Proteomic analysis of epicardial and subcutaneous adipose tissue reveals differences in proteins involved in oxidative stress

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Epicardial adipose tissue (EAT) is an endocrine organ adjacent to coronary arteries and myocardium without anatomy barriers. Locally produced adipokines may reflect or affect to cardiovascular physiology and pathology. Our aim was to study the protein expression profiles of EAT and subcutaneous adipose tissue (SAT) to identify local candidate molecules characterizing EAT in patients with cardiovascular disease. EAT and SAT samples were collected from 55 patients undergoing heart surgery. Proteins from these tissues were separated by two-dimensional (2D) gel electrophoresis, and differences between them were identified by MALDI-TOF/TOF spectra. Differences in protein levels were further investigated by real-time RT-PCR and Western blots, and production of reactive oxygen species (ROS) in EAT and SAT was evaluated by nitroblue tetrazolium chloride assays. ROS production was higher in EAT than SAT. We have found mRNA differences for catalase, glutathione S-transferase P, and protein disulfide isomerase, and 2D Western blots additionally showed post-translational differences for phosphoglycerate mutase 1; all four are related to oxidative stress pathways. EAT suffers greater oxidative stress than SAT in patients with cardiovascular diseases and exhibits associated proteomic differences that suggest the possibility of its association with myocardial stress in these patients.

Obesity is one of the main causes of metabolic diseases. It is associated with a chronic inflammatory response and cardiovascular diseases (34). However, risk depends significantly on the distribution of adipose tissue in the body; for example, metabolic risk factors were more associated with omental adipose tissue (OAT) than with subcutaneous adipose tissue (SAT) among 3,001 participants in the Framingham Heart Study (6). These differences are plausibly due to different adipose tissues differing in adipokine expression and the production of inflammatory reactive oxygen species (ROS), which are known to be associated with cardiovascular disease (8); OAT is associated with more circulating substances related to inflammation and oxidative stress than SAT is (24). Epicardial adipose tissue (EAT) is a visceral fat pad adjacent to coronary arteries and the myocardium (11). The amount of EAT is related to left ventricle mass (13) and to the amount of intra-abdominal visceral fat, and visceral adiposity can in fact be evaluated by means of its echocardiographic measurement (10). Like other adipose tissues, EAT is now recognized as an endocrine organ, and its proximity to myocardium and coronary arteries suggests the possibility of paracrine action on these structures. In fact, in patients with coronary artery disease (CAD) (5, 12) and hypertension (32), EAT produces lower levels of the anti-inflammatory adipokine adiponectin and higher levels of the proinflammatory cytokines IL-6 and leptin than does the EAT of non-CAD or nonhypertensive patients undergoing cardiac surgery, whereas the levels of adiponectin in SAT do not differ between hypertensive and nonhypertensive patients (32), and proinflammatory cytokines are higher in the EAT than the SAT of CAD patients (20).

In morbidly obese women with polycystic ovary syndrome, proteomic analyses of OAT by means of two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS) have identified alterations in the levels of proteins involved in lipid and glucose metabolism, oxidative stress, and adipocyte differentiation (3). Proteomic differences between OAT and SAT have also been reported (23). As far as we know, however, no proteomic studies have hitherto compared SAT and EAT in patients with cardiovascular disease.

Thus we thought that locally produced adipokines by EAT might be reflecting or affecting to cardiovascular physiology and pathology due to its proximity to coronary arteries and myocardium without anatomy barriers. Accordingly, we studied the protein expression profiles of EAT and SAT to identify local candidate molecules characterizing EAT in patients with cardiovascular disease. In view of the above-noted precedents, special attention was paid to proteins related to oxidative stress.

METHODS

Patients

We studied material from a total of 55 patients undergoing heart surgery (valve replacement or coronary artery bypass graft). Means ± SD age was 71 ± 9 yr, and body mass index (BMI) was 28 ± 4 kg/m²; none had previous heart surgery or suffered severe infective disease. All gave written informed consent, and the study protocol was approved by the Galician Clinical Research Ethics Committee and carried out in accordance with the Declaration of Helsinki. Table 1 summarizes the clinical characteristics of the patients whose material was used in the various experiments described below.

Biological Material

EAT (−0.2–0.5 g wet weight) was obtained from above the right ventricle, and SAT (−2 g wet weight) was obtained from the thorax. Samples were placed in 5 ml of physiological salt solution (PSS) containing (in mM) 0.5 EDTA, 5 KCl, 10 HEPES, 2 MgCl₂, 10
NaHCO₃, 0.5 KH₂PO₄, 0.5 NaH₂PO₄, 10 glucose, 110 NaCl, and 0.16 CaCl₂ (pH 7.4) and were then centrifuged at 300 g for 15 min to remove blood before freezing in liquid nitrogen and storage at −80°C.

Extraction of Protein

Adipose tissue samples weighing 100–150 mg were rinsed in 5 ml of PSS, centrifuged for 1 min at 300 g to remove residual blood, placed in 1 mg:ml weight:volume ratio in a lysis buffer containing 125 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 100 mM diethiothreitol (DTT; from Sigma-Aldrich) and anti-protease cocktail (from Sigma-Aldrich), and sheared with a pestle using a sample grinding kit (GE Healthcare) following the manufacturer’s protocol and were then resuspended in sample solution (7 M urea, 2 M thiourea, 4% CHAPS, and 40 mM DTT) at a final concentration corresponding to 1 mg of homogenized tissue per μl.

Two-dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional (2D) electrophoresis (2DE) was performed on paired EAT and SAT samples from 11 patients (age, >60 yr in all cases, BMI, 20–30 kg/m²). Proteins were separated in the first dimension in 13 cm IPG strips (GE Healthcare; pH 3–10 NL) and was applied to the strips in the course of 10 h active rehydration with 0.5% IPG buffer and 1.2% DeStreak solution; GE Healthcare). Proteins were precipitated with a 2-D Clean-Up kit (GE Healthcare) following the manufacturer's protocol and were then extracted-homogenized tissue, prepared as described above, were separated and were then resuspended in sample solution (7 M urea, 2 M thiourea, 4% CHAPS, and 40 mM DTT) at a final concentration corresponding to 1 mg of homogenized tissue per μl.

n, number of patients included in the analysis. NBT, nitroblue tetrazolium chloride.

<table>
<thead>
<tr>
<th>Total n</th>
<th>Gender, Male</th>
<th>Hypertension</th>
<th>Diabetes Mellitus</th>
<th>Coronary Artery Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteomic analysis</td>
<td>11</td>
<td>6</td>
<td>54.5</td>
<td>9</td>
</tr>
<tr>
<td>Real time PCR</td>
<td>24</td>
<td>14</td>
<td>58.3</td>
<td>21</td>
</tr>
<tr>
<td>Western blot analysis</td>
<td>24</td>
<td>15</td>
<td>62.5</td>
<td>19</td>
</tr>
<tr>
<td>NBT assay</td>
<td>10</td>
<td>5</td>
<td>50.0</td>
<td>7</td>
</tr>
</tbody>
</table>

| All patients | 55 | 28 | 50.9 | 37 | 67.3 | 12 | 21.8 | 24 | 43.6 |

Table 1. Characteristics of patients

motography (HPLC) grade, Scharlau), dehydrated by addition of acetone (HPLC grade, Scharlau), dried in a SpeedVac, and digested with Promega modified porcine trypsin (20 ng/μl in 20 mM ammonium bicarbonate) for 16 h at 37°C. Peptides were extracted by three 20-min incubations in 40 μl of 60% acetone in 0.5% formic acid, and the pooled extracts were concentrated in a SpeedVac and stored at −20°C.

Mass spectrometric analysis. Dried peptide samples were dissolved in 4 μl of 0.5% formic acid, and 0.5 μl of this solution was mixed with an equal volume of matrix solution composed of 3 mg of α-cyano-4-hydroxycinnamic acid in 50% acetone containing 0.1% of trifluoroacetic acid. This mixture was deposited by the thin layer method in an Opti-TOF 384-well MALDI plate (Applied Biosystems). Mass spectrometric data were obtained in an automated analysis loop in an Applied Biosystems 4800 MALDI-TOF/TOF analyzer, MS data using positive ion reflector mode, an Nd:YAG laser (λ = 355nm), an average 1,000 laser shots and at least three trypsin autolysis peaks for internal calibration, and MS/MS data using precursors selected with a relative resolution of 300 (FWHM) and suppression of ions resulting from the dissociation of metastable species. Data were analyzed with 4000 Series Explorer Software V3.5. MS, and MS/MS data were fed to Mascot v2.1 (Matrix Science) via GPS Explorer v3.6 (Applied Biosystems) to search a nonredundant database (SwissProt release 56.0) with 30 ppm precursor tolerance, 0.35 Da MSMS fragment tolerance, and one missed cleavage allowed. All spectra and database results were inspected in detail using the above software.

mRNA extraction and real-time RT-PCR. With the use of Oligotex Direct mRNA Kits (Qiagen GmbH), mRNA was isolated from pairs of ~100 mg EAT and SAT samples from 24 patients (age 70 ± 10 yr, BMI 28 ± 5 kg/m²); the final concentration of each sample corresponded to 1 μg of tissue per μl of solution. mRNA solution (4.14 μl) was transcribed using 200 U of MMLV reverse transcriptase (Invitrogen) in 30 μl of a pH 8.4 solution containing 20 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂, 2.5 mM dNTPs, 20 U of RNase inhibitor, and random primers; the program followed was 50 min at 37°C, 10 min at 42°C, and 5 min at 95°C. Real-time quantitative PCR was performed using 8 μl (4 μl per each duplicate) of complementary DNA, Fast Start SybrGreen (Roche Diagnostics) as fluorochrome, and specific primers at a concentration of 300 nM. The genes studied are listed in Table 2 together with the corresponding primers and amplification conditions; the housekeeping gene used as reference was ACTB. Fluorescence curves were analyzed using the program Chromo 4 (MJ Research); the relative expression of each gene of interest was calculated with respect to actin expression. The validity of the amplification was checked by examining melting curves. All experiments were performed in duplicate.

Western blot analyses. One-dimensional (1D) Western blot analyses were performed on paired samples of EAT and SAT from 24 patients (age 72 ± 8 yr, BMI 28 ± 4 kg/m²), and 2D analyses on samples from three of these patients. In the 1D case, 6 μg of protein extract-homogenized tissue, prepared as described above, were separated in 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane over 45 min at 280 mA; 2D was performed as described above, and the resulting protein spots were

Identification of Proteins by Mass Spectrometry

In-gel digestion and peptide extraction. Protein spots chosen for mass spectrometric analysis were excised from the gels and digested as per Shevchenko et al. (30), with minor modifications. Gel pieces were successively reduced with 10 mM DTT in 50 mM amionium bicarbonate (Sigma-Aldrich), alkylated with 55 mM iodoacetamide (Sigma-Aldrich) in the same medium, rinsed with 50 mM ammonium bicarbonate in 50% methanol [high-performance liquid chro
Table 2. Primers and conditions of real time PCR

<table>
<thead>
<tr>
<th>Gene/Protein</th>
<th>Accession Number</th>
<th>Primers, Foward/Reverse (5’→3’)</th>
<th>AT, °C</th>
<th>Ext, °C</th>
<th>bp</th>
<th>MT, °C</th>
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<tr>
<td>ACTB/ACTB</td>
<td>NM_001101.2</td>
<td>TTCTGACCCGATGCGCAGAT</td>
<td>HK</td>
<td>HK</td>
<td>198</td>
<td>87</td>
</tr>
<tr>
<td>CAT/CATA</td>
<td>NM_001572.2</td>
<td>GCCCGGACCCCGATAATCTTT</td>
<td>58</td>
<td>72, 1'</td>
<td>203</td>
<td>82</td>
</tr>
<tr>
<td>GSTP1/GSTP1</td>
<td>NM_000852.2</td>
<td>CCACCTAAACGTCCTGCTGCTA</td>
<td>60</td>
<td>No</td>
<td>188</td>
<td>84</td>
</tr>
<tr>
<td>P4HB/PDIA1</td>
<td>NM_000918.3</td>
<td>CTGCAAGAAGATGCGTTGTT</td>
<td>60</td>
<td>No</td>
<td>193</td>
<td>80</td>
</tr>
<tr>
<td>PGAM1/PGAM1</td>
<td>NM_002629.2</td>
<td>CGAGGGGAGACTGCTACTGA</td>
<td>60</td>
<td>No</td>
<td>183</td>
<td>81</td>
</tr>
</tbody>
</table>

AT, annealing temperature; Ext, extension step; bp, amplicon length in base pairs; MT, melting temperature; HK, housekeeping, used at all conditions; CATA, catalase; GSTP1, glutathione S-transferase P; PDIA1, protein disulfide isomerase; PGAM1, phosphoglycerate mutase 1.

Electroblotted on PVDF membrane over 45 min at 600 mA. The PVDF membranes were blocked for 2 h at room temperature with 5% of milk in TBST containing 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20; exposed for 3 h to primary antibodies [rabbit catalase (CATA; 1:2,000 dilution, Abcam), mouse GSTP1 (1:1,000), mouse phosphoglycerate mutase 1 (PGAM1; 1:1,000), mouse ACTB (1:1,000), or mouse protein disulfide isomerase (PDIA1; 1:750); all from Santa Cruz Biotechnology] and treated with peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (ECL; from Pierce), and in the ID analyses blots were quantified densitometrically using ImageJ software, and target protein levels were expressed relative to housekeeping ACTB. All experiments were performed in duplicate.

Immunohistochemistry. Samples of EAT and SAT were fixed in formalin, embedded in paraffin, and cut in 4 μm sections. Antigens in deparaffinized, rehydrated sections were unmasked by 20 min in 0.25 mM Tris-EDTA (pH 9) in a water bath at 99°C, followed by cooling to room temperature over a further 20 min. The sections were then incubated with CATA antibody (dilution 1:100), followed by LSAB + Peroxidase (Dako Diagnostics) as streptavidin-biotin conjugated secondary antibody, and labeled antigen was visualized with 3,3’-diaminobenzidine tetrahydrochloride (Dako Diagnostics). Haematoxylin staining was performed using standard procedures. Negative controls were processed without the primary antibody.

Nitroblue tetrazolium chloride reduction in adipose tissue. The NADPH-induced production of ROS by cellular oxidases in paired EAT and SAT samples from 10 patients was determined by measuring the reduction of nitroblue tetrazolium chloride (NBT) to the corresponding insoluble diformazan salt. Samples (100 mg) were washed in PSS, homogenized in 300 μl of PSS-Ca²⁺ (PSS containing 0.16 mM CaCl₂), and centrifuged for 10 min at 13,000 g for removing rests of homogenate tissue such as membranes or lipids. A volume corresponding to 10 mg of homogenized tissue such as membranes or lipids. A volume corresponding to 10 mg of homogenized tissue such as membranes or lipids. A volume corresponding to 10 mg of homogenized tissue such as membranes or lipids.

RESULTS

2DE and Mass Spectrometry

2DE gels of EAT samples showed 270 ± 43 protein spots matched to the corresponding SAT sample, and gels of SAT samples showed 250 ± 26. Figure 1 shows representative EAT and SAT gels and, for each of the seven spots showing significant differences in intensity between EAT and SAT, the intensity of the spot in each sample. Five spots were more intense in EAT than in SAT, and two more were intense in SAT than in EAT (see Table 3 for fold differences). Except for SSP_8806, all these spots appeared in either the EAT or SAT sample of at least eight patients; SSP_8806 showed up in only six patients.

Mass spectrometry identified eight proteins: two in spot SSP_0207, and one in each of the other six (see Table 3). All had a MASCOT score greater than 100, but one of those attributed to spot SSP_0207, ribosomal protein SA, was ruled out because its molecular weight does not agree with the position of the spot in the gel. Of the remaining seven, five are related to oxidative stress: carbonic anhydrase 1 (CAH1), CATA, glutathione S-transferase P (GSTP1), protein disulfide isomerase (PDIA1), and phosphoglycerate mutase 1 (PGAM1). One of the other two, vimentin, is structural, and the other, aconitase, is a metabolic intermediate. The two with higher levels in SAT than EAT were CAH1 and CATA.

Real Time RT-PCR

Real Time RT-PCR was performed for the four stress-related proteins that were detected in almost all samples: CATA (gene: CAT), GSTP1, PDIA1 (gene: P4HB), and PGAM1. Relative to ACTB, CATA mRNA levels were significantly lower in EAT than SAT (8.81 ± 0.7 as against 9.35 ± 1.2, in arbitrary units; P = 0.022), whereas GSTP1 and PDIA1 mRNA levels were significantly higher in EAT than SAT (7.79 ± 0.6 as against 7.55 ± 0.5, and 8.99 ± 0.5 as against 8.76 ± 0.5; P = 0.016 and P = 0.027, respectively), and PGAM1 mRNA levels were significantly higher in EAT than SAT (7.79 ± 0.6 as against 7.55 ± 0.5, and 8.99 ± 0.5 as against 8.76 ± 0.5; P = 0.016 and P = 0.027, respectively).
levels showed no significant difference (8.56 ± 0.6 in EAT, and 8.35 ± 0.7 in SAT; *P* = 0.128; see Fig. 2A). Interestingly, whereas CATA mRNA levels were lower in EAT than SAT in all 24 subjects, this was not so for the other mRNAs (Fig. 2B).

Moreover, we evaluated the GSTP1, PDIA1, PGAM1, and CATA mRNA expression differences between EAT and SAT in the CAD patients group (*n* = 12). We found that GSTP1, PDIA1, and PGAM1 mRNA levels were significantly higher in EAT than SAT (7.72 ± 0.6 as against 7.40 ± 0.6; *P* = 0.015, 9.02 ± 0.5 as against 8.60 ± 0.4; *P* < 0.001, and 8.53 ± 0.6 as against 8.10 ± 0.6; *P* = 0.002, respectively). However, there was no difference concerning CATA mRNA expression (8.72 ± 0.7 as against 9.31 ± 1.54; *P* = 0.135). After the mRNA expression analysis in non-CAD group (*n* = 12), we detected that GSTP1, PDIA1 and PGAM1 levels were similar in EAT and SAT. Moreover, differences were not detected between CAD and non-CAD in EAT or SAT (supplementary Fig. 1; all supplemental material can be found with the online version of this article).

**Western Blots Analyses and Immunohistochemistry**

Western blot analyses were performed for the same proteins as real time RT-PCR. Figure 3A shows 1D result for a representative patient. For CATA and PGAM1, the quantitative Western blot results were qualitatively in keeping with the real time RT-PCR results, CATA levels relative to ACTB being lower in EAT than SAT (3.1 ± 0.7 as against 5.1 ± 0.4; *P* = 0.003) and PGAM1 showing no significant difference (2.1 ± 1.6 as against 2.2 ± 1.1; *P* = 0.800). Afterward, we evaluated CATA protein contents between EAT and SAT in patients with or without CAD. Thus CATA levels were lower in EAT than SAT in patients without CAD (2.5 ± 1.7 as against 6.1 ± 2.8; *P* = 0.012). However, these changes were not detected in the CAD group (3.5 ± 4.7 as against 4.4 ± 5.2; *P* = 0.103). By contrast, the 1D Western blot results for GSTP1 and PDIA1 apparently differed from the RT-PCR results, both GSTP1 and PDIA1, like PGAM1, showing no significant EAT-SAT difference (GSTP1, 2.7 ± 3.4 as against 2.9 ± 2.8, *P* = 0.745; PDIA1, 1.6 ± 2.8 as against 2.2 ± 2.8, *P* = 0.745).

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**Table 3. Proteomic analysis and identification**

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein Name</th>
<th>Accession Name</th>
<th>Molecular Weight, Da</th>
<th>Isoelectric Point</th>
<th>Fold Change</th>
<th>Sequence Covering, %</th>
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<tbody>
<tr>
<td>0207</td>
<td>Vimentin/40S ribosomal protein SA</td>
<td>VIME_HUMAN/RSSA_HUMAN</td>
<td>53520/32722</td>
<td>5.06/4.79</td>
<td>+7.051</td>
<td>54.84/54.42</td>
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<tr>
<td>0607</td>
<td>Protein disulfide isomerases</td>
<td>PDIA1_HUMAN</td>
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<td>4.69</td>
<td>+2.565</td>
<td>57.23</td>
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<tr>
<td>2004</td>
<td>Glutathione S-transferase P</td>
<td>GSTP1_HUMAN</td>
<td>23224</td>
<td>5.44</td>
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<tr>
<td>6103</td>
<td>Carbonic anhydrase I</td>
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<td>8105</td>
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<td>8601</td>
<td>Catalase</td>
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<td>6.95</td>
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<td>65.21</td>
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<tr>
<td>8806</td>
<td>Aconitate hydratase, mitochondrial</td>
<td>ACON_HUMAN</td>
<td>82425</td>
<td>6.85</td>
<td>+32.177</td>
<td>35.33</td>
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</table>
and PDIA1, 1.4 ± 1.4 as against 1.8 ± 1.5, P = 0.508; see Fig. 3B). Moreover, we found that CAD was independent of GSTP1, PDIA1, and PGAM1 protein levels.

The results for CATA were in keeping with the immunohistochemical observation that EAT adipocytes were less reactive than SAT adipocytes to CATA antibody (Fig. 3C).

Figure 3D shows the 2D Western blots that most clearly illustrate the observed 2D behavior. The pattern spot of PDIA1 was different in both tissue EAT and SAT. Moreover, three spots corresponding to GSTP1, one is considerably more intense in EAT than in SAT, and EAT shows four PGAM1 spots as against three for SAT. These EAT-SAT differences in post-translational profile may explain the apparent discrepancies between the proteomic analysis and 1D Western blot results.

**Oxidative Stress Assay**

Greater NADPH-induced production of ROS by EAT than by SAT was shown by comparing the slopes of plots, for each individual patient, of absorbance by diformazan against time: average slope was significantly greater for EAT than for SAT (5.61 ± 10^−4 as against 1.26 ± 10^−4 /min, P = 0.0037; see Fig. 4). DPI inhibited the reaction, reducing slopes signifi-
cantly ($P < 0.001$) in both EAT ($3.12 \times 10^{-4}$/min) and SAT ($1.3 \times 10^{-6}$/min). DPI, DMSO, and reaction mixture alone had no influence on absorbance (data not shown).

### Immunoprecipitation

2DE Western blot shows that GSTP1, PDI, and PGAM1 were represented by more than one spot. One of the common post-translational modifications of all proteins is serine or/and tyrosine phosphorylation. In this way, after phosphoserine or phosphotyrosine immunoprecipitation of EAT and SAT we have detected that CAT-A, GSTP1, PDIA1, and PGAM1 are proteins that can be phosphorylated at least in tyrosine or serine (supplementary Fig. 2).

### DISCUSSION

Although there have been several proteomic studies of visceral adipose tissue, OAT, and SAT (1, 3, 23), it is the first time that EAT and SAT from patients with cardiovascular disease have been compared by proteomic analysis. We found evidence that in cardiac patients catalase levels are lower in EAT than in SAT and that in these patients SAT and EAT differ in the post-transcriptional profiles of oxidative stress-involved proteins such as PDIA1, GSTP1, and PGAM1. Additionally, EAT presents higher oxidative stress than SAT in patients with cardiovascular disease.

In this study, SAT-EAT proteome differences were initially detected by staining with Coomassie brilliant blue, one of the most common SDS-PAGE gel stains (21). This method is considered generally to be less sensitive than silver or fluorescent staining techniques, but is less susceptible to between-gel variability than silver stain (25). Moreover, enhanced sensitivity has been reported when near-infrared fluorescence imaging has been used for detection (19) in which case Coomassie brilliant blue is comparable with Sypro ruby stain (9).

Catalase is an antioxidant enzyme that protects cells against potentially harmful effects of the ROS hydrogen peroxide by converting it to oxygen and water: transgenic mice producing human catalase in mitochondria live longer (28), and the adipose tissue of obese mice has low catalase levels and high hydrogen peroxide levels (7). Like other ROS, hydrogen peroxide triggers the production of several inflammatory factors, including tumor necrosis factor-α, interleukin-8 (4), and neutrophil-binding adhesion molecules (17). Among these last, VCAM-1 binds monocytes and T lymphocytes, which are precisely the types of leukocyte found in early human and experimental atheroma. Atherosclerosis being the main cause of ischemic cardiomyopathy, this process thus constitutes one of the mechanisms that make obesity one of the most important risk factors for cardiovascular disease (16). In relation to our previous finding that lower levels of adiponectin and higher levels of IL-6 in EAT are associated with the extent of CAD (5), it may be noted that both adiponectin and catalase are regulated by the transcription factor PPAR-γ (22, 33), which also regulates glucose and lipid metabolism (14, 18). However, further work is necessary to address the relative importance of lower catalase expression in EAT and its mechanism of regulation. Thus we think that lower catalase expression could be helping the oxidative stress in EAT rather than in SAT according to its antioxidative activity and the high levels of ROS product detected in EAT.

Other proteins with respect to which EAT and SAT differed in this study were GSTP1 and PDIA1. In these cases, EAT and SAT differed in the corresponding mRNA levels (EAT having higher levels in both cases), but not in protein levels as determined by 1D Western blots. Even though mRNA differences between these fat tissues were higher in patients with CAD, we did not find changes in protein levels. That this apparent discrepancy between mRNA and protein expression may be due to differences in post-translational modification is suggested by the 2D Western blot results, which showed EAT-SAT differences in the profiles of these proteins. Moreover, as Sam et al. (27) has described, the increased mRNA levels of several antioxidant enzymes may suggest an effort to normalize levels of oxidative stress and decrease in protein expression may suggest a post-translation modification with accelerated degradation.

The detoxifying enzyme GSTP1 is present in the cytosol, nucleus, and mitochondria of cells of all aerobic organisms, which it protects against oxidative stress (8); its known post-translational modifications are the glycosylation and phosphorylation of serine and threonine residues (26). Our immunoprecipitation assays have shown that this protein can be phosphorylated in EAT and SAT. In this study it appeared as three 2D Western blot spots, one of which was more intense in EAT than SAT. This is the most cationic spot that can be the

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**Fig. 4.** Nitroblue tetrazolium chloride (NBT) assay of reactive oxygen species (ROS) production in samples from 10 patients. A: time course of optical density (OD) at 550 nm (means, with SEMs shown by error bars) in EAT and SAT, with and without addition of diphenylene iodonium (DPI) to the medium. B: box-and-whisker diagrams of the slopes of the OD-time plots for each patient. For statistical results, see text. **$P < 0.01$, between EAT and SAT. ***$P < 0.001$, between with and without DPI in EAT or SAT assay.
unphosphorylated isoform. In this way, we had observed higher mRNA GSTP1 expression levels in EAT than SAT. Conversely, 1D Western blot was not able to be enough quantitative assay for detecting GSTP1 levels changes between the tissues; however, 2D Western blot has shown those differences.

PDIA1 is a protein disulfide isomerase (PDI) that belongs to the unfolded protein response (UPR) pathway, which in principle alleviates endoplasmic reticulum stress. Mice with ischemic heart disease that overproduce PDIs show a marked reduction in infarct size and a significant reduction of cardiomyocyte apoptosis in the peri-infarct region (29). However, prolonged activation of the UPR pathway can cause a toxic accumulation of ROS within the cell. In this study, 2D Western blots showed different spot pattern of PDIA1 for EAT and SAT, suggesting the occurrence of a post-translational modification in EAT that does not occur in SAT. It is tempting to speculate that this modification is the S-nitrosylation that has been reported to take place in endothelial cells during transient hypoxia (2). However, we could also show by immunoprecipitation assays that PDIA1 can be phosphorylated in this tissue.

Although PGAM1 may contribute to an increase in glycolytic flux in response to hypoxia, PGAM1 levels increasing in fibroblasts cultured in hypoxic conditions (31), increased glycolysis has also been associated with protection against oxidative stress (15). PGAM1 has several phosphorylation sites. In this study we found no differences between EAT and SAT in either PGAM1 or PGAM1 mRNA levels, but 2D Western blots showed four spots for EAT as against only three for SAT.

The significance of the above results is limited by the fact that, due to shortage of EAT, not all assays were performed on samples from the same patients, although the general clinical characteristics of all groups were similar. In addition, we could not obtain EAT from patients without cardiovascular disease matched for hypertension, diabetes mellitus, and hyperlipidemia undergoing a sternotomy incision due to ethical concerns. For this reason, we could not study expression differences between patients with and without cardiovascular disease, which would be very interesting.

This is the first demonstration of EAT presenting higher oxidative stress than SAT in patients with cardiovascular disease. Proteomic analysis has determined post-translational modifications of antioxidant enzymes: GSTP1, PDIA1, and PGAM1 and lower CATA expression in epicardial fat with respect to SAT. Our findings suggest a possible relationship among these enzymes and oxidative stress. The mRNA changes in the expression of both fat pads may be explained in part to cell composition, cardiovascular disease, or both. However, although future mechanistic insights are necessary, we observed higher oxidative stress in EAT adipocytary fraction than SAT, whereas no differences were detected between both stromal vascular fractions (supplementary Fig. 3). Since EAT extends from the epicardial surface into the myocardium, often following the adventitia of the coronary artery branches without any fascial structures separating it from myocardial tissue (11), it seems possible that enhanced oxidative stress in EAT may parallel a similar situation in adjacent coronary or myocardial tissue, possibly with the involvement of paracrine interactions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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