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Large, Sustained Cardiac Lipid Peroxidation and Reduced Antioxidant Capacity in the Coronary Circulation After Brief Episodes of Myocardial Ischemia

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OBJECTIVES We sought to investigate whether a brief episode of myocardial ischemia produces a detectable cardiac oxidative stress in patients undergoing elective coronary angioplasty (PTCA).

BACKGROUND Although cardiac oxidative stress has been clearly demonstrated in experimental models of ischemia-reperfusion, its presence in patients after transient myocardial ischemia is still unclear.

METHODS In order to evaluate oxidative stress in ischemic cardiac regions, plasma conjugated dienes (CD), lipid hydroperoxides (ROOHs) and total antioxidant capacity (TRAP), independent indexes of oxidative stress, were measured in the aorta and great cardiac vein (GCV) before (t_0), 1, (t_1), 5 (t_5) and 15 min (t_{15}) after first balloon inflation in 15 patients undergoing PTCA on left anterior descending coronary artery (Group 1); six patients with right coronary artery stenosis (Group 2), which is not drained by the GCV, were studied as controls.

RESULTS In Group 1 at baseline, CD and ROOHs levels were higher in GCV than in aorta ($p < 0.01$ for both), and TRAP levels were lower ($p < 0.01$). Aortic levels of CD, ROOHs and TRAP did not change at any time after t_0; venous levels of CD and ROOHs levels markedly increased at t_1, at t_5 and remained elevated at t_{15} ($p < 0.01$ for all comparisons vs. t_0); venous levels of TRAP decreased at t_1 and t_5 ($p < 0.01$ vs. t_0) and returned to normal at t_{15}. In Group 2, CD, ROOHs and TRAP levels were similar in the aorta and GCV and did not change throughout the study.

CONCLUSIONS Short episodes of myocardial ischemia during PTCA induce a sustained oxidative stress, which is detectable in the venous effluent of reperfused myocardium. (J Am Coll Cardiol 2000;35:633–9) © 2000 by the American College of Cardiology

Lipid peroxidation of membrane polyunsaturated fatty acids by reactive oxygen species (ROS) is considered the major mechanism of ischemia-reperfusion injury (1). An enhanced cardiac oxidative stress has consistently been demonstrated in experimental ischemia-reperfusion models (2,3) and after severe myocardial ischemia in patients with acute myocardial infarction (4–6) or during extracorporeal circulation (7,8); however, its presence in patients undergoing brief episodes of myocardial ischemia is still controversial (9–15), partially because of the intrinsic limitations in the available methods of in vivo measurement. Since direct assessment of ROS (2,3) is not applicable in humans, plasma levels of lipid peroxidation products and of antioxidants are the most commonly investigated markers of oxidative stress in clinical studies (16–19). Conjugated dienes (CD) and lipid hydroperoxides (ROOHs) are two independent and stable indexes of in vivo ROS production (18,19); they are generated at intermediate stages of lipid peroxidation cascade and are considered to be more specific than other oxidative products, such as malondialdehyde (18–20). Plasma antioxidant capacity (TRAP) is an accurate index of oxidative stress, which provides a measure of total plasma defenses against ROS (21).

Therefore, in order to investigate cardiac oxidative stress and its response to transient episodes of myocardial ischemia, we measured levels of CD, ROOHs and TRAP in the

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Abbreviations and Acronyms

A.U.	= arbitrary units
CD	= conjugated dienes
GCV	= great cardiac vein
LAD	= left anterior descending coronary artery
PTCA	= percutaneous transluminal coronary angioplasty
RCA	= right coronary artery
ROOHs	= hydroperoxides
ROS	= reactive oxygen species
TRAP	= plasma antioxidant capacity
t_0	= before first balloon inflation
t_1	= 1 min after first balloon deflation
t_5	= 5 min after first balloon deflation
t_{15}	= 15 min after first balloon deflation

coronary circulation of anginal patients undergoing elective percutaneous transluminal coronary angioplasty (PTCA), a clinical model of controlled ischemia-reperfusion.

METHODS

Patients. Twenty-one patients with a single, discrete coronary stenosis undergoing elective PTCA were studied (Table 1): 15 had a proximal stenosis of the left anterior descending coronary artery (LAD) (Group 1) and six had a proximal stenosis of the right coronary artery (RCA) (Group 2). Patients with angina episodes in the last 12 h before the procedure, myocardial infarction in the last three months, complex or severe (>90%) coronary stenosis, angiographically detectable collateral circulation or intercurrent inflammatory process were excluded. At the time of PTCA, all patients were on oral aspirin; 17 patients were on oral calcium-antagonists, 12 on oral nitrates and 9 on beta-adrenergic blocking agents. No patient was on vitamins.

The protocol was approved by the Ethics Committee of the Catholic University of Rome; all patients gave informed consent.

Protocol. In a fasting state, a coronary guiding catheter was advanced in the aorta, and it was utilized to perform PTCA and arterial sampling; a multipurpose catheter was positioned, through femoral access, into the great cardiac vein (GCV). As the GCV selectively drains blood from left coronary territory only, in Group 1 patients, who had PTCA on LAD, blood samples were collected from the cardiac regions undergoing ischemia-reperfusion; conversely, in Group 2 patients, who had PTCA on the RCA, venous blood was collected from cardiac regions that were not jeopardized by ischemia-reperfusion. Six Group 1 and all six Group 2 patients underwent aorta and GCV sampling before the first balloon inflation (t_0) and at 1 (t_1), 5 (t_5) and 15 min (t_{15}) after first balloon deflation. In the remaining nine Group 1 patients, blood samples were collected at t_0 from the aorta and GCV, and at t_1, t_5 and t_{15} from the GCV only. Blood was collected in heparinized syringes for measurement of oxygen saturation and for extraction of plasma, which was stored at −70° and used within one month. Arterial blood pressure, heart rate and three ECG leads were continuously monitored throughout the study. Endovenous heparin was given at baseline (100 U/kg) and throughout PTCA in order maintain the activated coagulation time ≥300 s. Anginal pain (scored from 0 to 10) and maximal ST segment shift were regarded as indexes of balloon induced ischemia and the increase of oxygen saturation in the GCV as a marker of postischemic reactive hyperemia.

Table 1. Clinical and Procedural Characteristics

	Group 1 15 Patients (%)	Group 2 6 Patients (%)	p Value
Age (yr)	63 ± 9	62 ± 8	NS
Men (n)	13 (87)	3 (50)	NS
Diabetes (n)	3 (20)	1 (17)	NS
Hypertension (n)	8 (53)	2 (33)	NS
Cigarette smoking (n)	2 (13)	1 (17)	NS
Plasma cholesterol >200 mg/dl (n)	6 (40)	2 (33)	NS
Previous MI (n)	6 (40)	3 (50)	NS
Stable/unstable angina (n)	8/7	3/3	NS
CCS I–II/III–IV (n)	6/2	1/2	NS
Braunwald classification IB/II–IIIB (n)	0/7	0/3	NS
Diameter stenosis (%)	80 ± 9	78 ± 4	NS
Duration of first balloon inflation (s)	111 ± 43	156 ± 35	NS
ST-segment shift (mm)	1.3 ± 0.8	0.9 ± 0.7	NS
Angina score (from 0 to 10)	6 ± 4	5 ± 4	NS

CCS = Canadian Cardiovascular Society classification of angina; MI = myocardial infarction; n = number; NS = not significant.

Measurement of lipid peroxidation products. Plasma lipids were extracted by a modification of the Folch method (22). Conjugated dienes (CD) were measured by second derivative spectrophotometry (18). Minima at 232 nm and 246 nm were ascribed to the trans-trans and cis-trans conjugated dienes isomers, respectively, and quantified in arbitrary units as $d^2A/d\ lambda^2$, which represent the measurement from minima to adjacent maxima at the higher wavelength. Intra- and interassay coefficients of variation for this method were 7.5% and 10.2%, respectively. Hydroperoxides were measured with the FOX Version II assay for lipid ROOHs (19). They were determined as a function of the mean absorbance difference of samples with and without elimination of ROOHs by triphenylphosphine. Intra- and interassay coefficients of variation for this method were 5.0% and 7.5%, respectively.

Measurements of plasma antioxidant capacity. Plasma levels of TRAP were measured by the assay previously described by Rice-Evans and Miller (21), which is based on the quenching of the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid [ABTS]) (Sigma, St. Louis, Missouri) radical cation by antioxidants. ABTS radical cation formation was continuously monitored by absorbance increase at 734 nm, at 20° C. The assay was standardized using Trolox (Fluka Chemie, Buchs, Switzerland), a water soluble vitamin E analog. Intra- and interassay coefficients of variation for this method were 7.5% and 9.1%, respectively.

Statistics. Levels of CD, ROOHs and TRAP and their venous-arterial differences were normally distributed and are expressed as mean ± SD. Arterial and venous levels of CD, ROOHs and TRAP and their venous-arterial differences at different time-points were compared by analysis of variance, and for a $p < 0.05$ comparisons were carried out using *t* test with Bonferroni correction. Analysis of variance and *t* test with Bonferroni correction was also used to compare oxygen saturation in the GCV. Linear regression analysis was used to define the relationship between indexes of ischemia and markers of peroxidation. A p value <0.05 (two-tailed) was considered significant.

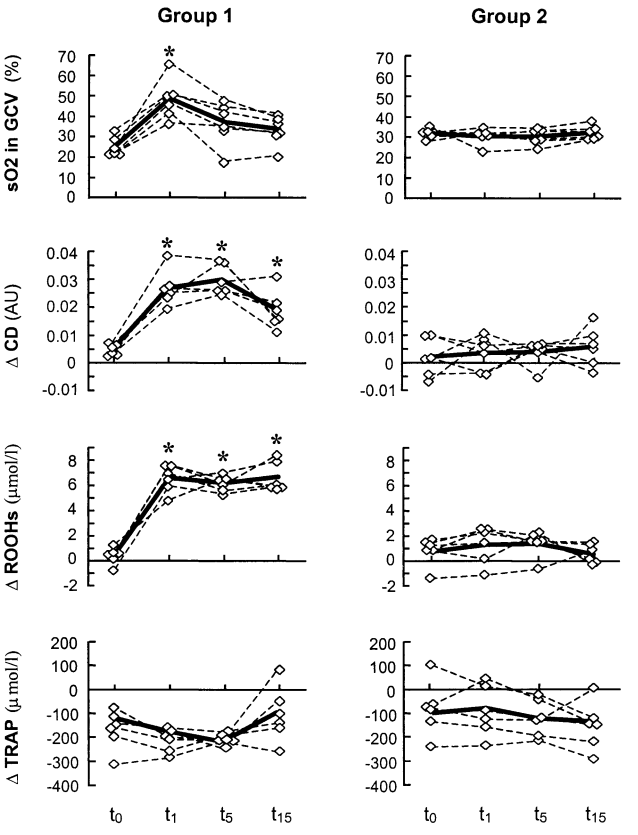

Figure 1. The graphs refer to the six Group 1 and six Group 2 patients in whom paired blood samples from great cardiac vein (GCV) and aorta were obtained throughout the study. Blood oxygen saturation (sO2) significantly increased in the GCV 1 min after balloon deflation (t_1) compared with baseline (t_0); no increase was observed in Group 2 patients. In Group 1 patients, differences between concentration of conjugated dienes (CD) in GCV and aorta (delta CD) consistently increased 1 (t_1) and 5 min (t_5) after balloon deflation and were still elevated at 15 min (t_{15}) compared with t_0; differences between concentration of hydroperoxides (ROOHs) in GCV and aorta (delta ROOHs) showed similar changes. In Group 2 patients, delta CD and delta ROOHs did not change throughout the study. Differences between plasma antioxidant capacity (TRAP) values in GCV and aorta (delta TRAP) did not change at t_1, t_5 and t_{15} in both groups. **Thick lines** represent mean values; **dashed lines** represent individual data. *$p < 0.01$ vs. baseline.

RESULTS

Clinical and procedural characteristics were not different in the two groups of patients (Table 1).

Blood oxygen saturation. In Group 1, undergoing PTCA on the LAD, oxygen saturation in the GCV was unchanged immediately before balloon deflation (30.1 ± 8.6% vs. 28.0 ± 5.5% at t_0, p = NS) but increased markedly at t_1 (49.3 ± 7.8%, $p < 0.01$ vs. t_0), demonstrating postischemic reactive hyperemia; conversely, no significant change was observed in Group 2, undergoing PTCA on the RCA (29.4 ± 5.0 at t_1 vs. 30 ± 4.0 at t_0, p = NS) (Fig. 1).

Lipid peroxidation at baseline. In Group 1 patients, CD and ROOHs at t_0 were significantly higher in the GCV than in the aorta (0.035 ± 0.001 vs. 0.031 ± 0.002 arbitrary units [A.U.], $p < 0.01$ and 3.41 ± 0.9 vs. 2.35 ± 0.03 μmol/l, $p < 0.01$, respectively), while TRAP was lower (809 ± 52 vs. 952 ± 66 μmol/l, $p < 0.01$); conversely, in Group 2 patients, CD (0.036 ± 0.004 vs. 0.035 ± 0.004 A.U.), ROOHs (3.26 ± 0.80 vs. 2.58 ± 0.82 μmol/l) and TRAP levels (904 ± 112 vs. 989 ± 35 μmol/l, P = NS) at t_0 were similar in the GCV and aorta.

Lipid peroxidation after ischemia-reperfusion. *Group 1 patients.* In the six Group 1 patients, who had blood sampling in both the aorta and GCV at all time-points, arterial levels of CD, ROOHs and TRAP did not change at

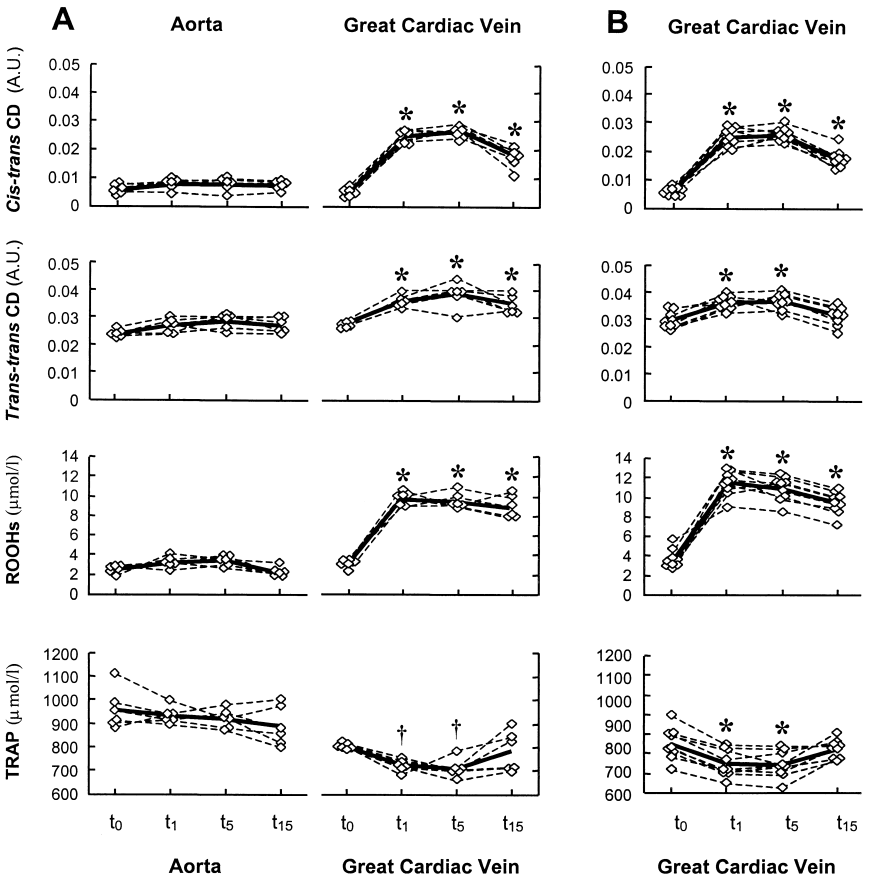

Figure 2. Levels of cis-trans conjugated dienes (CD), trans-trans CD, hydroperoxides (ROOHs) and total antioxidant capacity (TRAP) in the six Group 1 patients in whom blood samples were obtained from GCV and aorta **(A)** and in the nine Group 1 patients sampled from GCV only but not from aorta **(B)**. Venous levels of CD isomers and ROOHs markedly increased and TRAP values decreased 1 (t_1) and 5 (t_5) min after balloon deflation; levels of CD isomers and ROOHs were still elevated, whereas TRAP values returned to baseline levels 15 min after balloon deflation (t_{15}). No changes of CD, ROOHs or TRAP levels were observed in aorta. **Thick lines** represent mean values; **dashed lines** represent individual data. *$p < 0.01$ vs. baseline. †$p < 0.05$ vs. baseline.

any time-point after t_0 (p = NS at each time-point vs. t_0 for both CD, ROOHs and TRAP). In the same patients, venous levels of CD and ROOHs markedly increased at t_1 and at t_5 and remained elevated at t_{15} ($p < 0.01$ at each time-point vs. t_0, for both CD and ROOHs); TRAP significantly decreased at t_1 ($p < 0.05$ vs. t_0), and t_5 ($p < 0.05$ vs. t_0) and returned to baseline at t_{15} (p = NS vs. t_0) (Fig. 2A). Similar changes of CD, ROOHs and TRAP were observed in the GCV in the remaining nine Group 1 patients, who had venous but not arterial sampling following t_0 (Fig. 2B). In all Group 1 patients, although both venous levels of cis-trans and trans-trans CD increased after balloon deflation, cis-trans CD showed a larger increase (peak increase 386 ± 127% vs. t_0) than trans-trans CD (30 ± 15% vs. t_0; $p < 0.01$ vs. cis-trans CD increase) (Fig. 2). In the same patients, the peak increases in the GCV of total CD ($r = -0.83$, $p < 0.001$), cis-trans CD ($r = -0.90$, $p < 0.001$), trans-trans CD ($r = -0.75$, $p = 0.001$) and ROOHs ($r = -0.92$, $p < 0.001$) were inversely correlated with their baseline levels; conversely, they were not related to severity of angina, ST segment shift or reactive hyperemia (Fig. 3).

In the six Group 1 patients, who had blood sampling in both the aorta and GCV at all time-points, venous-arterial differences of CD significantly increased at t_1 ($p < 0.01$) and at t_5 ($p < 0.01$ vs. t_0) and remained elevated at t_{15} ($p < 0.01$ vs. t_0) (Fig. 1); venous-arterial differences of cis-trans CD markedly increased at t_1 and t_5, and they remained elevated at t_{15}, ($p < 0.01$ at each time-point vs. t_0), whereas a milder increase of venous-arterial differences of trans-trans CD was observed at t_1 (p = NS vs. t_0) and t_5 ($p < 0.05$ vs. t_0). Venous-arterial differences of ROOHs showed similar changes ($p < 0.01$ at each time-point vs. t_0) (Fig. 1). Venous-arterial differences of TRAP mildly decreased at t_1 and t_5 without achieving statistical significance (Fig. 1).

Group 2 patients. Aortic and venous levels of CD, ROOHs and TRAP were remarkably stable at all time-points following t_0 (p = NS at each time-point vs. t_0 for both arterial and venous CD, ROOHs and TRAP levels) (Fig. 4). In the same group of patients, venous-arterial

differences of CD, ROOHs and TRAP did not change at any time (Fig. 1).

DISCUSSION

In our study, the use of three independent markers of oxidative stress and selective GCV sampling allowed us to demonstrate a marked and sustained cardiac oxidative stress after short episodes of ischemia-reperfusion in anginal patients undergoing elective PTCA on the LAD. Conversely, no transcardiac changes of oxidative stress markers were observed in the GCV of patients undergoing PTCA of the RCA. The presence of reactive hyperemia (assessed by measuring sO2 in the GCV) in patients who underwent PTCA of the LAD, but not in those who underwent PTCA of the RCA, confirmed that the catheter tip was correctly positioned in the GCV, which selectively drains blood from myocardial regions perfused by the LAD. Therefore, a brief episode of myocardial ischemia caused a marked oxidative stress in the coronary circulation of ischemic regions, but not in normal myocardial regions nor in the systemic circulation.

Detection of oxidative stress in humans. Previous studies showed absence of (13–15), or mild, oxidative stress (6,9–12) after transient episodes of ischemia-reperfusion, probably because of low sensitivity and specificity of the assays (5,9–15) and of peripheral blood sampling (14,15).

Direct measurement of oxygen free-radical generation by electron spin resonance is accurate in animal models (2,3), but it is not applicable in humans; therefore, ex vivo spin-trapping techniques have been utilized in clinical studies (4), but they are limited by the ex vivo production of secondary oxidative species. Malondialdehyde, an end product of lipid peroxidation, has been largely investigated by the thiobarbituric acid assay, which is now questioned because it underestimates the extent of lipid peroxidation and has a limited specificity (20). Isoprostanes are free-radical catalyzed products of arachidonic acid, and they have been recently proposed as noninvasive markers of in vivo oxidative stress (5,6,17). Their urinary levels show a sharp increase in patients with acute myocardial infarction a few hours after reperfusion, either by thrombolysis or primary PTCA (5,6). Yet only a mild increase (20% to 30%) was observed in anginal patients 6 h after diagnostic coronary arteriography and elective PTCA (6). Of note, in our study, markers of oxidative stress increased by 80% to 230% in the GCV after a single, brief balloon inflation. Therefore, although isoprostanes are attractive noninvasive markers, their accuracy is insufficient to detect the oxidative stress resulting from brief episodes of myocardial ischemia in humans. To this purpose, a selective cardiac blood sampling is necessary in order to avoid false negative results (14,15); indeed, lipoperoxide levels were remarkably stable throughout the study, both in the aorta and in the venous blood of nonischemic cardiac regions, suggesting that, in such a clinical setting, peroxidative products were promptly diluted and scavenged in the peripheral circulation.

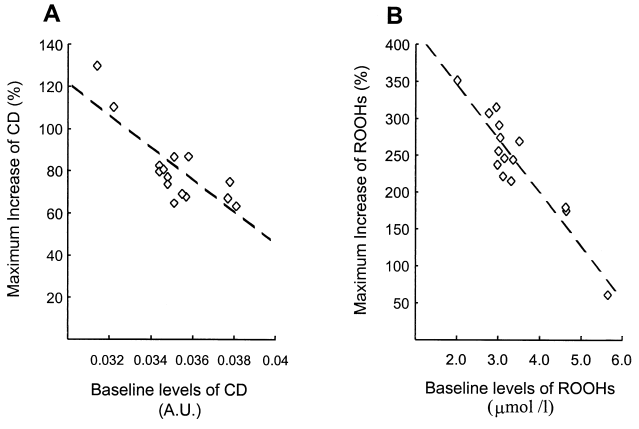

Figure 3. Simple linear regression analysis of the relationship between: **(A)** maximum increase of conjugated dienes (CD) and their baseline levels in the GCV ($r = -0.83$, $p < 0.001$); **(B)** maximum increase of hydroperoxides (ROOHs) and their baseline levels in the GCV ($r = -0.92$, $p < 0.001$).

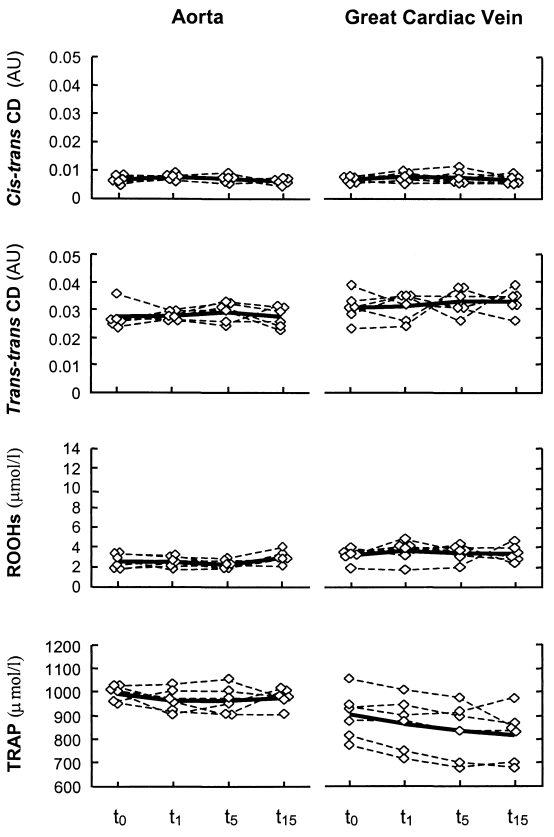

Figure 4. Levels of cis-trans conjugated dienes (CD), trans-trans CD, hydroperoxides (ROOHs) and total antioxidant capacity (TRAP) in the blood samples from aorta and great cardiac vein (GCV) of six Group 2 patients. Levels of CD isomers, ROOHs and TRAP did not change at 1 (t_1), 5 (t_5) nor 15 min (t_{15}) after balloon deflation, both in aorta and GCV. **Thick lines** represent mean values; **dashed lines** represent individual data.

In our study we chose two accurate methods for measuring lipid peroxide generation (18,19). Second derivative spectroscopy for CD determination provides greater sensitivity compared with simple absorption spectroscopy and gives additional information on the redox status as it discriminates between two different CD configurations (e.g., cis-trans and trans-trans); cis-trans CD are formed in the presence of adequate levels of antioxidants; conversely, with low antioxidant levels, the kinetic equilibrium of oxidation is shifted towards trans-trans isomers, indexes of irreversible oxidative damage (18). The FOX Version II assay outperforms other assays for ROOHs assessment for its simplicity and reproducibility, providing a measurement of all classes of hydroperoxides in plasma (19).

Previous studies evaluated changes of single antioxidants during PTCA with conflicting results (10,14); TRAP is more likely to represent the real status of plasma defenses, as it is largely determined by the overall effect of water-soluble antioxidants (uric acid, ascorbic acid, protein thiols and bilirubin) and lipid-soluble antioxidants (alpha-tocopherol, beta-carotene, ubiquinol) (21).

Mechanisms of lipid peroxide generation after myocardial ischemia-reperfusion. Our results consistently demonstrated a marked and sustained increase of lipid peroxidation and a transient decrease of antioxidant defenses, which selectively affected the cardiac regions undergoing transient episodes of ischemia-reperfusion. The simultaneous increase of oxygen saturation and of peroxidative markers in GCV, peaking 1 min after balloon deflation, suggested a ROS-mediated oxidative stress caused by sudden reoxygenation of a previous ischemic tissue (2). Both CD isomers consistently increased in the GCV; however, the larger increase of cis-trans CD isomers compared with that of trans-trans isomers suggested that a single, brief episode of ischemia-reperfusion generated a marked but reversible oxidative injury. The simultaneous reduction of TRAP values and the increase of ROOHs levels further support a reperfusion-mediated oxidative stress; indeed, ROOHs are generated, following CD formation, by oxygen incorporation and further propagation of lipoperoxidative reactions (19).

Timing of lipid peroxide generation in our study is similar to that reported by previous studies in a similar clinical setting with a peak lipid peroxide production between 1 and 5 min after reperfusion (3,10–12). However, we observed a sustained release of both CD and ROOHs up to 15 min after balloon deflation, rather than a return to baseline levels, as reported by previous studies. This result may be explained by longer balloon inflation (111 ± 43 s instead of the 60 s reported in previous studies) or a higher sensitivity of our markers of oxidative stress (18–20). Mitochondrial respiratory chain and endothelial xanthine oxidase are the most likely metabolic pathways of ROS generation after brief episodes of myocardial ischemia-reperfusion. Activated neutrophils can also contribute to ROS generation (23); platelet derived ROS have been recently demonstrated in vitro after 30 min of anoxia-reperfusion (24). However, at least in our clinical model of a single, 2 min episode of ischemia-reperfusion, both platelets and neutrophils are unlikely to play a major role, as experimental studies have shown that longer anoxic-reoxygenation periods are needed in order to release detectable amounts of ROS by these cell types (24,25). Further clinical studies are warranted in order to clarify the relative contribution of these potential sources of ROS following in vivo brief episodes of ischemia-reperfusion.

Oxidative stress at baseline. We also observed a slight but significant cardiac release of lipoperoxides and a reduced plasma antioxidant capacity at baseline, in the coronary circulation containing coronary stenosis (Group 1 patients), in the absence of clinical evidences of recent episodes of myocardial ischemia (last 12 h). The mechanisms responsible for this mild baseline transcardiac production of lipoperoxides cannot be deduced from the results of our study. However, the absence of a transcardiac oxidative stress at baseline in normal cardiac regions suggested that repeated ischemia-reperfusion episodes and/or the atherosclerotic process may cause a persistent enhancement of ROS generation by locally activated vascular cells (26,27).

Of note, this baseline oxidative stress was inversely correlated with the peak increase of CD and ROOHs after balloon deflation. This result is consistent with recent experimental studies showing that an enhanced oxidative stress contributes to cardiac protection from further oxidative injury through an up-regulation of cellular antioxidant enzymes (28,29). However, baseline levels of TRAP were not related to the increase of lipoperoxides, probably because they mainly reflect plasma content of chain-breaking antioxidants instead of the cellular content of inducible enzymatic defenses (10,21).

Conclusions. Plasma levels of CD, ROOHs and TRAP represent sensitive markers of cardiac oxidative stress in humans. A baseline oxidative stress is detectable only in venous blood draining the stenosed vessel. Short episodes of myocardial ischemia induced a consistent and sustained lipid peroxidation, which is detectable in the venous effluent of reperfused myocardium. These findings suggest that cardiac lipoperoxidation may be a common event following brief episodes of myocardial ischemia and support a role for antioxidant therapy in patients with ischemic heart disease.

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