Plasma heme oxygenase-1 is decreased in peripheral artery disease patients

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Abstract. Peripheral artery disease (PAD) is a common manifestation of atherosclerosis. A number of emerging risk factors, including oxidative stress biomarkers, free radicals and heat shock proteins, may add to the established risk factors for cardiovascular disease (CVD). The present study assessed surrogate markers of oxidative stress, including total reduced glutathione (GSH), lipid hydroperoxides (LOOH), isoprostanes, heme oxygenase-1 (HO-1) and metabolic biomarkers, such as adiponectin and lactate, in PAD patients (n=27). Healthy age-matched volunteers (n=27) served as controls. GSH and LOOH were evaluated by measuring total thiol groups and iron oxidation, respectively, by spectrophotometric analysis. Adiponectin, isoprostanes and HO-1 levels were determined using commercially available ELISA kits and lactate level was determined colorimetrically. Results from patients with PAD demonstrated no significant difference in GSH content and LOOH formation when compared with healthy controls (5.1 ± 7.6 vs. 6.9 ± 9.1 nmol/ml and 6.8 ± 14.2 vs. 8.3 ± 14.9 nmol/ml, respectively), however, isoprostanes were demonstrated to be significantly reduced (3.8 ± 4.8 pg/ml vs. 120 ± 91 pg/ml; $P < 0.001$). Furthermore, HO-1, a protective heat shock protein, was significantly reduced in PAD patients (0.8 ± 0.7 vs. 3.4 ± 1.3 ng/ml; $P < 0.001$). Adiponectin, an antiatherogenic adipokine, was not significantly different between the two groups (1.4 ± 0.2 vs. 1.5 ± 0.5 μ g/ml), whereas serum lactate was significantly higher in PAD patients compared with controls (0.11 ± 0.01 vs. 0.1 ± 0.01 mM; $P < 0.05$). Using multivariate analysis, HO-1, hypertension, smoking and dyslipidemia were indicated to be independently associated with the presence of PAD, while only ankle-brachial

index was an independent predictor of severity of PAD. The oxidative pathway may be partially involved in the onset and progression of PAD and may represent a target to reduce the risk of ischemic events.

Introduction

Peripheral artery disease (PAD) affects 3-20% of the population >50-55 years old and is associated with a high cardiovascular (CV) mortality (1). PAD remains a major clinical problem as it is considered a marker of the extent of atherosclerosis. Furthermore, a large number of PAD patients suffer from multiple arterial co-morbidities leading to high CV mortality or a poor prognosis within a short time frame (2-4).

In the United States of America, Medicare spends ~3.9 billion dollars annually on treatment associated with PAD; this is more than that spent on other CV diseases, including myocardial infarction, angina, aortic aneurysm and carotid disease, or on high blood pressure, cigarette smoking, diabetes mellitus and hypercholesterolemia (3). Different pathophysiological mechanisms underlie the development of atherosclerotic plaque and the subsequent development of CV disease, these mechanisms include inflammation, platelet activation, endothelial damage, the proliferation/apoptosis balance of smooth muscle cells, and oxidative stress (5). The association between oxidative stress and CV disease has been demonstrated by numerous previous studies (6,7). These studies have demonstrated that oxidative stress may have an etiological role and be used as a biomarker for atherosclerosis. When oxidative stress occurs, cells trigger a series of biochemical cascades in an attempt to counteract the altered redox balance and restore homeostasis (8). Among these biochemical responses, the heme oxygenase (HO) system has been suggested to be key (7,9). HO-1 is an intracellular enzyme that catalyzes the breakdown of heme to carbon monoxide, ferrous iron and biliverdin (10). However, numerous other effects not directly associated with erythrocyte metabolism but of potential relevance to the CV system have been reported, including protection from ischemia/reperfusion (10,11), blood pressure regulation (12), inflammation (13-15) and in angiogenesis (16,17). In addition, the presence of HO-1 has been demonstrated in various intracellular and extracellular compartments, suggesting that this

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protein may have other properties independent of its known enzymatic activity (18-20). This is important as HO-1 is an intracellular enzyme and the role of its presence in the plasma is unclear. One physiological role may be in degrading excess free heme in association with hemopexin and transferrin that may result from a hemolytic or other process. Alternatively, HO-1 may be released into the plasma from smooth muscle cells, cardiomyocytes, leukocytes, monocytes/macrophages and/or endothelial cells that are damaged by the effect of hypertension, oxidative stress and/or chronic inflammation (8).

The observations described above suggest that HO-1 has a role in CV disease pathogenesis. However, the role of HO-1 in PAD remains to be elucidated.

Thus, the present study aimed to measure serum levels of HO-1 and other surrogate markers of oxidative stress, including reduced glutathione, lipid hydroperoxides and isoprostanes, in patients with PAD at the time of their first diagnosis.

Patients and methods

Patients (n=27; male; mean age, 66±8 years) were enrolled consecutively and examined in the non-invasive vascular laboratory of the Medical Angiology Unit (Garibaldi Hospital, Catania, Italy). Patients were diagnosed with PAD based on their medical history of intermittent claudication and/or vasodilatation therapeutic agents, and an ankle/brachial index (ABI) ≤0.9. The ABI was obtained by measuring the arterial pressure at the posterior or anterior tibial of the lower limbs and was divided by the brachial arterial pressure. A pocket-sized Doppler equipped with an 8 MHz pencil style probe (Sonomed SRL, Rome, Italy) was used to record the ABI. The lowest value observed in one of the two peripheral arteries of the lower limbs was considered. All the enrolled patients met the criteria of stage II according to the Fontaine classification of PAD, in which pain due to walking is intermittent claudication (21). The mean value of the free walking distance in all PAD patients was 347±170 m. The mean ABI value of the PAD patients was 0.83±0.08; 0.85±0.7 in less severe patients (stage IIa) and 0.78±0.5 in the more severe patients (stage IIb). None of the PAD patients exhibited a higher ABI (>1.3) than the previously mentioned values. Healthy male volunteers (n=27) served as age-matched controls.

Controls were recruited from healthy volunteer blood donors, regularly attending the transfusion center of the University of Catania Hospital (Catania, Italy). Body mass index (BMI) was calculated, for patients and controls, as kg/m². Obesity was diagnosed when the BMI was >30. Controls were healthy individuals with no known risk factors for PAD, including diabetes or dyslipidaemia.

The adopted procedures were in agreement with the Helsinki Declaration of 1975, as revised in 1983 and were approved by the Ethics Council and Institutional Review Boards of the Garibaldi Hospital, University of Catania. All subjects provided informed consent. Participants did not suffer from recent coronary acute syndrome, heart failure, chronic or acute renal failure, active cancer, chronic liver disease or immunologic diseases.

Blood sampling. Venepuncture was conducted using an antecubital vein at the time of diagnosis and enrollment. The

blood was collected in vacutainers and distributed in 0.5 ml aliquots. The serum samples, obtained by centrifugation at 3,000 x g for 15 min at 4°C, were stored at -80°C until analysis (22).

Lipid hydroperoxide (LOOH) determination. LOOH levels were evaluated following the oxidation of Fe²⁺ to Fe³⁺ in the presence of xylenol orange (23). The assay mixture contained, in a total volume of 1 ml/100 ml of plasma sample, 100 mmol/l xylenol orange, 250 mmol/l ammonium ferrous sulfate, 90% methanol, 4 mmol/l butylated hydroxytoluene and 25 mmol/l H₂SO₄. Following incubation for 30 min at room temperature, the absorbance was measured using a U2000 Hitachi spectrophotometer (Hitachi, Ltd., Tokyo, Japan) at a wavelength of 560 nm. Calibration was obtained using hydrogen peroxide (0.2-20 mmol/l). The limit of detection for this assay is ~0.25 nmol/l.

Measurement of glutathione (GSH). Plasma levels of GSH were measured using a spectrophotometric assay based on the reaction of thiol groups with 2,2-dithio-bis-nitrobenzoic acid at a wavelength of 412 nm (εM=13,600 M⁻¹ cm⁻¹, where εM is a wavelength-dependent molar absorptivity coefficient) (23). Measurements were performed in duplicate.

Measurement of HO-1, adiponectin and isoprostanes. A commercially available HO-1 (human) ADI-EKS-800 ELISA kit (Stressgen Biotechnologies Corporation, Victoria, BC, Canada) was used to measure HO-1 concentration. The assay was performed according to the manufacturer's protocol as previously described (24,25). Briefly, each plasma sample was incubated with anti-HO-1, anti-rabbit immunoglobulin G and horseradish peroxidase conjugates, in successive order. Absorbance was measured at a wavelength of 450 nm, and HO-1 concentration was calculated from a standard curve generated with purified proteins. The limit of detection as specified by the manufacturer was 0.78±0.65 ng/ml. Each measurement was performed in triplicate, and means were reported. Similarly, isoprostanes and adiponectin were determined using the 8-Isoprostane ELISA kit and the adiponectin (Human) EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol.

Statistical analysis. Statistical analyses were performed using Statview® 5.0 (SAS Institute Inc., Cary, NC, USA). Univariate analyses were performed using Student's unpaired t-test for numeric variables, whereas the differences in the prevalence for nominal variables were analyzed using the χ² test. Correlation analyses were performed using the Pearson rank correlation method.

Multivariate analysis (by multiple regression model) was performed in order to determine the effect of clinical and laboratory parameters on the presence or severity of PAD. Two different models of multivariate analysis for the presence and for the severity of PAD were built, aiming to test whether HO-1 was an independent predictor of the presence or the severity of PAD, respectively, with the following dependent variables: HO-1, hypertension, diabetes, ABI, smoking, age, dyslipidemia, obesity and free walking distance. All data are

Table I. Clinical and laboratory data in PAD patients and controls. Data are expressed as the mean $\pm$ standard deviation or as percentages.

Parameter	PAD (n=27)	P=	Controls (n=27)
Age, years	66 $\pm$ 8	ns	65 $\pm$ 9
BMI (kg/m ²)	29 $\pm$ 1	ns	29 $\pm$ 2
Obesity (%)	9 (33)	ns	9 (23)
Diabetes, n (%)	10 (37)	-	/
Smoking, n (%)	22 (81)	ns	20 (74)
Dyslipidemia, n (%)	16 (59)	-	/
Hypertension, n (%)	19 (70)	-	/
Use of statins, n (%)	15 (56)	-	/
Use of aspirin, n (%)	24 (89)	-	/
PAD stage IIa, n (%)	13 (48)	-	/
Pain-free walking distance (meters)	347 $\pm$ 170	-	/
Ankle-brachial index	0.83 $\pm$ 0.08	-	/
Lipid hydroperoxides (nmol/ml)	6.8 $\pm$ 14.2	ns	8.3 $\pm$ 14.9
Glutathione (nmol/ml)	5.1 $\pm$ 7.6	ns	6.9 $\pm$ 9.1
Heme oxygenase-1 (ng/ml)	0.8 $\pm$ 0.7	<0.0001	3.4 $\pm$ 1.3
Lactic acids (mM)	0.11 $\pm$ 0.01	0.0123	0.10 $\pm$ 0.01
Isoprostanes (pg/ml)	3.8 $\pm$ 4.8	<0.0001	120 $\pm$ 91
Adiponectin (μ g/ml)	1.4 $\pm$ 0.2	ns	1.5 $\pm$ 0.5

PAD, peripheral artery disease; ns, not significant.

Table II. Pearson correlations in peripheral artery disease patients.

Parameter	LOOH	Glutathione	HO-1	Lactic acids	Isoprostanes	Adiponectin
Age	-.074	-.111	-.125	.026	.112	-.457^a
BMI	.123	.220	.151	-.304	-.227	-.076
PfWD	.084	-.316	.258	-.100	-.238	-.178
ABI	.238	-.359	.057	.001	.104	-.095
LOOH	-	-.194	-.263	.100	.243	-.227
Glutathione	-.194	-	-.253	-.525^a	.045	.016
HO-1	-.263	-.253	-	-.061	.009	.190
Lactic acid	.100	-.525^a	-.061	-	.175	-.242
Isoprostanes	.243	.045	.009	.175	-	.008
Adiponectin	-.227	.016	.190	-.242	.008	-

In bold the relationships that reached statistical significance (*P<0.05). BMI, body mass index; PfWD, Pain-free walking distance; ABI, ankle-brachial index; LOOH, lipid hydroperoxides; HO-1, heme oxygenase-1.

expressed as mean and standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

The major clinical characteristics of the study subjects are summarized in Table I; the co-administration of other therapeutic agents for CV prevention was considerable, and included anti-hypertensive and lipid-lowering agents, in addition to aspirin. This was a potential limitation of the present study. As presented in Table I, no significant

differences in LOOH, GSH and adiponectin levels were observed between the PAD patients and controls. However, a significant increase in GSH level was observed in stage IIb patients when compared with stage IIa patients (Fig. 1; P<0.036). Furthermore, a significant decrease in plasma protein levels of HO-1 was observed in PAD patients when compared with the controls. Notably, this reduction was not dependent on the stage of the disease or levels of oxidative stress biomarkers. A multivariate analysis (multiple regression) was also performed in order to test whether HO-1 was an independent predictor of the presence or the severity of

Figure 1. Association between peripheral artery disease severity and GSH. Data are expressed as the mean $\pm$ standard error of the mean.

PAD. It was observed that HO-1, hypertension, smoking and dyslipidemia were independent predictors of the presence of PAD ($P < 0.0001$, $P < 0.0001$, $P = 0.0082$, and $P = 0.0013$ respectively). In addition, ABI was the only independent predictor of PAD severity ($P < 0.0001$).

Discussion

Oxidative stress is the result of an imbalance between the generation of reactive oxygen species and the antioxidant defense system. Although appropriate epidemiological markers to measure oxidative stress are lacking, certain markers have been examined specifically with respect to PAD. In the present study, various biomarkers were evaluated to detect the level of oxidative stress.

The results of the present study indicated there was no significant difference in GSH content in PAD patients when compared with healthy controls. These data may appear inconsistent with previous data that demonstrated a marked increase in oxidative stress markers in CV disease (26). However, it should be considered that oxidative stress triggers a series of compensatory biochemical mechanisms in order to maintain the redox balance. Thus, it may be that patients increase GSH synthesis via the upregulation of its enzymatic synthesis, such as GSH reductase and gamma glutamyl cysteine synthase. The findings of the present study are consistent with this hypothesis, they indicate that GSH is increased in the group of patients with more severe disease (stage IIb) compared with the milder form. Similarly, the level of isoprostanes in stage IIb patients are not significantly different when compared with stage IIa patients, suggesting that the more severe patients exhibit a balanced maintenance of the systemic redox balance accompanied by a concomitant upregulation of GSH production. Regarding lipid peroxidation, LOOH may notably be transformed into more oxidized products, including malondialdehyde, which is a downstream product of lipid peroxidation and is not measured by the spectrophotometric assay used in the current study. Isoprostanes, the other lipid peroxidation biomarker used in the present study, may appear inconsistent with data presented in previous studies (27,28). Since their identification, levels of F_2 -isoprostanes have been recommended as a reliable biomarker for measuring *in vivo*

lipid oxidation and oxidative stress (27). Previous studies demonstrated higher levels of urinary 8-iso-prostaglandin $F_{2\alpha}$ in patients with chronic lower limb ischemia compared with healthy controls (28,29). One previous study performed multivariate adjustment. Following adjustment for age, gender, diabetes, hypertension, BMI, creatinine, low-density lipoprotein, triglyceride, high-sensitivity C-reactive protein and homocysteine, an increment of every 10 pg/ml in plasma 8-iso-PGF $_{2\alpha}$ was associated with an increased risk of 11% for lower limb ischemia (28). Furthermore, the present study also evaluated the levels of adiponectin in PAD patients and healthy controls. Results from the present study indicated there was no significant difference in adiponectin levels between the two groups, however, a previous study (30) demonstrated that low adiponectin levels were associated with an increase in PAD incidence.

The current study indicated there was a significant reduction in HO-1 levels in PAD patients when compared with healthy controls. The protective properties of HO-1 are multifactorial and likely due to degradation of pro-oxidant heme, the generation of antioxidant biliverdin and bilirubin, and the production of carbon monoxide, which is a potent vasodilator with anti-inflammatory effects (7,9). Furthermore, previous studies have also observed reduced serum HO-1 in lung disease (31). The presence of HO-1 has recently been demonstrated in various intracellular and extracellular compartments, suggesting that this protein may exhibit other functions independently of its known enzymatic activity (18,20,32). This latter point is important as HO-1 is an intracellular enzyme, thus, the underlying reason for its presence in the plasma is unclear. Data from the present study demonstrates that HO-1 reduction is not dependent on other covariables and, in particular, oxidative stress parameters (Table II). In this context, it may be hypothesised that as our previous data indicated that HO-1 protein expression is reduced in experimental diabetes and as the majority of PAD patients in the current study also had diabetes, reduced HO-1 levels in PAD may reflect reduced intracellular HO-1 content in this group (33). In addition, previous studies have also reported that HO-1 deletion in mice evokes resistance to diet-induced insulin resistance and inflammation, markedly reducing secondary diseases, including steatosis and liver toxicity (34,35). Multivariate analyses were also performed in order to test whether HO-1 was an independent predictor of the presence or severity of PAD. It was observed that HO-1 was independently associated with the presence of PAD. Furthermore, hypertension, smoking and dyslipidemia were also independent predictors of the presence of PAD, while ABI was the only independent predictor of PAD severity. These findings suggest that HO-1 may be useful in determining the presence of PAD.

Therefore, it is possible to hypothesise that plasma HO-1 reduction may also be part of the compensatory mechanisms to maintain the cellular redox status. However, the sample size of the cohort in the present study is small and this may represent a potential limitation of the data, which is required to be confirmed on a larger population.

In conclusion, the results of the present study indicate that oxidative stress depends on the stage of PAD and that the condition is followed by a concomitant antioxidant response,

which, however, is only partially sufficient to maintain the redox balance. These findings suggest a biological basis for antioxidants, such as polyphenols, to be tested as therapeutic agents in appropriately designed prospective trials.

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