Oxidative stress in ischemia-reperfusion injury: assessment by three independent biochemical markers

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Key words:
Oxidative stress; Ischemia-reperfusion; Lipid peroxidation.

Background. **Oxidative stress plays a key role in ischemia-reperfusion injury, causing peroxidation of tissue lipids and proteins. However, it is debated whether brief ischemic episodes are sufficient to cause detectable oxidative stress in humans, since biochemical markers used so far in the setting of percutaneous transluminal coronary angioplasty (PTCA) gave conflicting results.**

Methods. **We determined lipid hydroperoxides (ROOHs), conjugated dienes (CD) and total radical-trapping antioxidant capacity (TRAP), three different independent markers of oxidative stress, in aortic and great cardiac vein blood of 5 patients undergoing PTCA before a single balloon inflation lasting 115 – 38 s (t0), and 1 min (t1), 5 min (t5), 15 min (t15) after balloon deflation (Group 1). ROOHs and CD were also determined in aortic and great cardiac vein blood of 5 patients with mitral valve disease (Group 2).**

Results. **In Group 1, great cardiac vein levels of ROOHs and CD at t1 increased by 219% and 79%, respectively, compared to t0 ($p < 0.01$); this sharp and consistent increase persisted up to t15 (+189% and +63%, respectively, compared to t0; $p < 0.01$). Great cardiac vein levels of TRAP were significantly lower than aortic levels at t0, and exhibited a further decrease at t1. No significant differences in aortic and great cardiac vein levels of ROOHs and CD at t0 were observed between Group 1 and Group 2.**

Conclusions. **The three methods we used showed a remarkable sensitivity for the detection of post-ischemic reperfusion injury in cardiac venous blood and may be useful for detecting small foci of ischemia-reperfusion injury in microvascular angina.**

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Reactive oxygen species (ROS) are key mediators of reperfusion injury, causing peroxidation of tissue lipids and proteins[1]. In humans, a large myocardial release of malondialdehyde, the most widely used index of lipid peroxidation, has been demonstrated during coronary bypass surgery, which causes large and prolonged episodes of myocardial ischemia[2].

However, it is still unclear whether brief ischemic episodes, such as those occurring during transient anginal attacks, are sufficient to cause detectable oxidative stress. This topic is of interest because, in animals, also relatively short episodes of ischemia followed by reperfusion have been shown to elicit the generation of ROS[3]. Moreover, increasing evidence suggests that ROS are important mediators of preconditioning[4,5], induce late protection against reperfusion-induced coronary endothelial injury[6], and may be involved in the inverse relation between antioxidant intake and acute coronary events observed in some studies[7].

In addition, markers of ischemia-reperfusion measured immediately after an ischemic episode may have a much greater sensitivity for the detection of subclinical ischemia than myocardial lactate, hydrogen and potassium ion release in the coronary sinus during ischemia.

Percutaneous transluminal coronary angioplasty (PTCA) is a clinical model of transient, brief myocardial ischemia-reperfusion, and biochemical markers used so far to investigate oxidative stress in this setting gave conflicting results, probably because of their inadequate sensitivity[8-13].

We report the results obtained in a group of patients with ischemic heart disease who underwent elective PTCA, using three different and independent markers of oxidative stress, i.e. lipid hydroperoxides (ROOHs), conjugated dienes (CD) and total radical-trapping antioxidant capacity (TRAP). These combined methods have recently been applied as a multiparametric assessment of oxidative stress in peripheral blood of insulin-dependent diabetics by Santini et al.[14]. ROOHs and CD are independent indices of lipid peroxidation, generated at dif-

ferent stages of the peroxidation cascade; when polyunsaturated fatty acids are attacked by free radicals, CD are formed and, in the presence of oxygen, ROOHs are generated through intermediate states.

ROOHs were assessed by the FOX2 version assay[15], a simple and reproducible method which has the distinct advantage to access and measure total available plasma ROOHs, whereas high-performance liquid chromatography (HPLC) based methods may fail to recover individual classes of lipid ROOHs during extraction and separation. CD were determined by second derivative spectrophotometry, which allows greater sensitivity compared to simple absorption spectroscopy since it discriminates between the different configurations of CD isomers, i.e. *cis-trans* and *trans-trans*, the latter being formed when the antioxidant capacity of plasma is reduced. This method, originally described by Corongiu and Banni[16] *in vitro*, has been refined and modified by Santini et al.[14] for *in vivo* use in clinical studies. Furthermore, in contrast to other lipid peroxidation products, CD isomer discrimination has been shown to assess not only ROS-induced cellular damage, but also antioxidant defenses; indeed, in diabetic patients, Santini et al.[14] found that the decreased ratio of *cis-trans/trans-trans* CD reflects an altered redox status of plasma and an impaired antioxidant capacity.

Plasma antioxidant capacity was assessed by measuring TRAP[17], which is determined by the overall effect of water-soluble antioxidants (uric acid, ascorbic acid, protein thiol and bilirubin) and lipid soluble antioxidants (α-tocopherol, β-carotene and ubiquinol). Since antioxidants seem to act cooperatively *in vivo*, this method could provide a more comprehensive assessment than the evaluation of individual antioxidants.

Methods

Preparation of plasma. Blood was collected into sampling vials containing heparin for preparation of plasma. Plasma samples were stored under nitrogen at -70 C and used within 1 month for analysis of TRAP and measurement of CD and ROOHs.

Measurement of lipid peroxidation products. Plasma lipids were extracted by a modification of the method of Folch et al.[18]: 3.8 ml of 2:1 (vol/vol) chloroform-methanol were mixed (with a vortex) for 2 min with 0.2 ml of plasma and 1.0 ml of acidified water (pH 2.5). The suspension was then centrifuged at 3000 rpm for 5 min at 4 C. The lower chloroform lipid layer was removed, dried under vacuum in a Savant RC 100 Speed-Vac and resuspended in 1.0 ml HPLC-grade methanol for CD assay or 100 µl HPLC-grade methanol for ROOHs measurement.

Hydroperoxides. Plasma ROOHs were determined with the FOX2 assay for lipid ROOHs[15], based on the ROOHs-mediated oxidation of Fe^{2} under acidic conditions. Fe^{3} forms a chromofore with xylenol orange, which absorbs strongly at 560 nm. FOX2 reagent was prepared by dissolving xylenol orange and ammonium ferrous sulfate in 250 mmol/l H_2SO_4 to final concentrations of 1 and 2.5 mmol/l, respectively. One volume of this concentrated reagent was added to 9 volumes of HPLC-grade methanol containing 4.4 mmol/l butylated hydroxytoluene to make the working reagent in 90% (vol/vol) methanol. Plasma lipid extracts (90 µl) in HPLC-grade methanol were transferred into 1.5 ml microcentrifuge vials. Triphenylphosphine in methanol (10 µl of 10 mmol/l) was added to the blank samples to selectively reduce ROOHs to hydroxy derivatives having no reactivity with Fe^{2}, while methanol (10 µl) was added to the test sample. All vials were then vortex-mixed and incubated at room temperature for 30 min. Then, 900 µl FOX2 reagent were added and again incubated at room temperature for 30 min. Finally, the vials were centrifuged at 12 000 rpm for 10 min and the absorbance of the supernatant read at 560 nm. ROOH content of samples was determined as the mean absorbance difference of samples with and without elimination of ROOHs by triphenylphosphine. Intra- and inter-assay coefficients of variation for this method were 6.5% and 9.2%, respectively.

Conjugated dienes. CD were measured by second derivative spectrophotometry[16], a technique which allows great sensitivity, since the CD shoulder at 234-240 nm that appears in the ordinary spectrum translates into sharp minimum peaks which are more easily measurable. Minima at 232 and 246 nm were ascribed to the *trans-trans* and *cis-trans* CD isomers, respectively and quantified in arbitrary units (AU) as $d^2A/d\lambda^2$, where $d^2A/d\lambda^2$ represents the measurement from minima to adjacent maxima at the higher wavelength. Intra- and inter-assay coefficients of variation for this method were 8.7% and 12.1%, respectively.

Measurements of plasma antioxidant capacity. The assay is based on the quenching of the ABTS radical cation ($ABTS^{\bullet+}$) by antioxidants[17]. $ABTS^{\bullet+}$ is generated by the interaction of the azo-compound, ABTS, with highly reactive ferrylmyoglobin radical species, produced by the activation of metmyoglobin with H_2O_2. Briefly, we mixed together ABTS (150 µmol/l), metmyoglobin (2.5 µmol/l) and plasma (25 µl); the addition of H_2O_2 (75 µmol/l) started the reaction. The absorbance increase at 734 nm, at 20 C, was indicative of $ABTS^{\bullet+}$ formation. The delay from the addition of H_2O_2 (time zero) and the onset of absorbance increase ($ABTS^{\bullet+}$ formation) was measured. The assay was standardized using a water-soluble vitamin E analog, Trolox™ (12.5 µmol/l).

Patients. *Group 1*. We studied 5 patients undergoing elective PTCA of a single left anterior descending (LAD) stenosis (> 70% lumen diameter). A single blood sample

was taken by needle puncture of the femoral vein; then, paired blood samples were taken from the aorta and great cardiac vein through Judkins and Multipurpose catheters, respectively, before (t0) and 1 min (t1), 5 min (t5) and 15 min (t15) after a single balloon inflation lasting 115 – 38 s (mean – SD). At the same time, great cardiac vein blood oxygen saturation was also measured.

Group 2. We also studied 5 patients undergoing cardiac catheterization for mitral valve disease; all had normal coronary arteries and no history of chest pain. Single blood samples from the femoral vein, aorta and great cardiac vein were collected as previously described for Group 1.

Statistical analysis. Normal distribution of data was assessed by Shapiro-Wilk W test. Continuous variables are presented as mean – SD and were analyzed by multivariate analysis of variance; *post-hoc* comparisons between groups were done by Newmann-Keuls test. Repeated measure design was introduced when required.

Results

The absence of significant differences between femoral vein and aortic levels of ROOHs and total CD (*trans-trans* + *cis-trans*) in Group 1 (2.49 – 0.57 vs 2.56 – 0.21 μmol/l, p = NS for ROOHs; 0.033 – 0.001 vs 0.029 – 0.002 AU, p = NS for CD) and in Group 2 (2.98 – 0.93 vs 2.72 – 0.75 μmol/l, p = NS for ROOHs; 0.029 – 0.003 vs 0.029 – 0.002 AU, p = NS for CD) demonstrated a good intra-individual reproducibility of the measurements and the absence of changes induced by catheter sampling. No significant differences between aortic and great cardiac vein levels of ROOHs and total CD (*trans-trans* + *cis-trans*) were observed either in Group 1 or in Group 2 at t0 (Table I).

In Group 1, aortic levels of ROOHs, CD and TRAP remained stable, but great cardiac vein levels of ROOHs and CD (both *trans-trans* and *cis-trans* isomers) consistently increased at t1 ($p < 0.01$ vs t0), and such increase lasted up to 15 min (t15, $p < 0.01$ vs t0). The mean percent increase at t1 was 219% for ROOHs and 79% for total CD, with a standard error of 12.8% and 4.2%, respectively, indicating a remarkably consistent and reproducible pattern of increase which persisted at t5 (+208% and +95% vs t0, respectively) and at t15 (+189% and +63% vs t0, respectively). Great cardiac vein TRAP levels were significantly lower than aortic levels at t0 ($p < 0.01$), and showed a further decrease at t1 ($p < 0.05$ vs t0) (Fig. 1). The detection of reactive hyperemia following LAD occlusion, demonstrated by the increase in great cardiac vein blood oxygen saturation from 26 – 5% at t0 to 48 – 8% at t1 ($p < 0.01$), proved that the venous blood actually drained the myocardium made ischemic by the balloon inflation in the LAD coronary artery.

Table I. Hydroperoxides, conjugated dienes and TRAP levels (mean – SD).

	Group 1				Group 2
	t0	t1	t5	t15	t0
Hydroperoxides (μmol/l)					
Femoral	2.49 – 0.57				2.98 – 0.93
Ao	2.56 – 0.21	3.18 – 0.63	3.24 – 0.51	2.19 – 0.49	2.72 – 0.75
GCV	3.11 – 0.13	9.91 – 0.66*	9.58 – 0.85*	9.04 – 1.14*	2.71 – 1.40
Conjugated dienes (AU) $d^2A/d\lambda^2$					
232 nm (*trans-trans* isomers)					
Femoral	0.025 – 0.010				0.024 – 0.003
Ao	0.023 – 0.010	0.027 – 0.002	0.028 – 0.002	0.028 – 0.002	0.025 – 0.002
GCV	0.027 – 0.001	0.035 – 0.001*	0.038 – 0.005*	0.035 – 0.003*	0.026 – 0.005
246 nm (*cis-trans* isomers)					
Femoral	0.0077 – 0.0004				0.0046 – 0.0010
Ao	0.0057 – 0.0017	0.0081 – 0.0009	0.0081 – 0.0010	0.0076 – 0.0011	0.0049 – 0.0010
GCV	0.0061 – 0.0015	0.0247 – 0.0021*	0.0269 – 0.0020*	0.0194 – 0.0018*	0.0046 – 0.0020
232 nm + 246 nm					
Femoral	0.033 – 0.001				0.029 – 0.003
Ao	0.029 – 0.002	0.035 – 0.002	0.036 – 0.002	0.034 – 0.003	0.029 – 0.002
GCV	0.033 – 0.001	0.060 – 0.001*	0.065 – 0.004*	0.054 – 0.050*	0.030 – 0.005
TRAP (μmol/l)					
Femoral	983 – 41				
Ao	966 – 91	939 – 35	927 – 36	904 – 80	
GCV	802 – 9	724 – 30	718 – 36	798 – 83	

Ao = aorta; GCV = great cardiac vein; TRAP = total radical-trapping antioxidant capacity. * $p < 0.01$ vs GCV at t0 and Ao at all times; $p < 0.01$ vs Ao at t0; $p < 0.05$ vs GCV at t0.

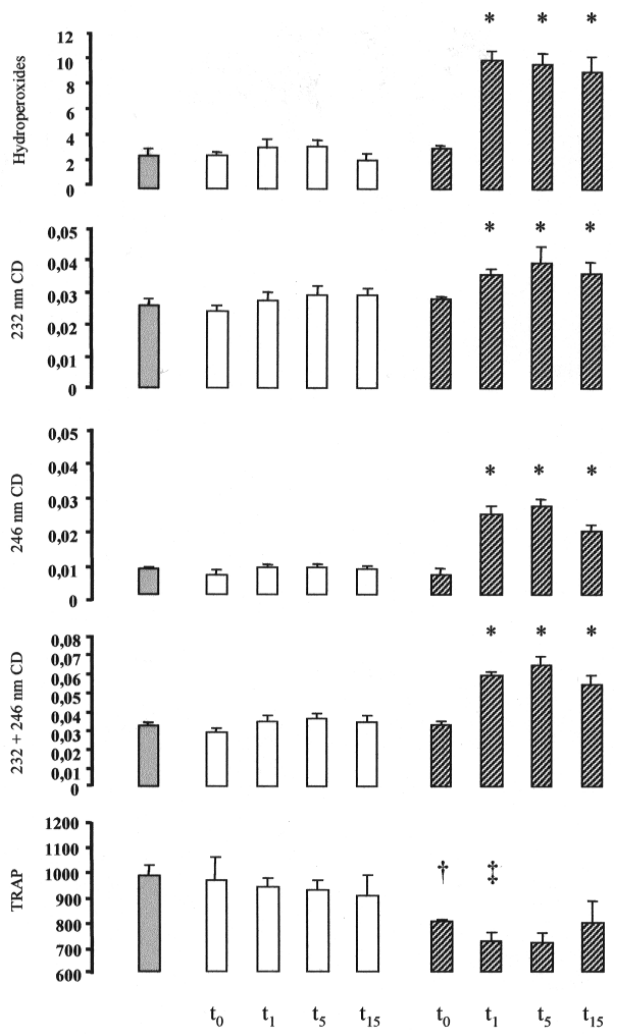

Figure 1. *Femoral vein (▒), aorta (□) and great cardiac vein (▨) plasma levels before (t0) and 1 min (t1), 5 min (t5) and 15 min (t15) after a single balloon inflation lasting 115 ± 38 s in Group 1 patients. Data are presented as mean + SD. Hydroperoxides are expressed as mmol/l, conjugated dienes (CD) as arbitrary units and total radical-trapping antioxidant capacity (TRAP) as mmol/l. CD isoforms are also reported; 232 nm CD indicates the* trans-trans *isomer, whereas 246 nm CD indicates the* cis-trans *isomer. * $p < 0.01$ vs great cardiac vein at t0; † $p < 0.01$ vs aorta at t0; ‡ $p < 0.05$ vs great cardiac vein at t0.*

Discussion

The combined use of these three sensitive, independent methods allowed us to show a remarkably consistent pattern of massive ischemia-reperfusion induced lipid peroxidation following a 2 min occlusion of the LAD, which persisted up to 15 min but was absent in controls. The simultaneous detection of reactive hyperemia shows that great cardiac vein blood drained from myocardial regions made ischemic during the balloon inflation. The possibility of catheter-induced artifacts was excluded by the absence of any difference between aortic and femoral vein samples.

Previous studies on oxidative stress in PTCA were based on the assessment of several biochemical markers of lipid peroxidation such as malondialdehyde[8-12], cholesterol hydroperoxides[19], linoleic acid CD ratio[20], or antioxidants such as uric acid, hypoxanthine[10] and glutathione peroxidase[12]. Most studies on malondialdehyde, determined as thiobarbituric acid reactive substances (TBARS), gave positive results, but the TBARS methods are the most criticized because of their ambiguity and underestimation of the extent of lipid peroxidation[21]; also, the analysis of peroxidation products of a single molecule (cholesterol, linoleic acid) may suffer from a lack of sensitivity and fail to adequately represent the peroxidation cascade.

As far as antioxidants are concerned, the determination of a single water- or lipid-soluble antioxidant is unlikely to assess the real free radical scavenging capacity, because antioxidants seem to act cooperatively *in vivo* with a reciprocal regeneration.

Two studies, to our knowledge, detected PTCA-induced oxidative stress by *ex-vivo* electron paramagnetic resonance spin trap technique[19,22], which is the gold standard in free radical detection but suffers from several limitations in human studies, because the spin trap cannot be injected in patients.

Isoprostanes are free radical-catalyzed products of arachidonic acid and have been proposed as specific, chemically stable, noninvasive indices of free radical generation *in vivo*[23,24]. Reilly et al.[24] found a consistent increase in urinary 8-epi PGF-2α, an isoprostane, after primary PTCA for acute myocardial infarction, but the increase after elective PTCA, although significant, was mild and not different from that observed following diagnostic coronary angiography. Moreover, 8-epi PGF-2α may also derive from platelets (cyclo-oxygenase 1) and monocytes (cyclo-oxygenase 2) in the absence of free radical stimulation, particularly in settings of marked cyclo-oxygenase activation such as myocardial infarction.

In our study, markers of oxidative stress in Group 1 increased by 80-220% at 1 min after reperfusion following a single balloon inflation of 115 – 38 s, remaining still elevated at 15 min; this increase is consistent and very large compared to that found in previous studies. Although the time of balloon inflation, i.e. the duration of ischemia, is commonly considered a major determinant of oxidative stress, previous studies suggest that this may not be necessarily always the case; Oldroyd et al.[11], indeed, found an increase in TBARS after only 60 s of LAD occlusion, whereas Paterson et al.[9] could not find any increase in TBARS and CD of linoleic acid after 90 to 360 s LAD occlusion.

In conclusion, the measurement of lipid peroxidation products and of total antioxidant capacity appears feasible by catheter sampling from the great cardiac vein blood. The magnitude and the persistence of changes observed after a 2 min coronary occlusion suggest that these measurements may have a much greater sensitivity for detecting mild or patchily distributed foci of ischemia than the measurement of myocardial lactate production during ischemia. Indeed, the elevated lactate concentration is by definition associated with reduced blood flow draining is-

chemic myocardium and it may be diluted by the flow draining normally perfused myocardium with normal lactate levels. Moreover, lactate production ceases as soon as ischemia is relieved; conversely, the production of markers of reperfusion injury is triggered by re-oxygenation and appears to last up to 15 min.

Therefore, these preliminary data show a remarkable sensitivity of the three methods employed for the detection of post-ischemic reperfusion injury in cardiac venous effluent blood; this approach may be useful for assessing the magnitude of myocardial oxidative stress and, possibly, also for detecting small foci of myocardial ischemia-reperfusion injury in patients with microvascular angina.

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