients without (Group 2Ai) and those with (Group 2Bi) heart failure during hospitalization.

**Table 3.** Cytokines in the Study Population

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls (n = 30)</th>
<th>Group 1 (n = 12)</th>
<th>Group 2 (n = 74)</th>
<th>p Value*</th>
<th>p Value†</th>
<th>p Value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-alpha (pg/ml)</td>
<td>18 ± 4</td>
<td>22.5 ± 9</td>
<td>37 ± 15</td>
<td>0.002</td>
<td>0.04</td>
<td>NS</td>
</tr>
<tr>
<td>sTNFR-I (pg/ml)</td>
<td>978 ± 775</td>
<td>1,297 ± 985</td>
<td>2,090 ± 1,082</td>
<td>0.003</td>
<td>0.02</td>
<td>NS</td>
</tr>
<tr>
<td>sTNFR-II (pg/ml)</td>
<td>1,957 ± 1,403</td>
<td>2,489 ± 1,413</td>
<td>3,278 ± 1,510</td>
<td>0.005</td>
<td>0.05</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>4.3 ± 7</td>
<td>3.4 ± 15</td>
<td>42 ± 78</td>
<td>0.001§</td>
<td>0.005§</td>
<td>NS§</td>
</tr>
<tr>
<td>IL-1ra (pg/ml)</td>
<td>224 ± 112</td>
<td>306 ± 237</td>
<td>1,224 ± 1,079</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Controls versus Group 2. †Group 1 versus Group 2. ‡Controls versus Group 1. §Mann-Whitney U test. All tested cytokines were significantly increased in Group 2 compared with Group 1 and controls. Data are presented as the mean value ± SD. Refer to Table 1 for a definition of Controls, Group 1, and Group 2.

IL-1ra = interleukin 1ra; IL-6 = interleukin-6; sTNFR-I = soluble tumor necrosis factor-alpha receptor I; sTNFR-II = soluble tumor necrosis factor-alpha receptor II; TNF-alpha = tumor necrosis factor-alpha.
The application of this technique to our patient population indicates that the acute phase of MI (first 24 to 48 h from symptom onset) is invariably associated with increased $\ddot{O}H$ generation, whose degree is not related to the extension of myocardial necrosis or to the progression to HF, whereas $\dot{O}H$ generation at peak is significantly inversely correlated with the percentage of vitamin E decrease during hospitalization. This would suggest that a decrease in vitamin E levels during hospitalization is a marker for ongoing oxidative stress in Group 2 patients, indicating that antioxidant reserve is being eroded during MI.

Antioxidant status could not differentiate patients with from those without HF in our study. This could reflect that antioxidant mechanisms are redundant in humans (32) and are likely to be differently affected.

Although peak oxidative stress was not correlated to the occurrence of HF, patients with HF as a complication of MI show a persistent elevation of $\dot{O}H$ generation in patients, irrespective of HF signs or symptoms. This would suggest that the degree of OSS in post-MI patients does not simply reflect LV systolic performance, as suggested (33), it could also reflect the interplay between cardiac function and the periphery (33,34).

It is thought that TNF-alpha is involved in the pathogenesis and progression of HF at both the central and peripheral levels. Levels of TNF-alpha are significantly elevated in patients with HF and exert a prognostic role independent of LV performance and clinical status (35). The ROS are downstream products of TNF-alpha-mediated signal transduction (36). It has been shown that TNF-alpha induces ROS generation in cardiac myocytes and peripheral muscles, and pretreatment with antioxidants abolishes TNF-alpha-induced cellular effects (37). Similarly, transgenic mice with cardiac-specific overexpression of TNF-alpha show increased OH generation by myocytes (38). Furthermore, ROS can sensitize cardiac myocytes to TNF-alpha–induced apoptosis through FLICE inhibitory protein (FLIP) expression modulation (39). Thus, there might be a two-way interaction between increased ROS generation and TNF-alpha activity in HF: the former is increased as a consequence of elevated TNF-alpha levels in part, whereas, at the same time, the latter is positively modulated in the setting of an altered cellular redox status. Therefore, increased TNF-alpha activity and ROS generation could play a synergic role in the progression to HF in patients with MI.

Our data show that patients with high levels of TNF-alpha and related soluble receptors also have increased $\ddot{O}H$ generation at both discharge and follow-up, suggesting a link between the two pathologic phenomena. Interestingly, it has recently been shown that secretion of TNF-alpha by TNF-alpha–converting enzyme (TACE), rather than transmembrane TNF-alpha, is responsible for the dilated cardiomyopathic phenotype in mice with cardiac-restricted overexpression of TNF-alpha (40). The findings that ROS can directly upregulate TACE activity (41) and subsequently increase TNF-alpha shedding provide a new working hypothesis on the TNF-alpha/ROS interaction in the pathogenesis and progression of HF.
Study limitations. Firstly, in the setting of HF, increased ROS production may arise from different enzymatic sources, including xanthine oxidase (42), mitochondria (43), cyclooxygenase, nitric oxide synthase, and nonphagocytic NAD(P)H oxidase (44). We did not investigate the possible source(s) of increased OH generation in patients with HF, as this was not the aim of the current study.

Secondly, we cannot exclude that ASA infusion itself could have partially affected absolute levels of inflammatory cytokines.

Finally, even though not at a significant level, the proportion of smokers in Group 2 was tendentially higher than that in Group 1 and Controls. This could be a possible confounder in our study. However, to specifically address whether smoking status could affect OSS and inflammatory status in our groups, we compared smokers versus non-smokers in Groups 1 and 2, and we could not find any differences in any of the recorded parameters. Furthermore, the proportion of smokers in Group 2a did not differ with respect to that in Group 2b (66% vs. 75%, p = NS).

Conclusions. Our data provide evidence for a distinct pattern of OH generation in post-MI patients showing progression to HF, with a persistent increase of OH production in the subacute and chronic phases in patients with an uncomplicated post-MI course. The finding that patients with HF display elevation of both ROS and sTNFR suggests that the interplay between OSS and inflammatory status should be specifically targeted as a possible mechanism for progression to LV dysfunction after MI.

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