REGENERATION IN VIVO IN RESPONSE to injury leads to relining of the intimal cell layer to ensure endothelial integrity (39, 40). However, endothelial regeneration also results in a long-term partial dysfunction with impaired endothelium-dependent relaxations mediated by pertussis toxin-sensitive Gi proteins (8, 9, 20, 35). The resulting reduced production of nitric oxide (NO) initiates the atherosclerotic process (9, 38, 39, 40). When endothelial regeneration and hypercholesterolemia are combined, endothelium-dependent relaxations are impaired further, suggesting an exacerbation of the pathological changes in regenerated endothelial cells by higher levels of plasma lipids (15, 20, 29, 38). By contrast, diets rich in polyunsaturated fatty acids (PUFA) improve endothelium-dependent relaxations in porcine coronary arteries whether covered with native or regenerated endothelium and curtail the deleterious effects of dyslipidemia (alone or in combination with regeneration) on endothelial function (13, 21, 22).

Experiments on primary cultures of native and regenerated endothelial cells demonstrated genomic changes in the latter related to vasomotor control, coagulation, oxidative stress, lipid metabolism, and extracellular matrix (20). However, the genomic changes caused by the combination of either dyslipidemia or supplementation with PUFA and endothelial regeneration are unknown.

The present experiments were designed to test the hypothesis that endothelial regeneration is affected differentially at the genomic level by exposure to either high cholesterol or PUFA-rich diets in vivo and that the definition of such differential genomic changes will help to identify genes contributing to the endothelial dysfunction in regenerated endothelium.

MATERIALS AND METHODS

Animals and Diets

This study was approved by the Institutional Committee on the Use of Live Animals in Teaching & Research of the University of Hong Kong. Female farm pigs (3–5 mo of age, before puberty; 30–40 kg) were randomly divided into three groups. The control group (n = 3) was fed with standard chow (cat. no. 5081; TestDiet, Brentwood, MO). The cholesterol (CHL) group (n = 6) was fed with modified LabDiet Laboratory 5081 Diet with 22% fat (cat. no. 5B8A, TestDiet). The fish oil (FO) group (n = 6) was fed with control diet supplemented with ω3-polyunsaturated fatty acids (ROPUFA 75 n-3 EE; Roche, Indianapolis, IN), which was equivalent to 3.5 g eicosapentaenoic acid (EPA)/day/animal and 2 g docosahexaenoic acid (DHA)/day/animal (Fig. 1) To prevent excessive weight gain and achieve the same daily caloric intake, the diet was restricted throughout the study period to 2.1% of the total animal body weight in the CHL group and to 3% of the total animal body weight in the FO group. The diets were initiated 2 wk before coronary angioplasty and continued until the animals were killed 4 wk later.

Coronary Angioplasty

The pigs were anesthetized with tiletamine plus zolezepam (20 mg/kg intramuscularly). Anesthesia was maintained with endotracheal isoflurane (2%) while the animals were ventilated mechanically. An intravenous line was placed in an ear vein to administer heparin (30 μg/kg). A coronary guide catheter (Cordis Webster, Diamond Bar, CA) was introduced into the femoral artery and advanced into the left coronary ostium under fluoroscopic guidance. A balloon catheter was introduced via a guide wire into the middistal left anterior descending (LAD) artery through the guide catheter. Part (~2 cm) of the LAD was subjected to endothelial denudation by inflating the balloon catheter four to five times (diameter, 3.0 mm; length, 22 mm; balloon/artery ratio, 1.2:1). Nitroglycerin was infused immediately after the injury into the LAD to prevent excessive coronary constriction and vasospasm. Twenty-eight days later, the animals were euthanized and their hearts removed (Fig. 1) (1, 11, 20).
Primary Cell Cultures

The hearts were collected and transferred into cold Earle’s balanced salt solution supplemented with 5% fetal bovine serum (FBS). Native (left circumflex artery) and regenerated (previously denuded part of the LAD) endothelial cells were collected as described (1, 11, 20). They were centrifuged at 1,250 rpm for five min and resuspended in Earle’s minimum essential medium containing 15% FBS in a 60 mm collagen-coated Petri dish (one culture dish per endothelial sample). The cells were cultured at 37°C in a humidified gas mixture (5% CO₂, 95% O₂). The medium was changed every 2 days to facilitate cell growth. The cultures derived from native and regenerated endothelial cells are termed native (N) and regenerated (R) cells, respectively. All cultures exhibited cobblestone cell morphology and positive staining for von Willebrand factor (data not shown).

RNA Extraction

Primary cultures (cells at passage zero) were used for microarray and RT-PCR analysis. Briefly, native and regenerated endothelial cells were harvested from parts of the coronary artery (of the same heart) lined with either native (left circumflex coronary artery) or regenerated (left anterior descending coronary artery) endothelium. Primary cultures of these cells were allowed to reach near confluence. They then were prepared for biochemical analysis. GeneChips microarray experiments were performed on “regenerated” and “native” samples and then analyzed by GeneSpring GX11 software, using two analytical algorithms (standard guided workflow and MAS 5.0). Genes with >1.8-fold change without any statistical calculation were also selected and discussed based on the list shown in Supplemental Table S1. In certain of the samples, the expression of genes of interest (quantitative real-time PCR, at passage 0) or protein presences (Western blotting, at passage 1 and 2) were also measured. To analyze the effects of diets per se, data obtained from primary cultures derived from native endothelium of either the CHL or the FO group were compared with those obtained in the native control cultures group using Student’s t-test for unpaired observations. The diet-induced changes in native cultures were compared also to those observed after regeneration in the control group. To analyze the effects of regeneration in each experimental diet group, the data obtained in native and regenerated cultures of the same pig hearts were compared (Student’s t-test for paired observations). Finally, the genomic changes induced by regeneration were compared between the 3 groups (Student’s t-test for unpaired observations). *The data obtained in the 3 additional control pigs were combined with earlier results obtained in 5 control animals (20) to form the control data base (Supplemental Table S1). N, native; R, regenerated.
The yield of RNA from each sample ranged from 600 ng to 17 μg. The ratio of absorption (260/280) of all samples was between 1.7 and 2.1. The integrity of RNA samples was assessed qualitatively (rRNA ratio for 28S/18S between 1.9 and 2.1). Samples were stored immediately at −70°C until use.

**Microarray Analysis**

High-quality RNAs from native and regenerated endothelial cells (250 ng) were examined with GeneChips Porcine Genome Arrays (Affymetrix, Santa Clara, CA), as described (20). The array contained 23,937 probe sets that interrogate 23,256 transcripts from 20,201 Sus scrofa genes for target hybridization. Probed arrays were scanned using an Affymetrix GeneChip Scanner 3000. The intensity for each feature of the array was captured with Affymetrix GeneChip Expression Analysis (GeneChip Operating System) Software. Comparison analysis of two single arrays from native and regenerated endothelial cells of the same heart was done to evaluate the differential gene expression in the regenerated cells. Expression values for each transcript in native and regenerated endothelial cells were normalized using GeneSpring GX11 Analysis Software (Agilent Technologies, Santa Clara, CA). Two analytical algorithms (standard guided workflow and MAS5.0) were used. The detailed description of each probe set ID of the GeneChip porcine genome array can be found at http://www.affymetrix.com/index.affx.

**Quantitative Real-time Polymerase Chain Reaction**

Quantitative real-time polymerase chain reaction (QPCR) analysis was used to verify (for 17 genes of interest in both CHL and FO groups) the mRNA expression changes observed in the microarray experiments. Total RNA (100 ng–1 μg) was added to the reverse transcription mixture [20 μl of first-strand buffer containing 10 mM DTT, 0.5 mM dNTPs, and 10 ng/μl Oligo(dT) (Gibco-BRL, Grand Island, NY); 1 unit/μl Rnasin; 1 unit/μl moloney murine leukemia virus reverse transcriptase (M-MLV RT; Gibco-BRL, Gaithersburg, MD)] and incubated for 10 min at room temperature followed by 37°C for 60 min to produce the first-strand cDNA. The product was denatured at 94°C for 7 min prior to PCR reactions. One microliter of the total reverse transcription product was added to the QPCR reaction mixture (20 μl) containing 10 μl 2X SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and the primers (sense and anti-sense, 1 μM). The specific primers were designed based on the entire coding region for each gene (Table 1). Standard reference curves were obtained concomitantly with the unknown samples for each real-time PCR experiment as described (20).

Unknown samples (same samples as used for microarray experiments) and gene-specific PCR products at different dilutions (1 × 10^−3–1 × 10^10) were amplified using the 7900HT Fast Real-Time PCR System (Applied Biosystems). The cycling condition was 50°C for 2 min (stage 1, ×1); 95°C for 10 min (stage 2, ×1); 95°C for 15 s, 60°C for 1 min (stage 3, ×40); and 95°C for 15 s (stage 4, dissociation). Each sample was measured in duplicate. The critical threshold (CT) was determined for each sample or dilution of the PCR products for regression analysis. GAPDH was also amplified as an internal control for quantification of genes of interests. Results were normalized to the copy numbers of GAPDH gene products in native and regenerated samples and expressed as average fold-change. The quality of the

**Table 1. Primer sequences used for real-time PCR measurements**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sense</th>
<th>Size</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX1</td>
<td>5'-GAAACATTTCAGCCGAGGCTGCTCAG-3'</td>
<td>99</td>
<td>AF207823</td>
</tr>
<tr>
<td>COX2</td>
<td>5'-TCCATAATAGTGGCCGAGGCTGCTCAG-3'</td>
<td>70</td>
<td>AF207824</td>
</tr>
<tr>
<td>FABP4</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>223</td>
<td>AF102872</td>
</tr>
<tr>
<td>eNOS</td>
<td>5'-GTGAGCAACAGGCTGATAAGA-3'</td>
<td>360</td>
<td>NM_214295</td>
</tr>
<tr>
<td>F2</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>188</td>
<td>NM_001122985.1</td>
</tr>
<tr>
<td>F2RL1</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>141</td>
<td>NM_005242.4</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-AAGGAAATCTCCAAGACGCTAC-3'</td>
<td>100</td>
<td>AF107097</td>
</tr>
<tr>
<td>GPX3</td>
<td>5'-GATCCATTTGCCTGATCCTGCT-3'</td>
<td>278</td>
<td>NM_00115155.1</td>
</tr>
<tr>
<td>IkappaB</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>95</td>
<td>NM_001005150</td>
</tr>
<tr>
<td>MMP7</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>435</td>
<td>AB031323.1</td>
</tr>
<tr>
<td>MMP23</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>188</td>
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<tr>
<td>OLR1</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
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<tr>
<td>PLA2G7</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>105</td>
<td>NM_00113013</td>
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<tr>
<td>Selectin L</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>276</td>
<td>NM_00112678.1</td>
</tr>
<tr>
<td>SOD2 (MnSOD)</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>236</td>
<td>CX062378</td>
</tr>
<tr>
<td>TF</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>161</td>
<td>553DIET AND ENDOTHELIAL DYSFUNCTION 553</td>
</tr>
<tr>
<td>TPPI</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>100</td>
<td>NM_001135258.1</td>
</tr>
<tr>
<td>TXAS1</td>
<td>5'-GAGAAGTTGCTTCAAAGTTG-3'</td>
<td>169</td>
<td>NM_214046.1</td>
</tr>
</tbody>
</table>
PCR products were tested after each run by melting curve analysis and visualized on 1.4% agarose gels using ethidium bromide to ensure that the PCR products produce a single band of the expected size. CT values obtained for the standard curve were plotted against the log of template amount (molecule/µl). The copy number was calculated based on the formula [(CT-Y)/slope] where Y is the y-intercept and slope derived from the standard curve.

Western Blotting

Protein lysates of cell cultures (at passage 1 and 2) of native and regenerated from the same hearts of both the CHL and FO groups were used to validate at the proteomic level changes. Because of the limited supply of experimental material, inherent to the experimental model studied, only two proteins of interest [endothelial nitric oxide synthase (eNOS) and F2] could be determined. Cells were washed twice with cold phosphate buffer solution and the lysates were collected in ice-cold lysis buffer (20 mM Tris·HCl, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate) and a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 ng/ml trypsin inhibitor, 20 µg/ml leupeptin, and 1 µM pepstatin). The protein concentration was determined using the Bradford assay method. Forty micrograms of total protein were separated on 10% or 12.5% polyacrylamide gel and blotted on nitrocellulose membranes (100 V, 2 h). The blot was incubated for 1 h in Tris-buffered saline, 0.1% Tween-20 containing 5% bovine serum albumin. Membranes were incubated overnight with either eNOS (BD Biosciences, Franklin Lakes, NJ) or F2 (Sigma, St. Louis, MO) antibodies at 4°C. This was followed by incubation of the horseradish peroxidase-labeled secondary antibody (Amersham, Freiburg, Germany) prior to image detection by enhanced chemiluminescence using a commercially available kit (Amersham). To reprobe, the membrane was incubated at 55°C for 30 min in a buffer containing Tris-HCl (67.5 mM, pH 6.8), β-mercaptoethanol (100 mM), and sodium dodecyl sulfate (SDS, 2%). After extensive washing, the membranes were incubated first in blocking buffer and subsequently with the primary antibody. For each run, β-actin or GAPDH was probed as an internal control for protein quantification. Data are expressed as percentage of the corresponding native control.

Lipid Profile

Blood was collected from the animals receiving the different diets on day 1 of the study (initiation of the diet intervention), day 14 (before coronary angioplasty) and day 42 (death). The plasma concentrations of lipids [triglycerides, low-density lipoproteins (LDL-C) and high-density lipoproteins (HDL-C)] were determined using enzymatic methods (WAKO, Osaka, Japan). Gas chromatography analysis of plasma EPA, DHA, and arachidonic acids (AA) was determined as described (13), by gas chromatography (GC) using a 6890N Network GC system (Agilent, Palo Alto, CA) attached to a 5973 Network Mass Selective Detector (Agilent).

Table 2. Weight, plasma lipids, and plasma fatty acid levels during the study period in pigs fed high cholesterol or PUFA-rich diets

<table>
<thead>
<tr>
<th></th>
<th>CHL</th>
<th>FO</th>
<th>CHL</th>
<th>FO</th>
<th>CHL</th>
<th>FO</th>
<th>CHL</th>
<th>FO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>31.75 ± 0.26</td>
<td>32.8 ± 0.53</td>
<td>35.45 ± 0.33*</td>
<td>32.3 ± 0.84</td>
<td>39.25 ± 0.23*</td>
<td>37.4 ± 1.01*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma level, mmol/dl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>0.35 ± 0.10</td>
<td>0.34 ± 0.09</td>
<td>0.29 ± 0.10</td>
<td>0.26 ± 0.06</td>
<td>0.28 ± 0.08</td>
<td>0.32 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C</td>
<td>0.86 ± 0.24</td>
<td>0.89 ± 0.24</td>
<td>5.49 ± 1.60*</td>
<td>1.02 ± 0.47</td>
<td>5.71 ± 1.04*</td>
<td>0.49 ± 0.12*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.27 ± 0.22</td>
<td>1.15 ± 0.16</td>
<td>2.05 ± 0.24*</td>
<td>0.88 ± 0.09</td>
<td>2.24 ± 0.22*</td>
<td>0.88 ± 0.08*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>0.86 ± 0.16</td>
<td>0.82 ± 0.13</td>
<td>3.08 ± 0.44*</td>
<td>0.88 ± 0.23</td>
<td>3.15 ± 0.57*</td>
<td>0.65 ± 0.12*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA, mg/dl</td>
<td>22.10 ± 4.17</td>
<td>23.04 ± 2.19</td>
<td>55.24 ± 8.84*</td>
<td>13.63 ± 2.14*</td>
<td>64.02 ± 2.78*</td>
<td>10.42 ± 0.78*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHA, mg/dl</td>
<td>n.m.</td>
<td>0.89 ± 0.63</td>
<td>3.62 ± 0.66*</td>
<td>n.m.</td>
<td>3.21 ± 0.45*</td>
<td>n.m.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPA, mg/dl</td>
<td>n.m.</td>
<td>0.83 ± 0.31</td>
<td>13.60 ± 0.67*</td>
<td>n.m.</td>
<td>12.44 ± 1.51*</td>
<td>n.m.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as means ± SE. CHL, high cholesterol; PUFA, polyunsaturated fatty acids; FO, fish oil; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C/HDL-C, LDL-C-to-HDL-C ratio; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; n.m., not measured. The asterisks denote statistically significant (P < 0.05) differences with the respective control on day 1.

RESULTS

Body Weight and Plasma Lipid Profile

During the 6 wk of diet, body weight increased significantly, but to a similar extent in the CHL and FO groups (Table 2). In the CHL group, the LDL-C, HDL-C, the AA levels, and the LDL-C-to-HDL-C ratio were increased significantly on day 42. In the FO group, the plasma levels of LDL-C, HDL-C, AA, and the LDL-C-to-HDL-C ratio were reduced significantly at the end of the study. Both plasma DHA and EPA increased significantly in the FO group (Table 2). The DHA and EPA levels were measured but were below detection level throughout the study period in the CHL group (data not shown).

Microarray Experiments

Control group. The study was initially designed to compare the results obtained in the high CHL and FO groups with the...
data obtained in an earlier study in pigs receiving a normal diet (20). In addition to five samples reported in the previous study (20), three more pigs receiving control diet were included in the present analysis (Fig. 1). Paired comparison analysis was performed between native and regenerated cells derived from these samples. A new list including 1,517 genes was generated to represent the significant changes between the native and regenerated endothelial cultures derived from pigs fed with control diet. Subsequent comparison with array samples derived from the CHL and FO groups was based on this list (Supplemental Table S1).1

High cholesterol diet. First, genes were sorted to define those that were modulated by the CHL diet in native cultures (Fig. 2). A number of annotated genes (63 upregulated and 128 downregulated) were altered by CHL diet in a similar way as by regeneration in the control group (Fig. 3, Supplemental Table S2). The comparison of the expression of those genes between regenerated cultures (relative to the respective native cells) derived from the FO and CHL treated pigs yielded changes in opposite direction (Fig. 4).

PUFA-rich diet. A second list of different genes not altered significantly by regeneration in the FO group, but significantly up- or downregulated in the regenerated cells of both the control and CHL groups, was generated (Supplemental Table S3). These genes were accepted to represent those protected by the PUFA-rich diet (Fig. 5). Although the changes in expression of those genes induced by the regeneration process in the FO group were not significant, in most cases they followed a similar pattern of either up- or downregulation as observed in regenerated cells derived from control and CHL-fed pigs (Fig. 6).

QPCR Experiments

In addition to the microarray experiment, QPCR was used for the evaluation of changes in expression of a number of other genes of interest (Fig. 7A and Table 3), selected in view of earlier work (20). The comparison between native and regenerated cell cultures from high cholesterol-fed pigs demonstrated downregulation of five (eNOS, GPX3, KsB, OLR1, and TFPI), upregulation of nine (FABP4, F2, F2RL1, MMP7, MMP23, PLA2G7, PTGS2/COX2, SELL, and TF) and no significant changes for three (PTGS1/COX-1, SOD2, and TXAS1) genes (Fig. 7A, Table 3). The QPCR comparison in native and regenerated cell cultures from PUFA-rich diet group demonstrated downregulation of six (eNOS, F2, OLR1, PTGS2/COX2, SELL, and TF), upregulation of two (FABP4 and MMP7) and no

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1 The online version of this article contains supplemental material.
significant changes for nine (F2RL1, GPX3, IκB, MMP23, PLAG2G7, PTGS1/COX-1, SOD2, TFPI and TXAS1) genes (Fig. 7A, Table 3).

Western Blotting

The protein presence of eNOS was reduced, while that of F2 was increased significantly in the regenerated cultures of the CHL group. No significant changes in eNOS and F2 protein presence were observed after regeneration in the FO group (Fig. 7B).

DISCUSSION

Rationale

Endothelial regeneration in vivo is a natural mechanism that leads to relining of the intimal cell layer to restore endothelial function and prevent neointimal formation after vascular injury (39, 40, 42). However, regenerated endothelium is partially dysfunctional, as illustrated by a reduction in the endothelium-dependent relaxation to aggregating platelets and serotonin (31, 38). This dysfunction is exacerbated by high cholesterol intake, leading to accelerated localized atherosclerosis (32, 35, 38). Under similar conditions of high cholesterol feeding, freshly collected regenerated endothelial cells from the porcine coronary artery exhibited a reduced quantity and an impaired function of pertussis toxin-sensitive G protein resulting in reduced endothelium-dependent relaxations (30). By contrast, diets rich in polyunsaturated fatty acids delay or prevent the endothelial dysfunction caused by endothelial regeneration and the ensuing atherosclerotic process (33, 34, 38). A previous study, using a microarray approach, defined the genomic changes in primary cell cultures derived from regenerated porcine coronary endothelial cells (20). The present study was undertaken to determine those caused by the in vivo administration (prior to and during the regeneration process) of either a cholesterol- or a PUFA-rich diet on the genomic patterns in primary cell cultures from regenerated porcine coronary endothelial cells, on the assumption that if differential changes were observed, they would help to explain the dysfunction resulting in impaired endothelium-dependent relaxations. The measurements of the lipid levels and of the polyunsaturated fatty acids in the plasma demonstrated that the administered diets had been successfully applied.

Methodology

The procedure to initiate endothelial regeneration in vivo is standard in the laboratory, as is the method to harvest and culture the endothelial cells from coronary arteries of the same heart lined with either native or regenerated endothelium (1,
The present study demonstrates a striking similarity between the genomic changes in native cell cultures caused by dyslipidemia and those induced by the regeneration process in normal pigs (20). Indeed, of the modified genes in regenerated control endothelial cell cultures (20) most were up- or downregulated in the same fashion in native cell cultures from hyperlipidemic pigs (10, 35). Hence, the present findings provide a genomic explanation for the similar blunting of endothelium-dependent relaxations in coronary arteries lined with regenerated endothelium and those harvested from hypercholesterolemic pigs and humans (10, 35). They indirectly reinforce the earlier interpretation that abnormal handling of lipids is a key factor leading to endothelial dysfunction following regeneration (11, 14, 20, 38). This abnormal handling of lipids in native cultures from cholesterol-fed pigs is exemplified by the appearance of FABP4. This gene is not expressed in native cultures of control pigs but expressed in their regenerated cells (20). The protein it encodes for may play a role in atherosclerosis (12) and endothelial dysfunction (21). Likewise, downregulation of genes (GPX3 and TXNIP) encoding for glutathione peroxidase and thioredoxin interacting protein, also observed in regeneration (20), suggests the presence of oxidative stress with the production of superoxide anions, which by scavenging NO contributes to endothelial dysfunction (17, 28, 38).

By contrast, supplementation with a PUFA-rich diet per se improves endothelium-dependent relaxations in coronary arteries of normal pigs by enhancing receptor-mediated release of NO (32, 33). The lack of genomic upregulation (indicated by the microarrays) of eNOS in the native cell cultures from PUFA-rich diet group and its comparable protein levels (demonstrated by Western blotting) in native and regenerated cultures of the FO groups makes it unlikely that the potentiation by FO of endothelium-dependent relaxations in coronary arteries with native endothelium (33) is due to a direct augmented presence of the enzyme, but rather to preservation of this enzyme’s activity and augmentation of the bioavailability of NO even after regeneration. Earlier work on cultured porcine endothelial cells also suggests a lack of direct effect of EPA on NO production (2).

**Regenerated Endothelial Cells**

When we analyzed the expression levels observed in regenerated cells of the high CHL group, it became obvious that most genes that were either down- or upregulated in the hyperlipidemic native cell cultures showed no further significant changes after endothelial regeneration. This then implies that the deleterious effect of hyperlipidemia on their expression is already maximal and that the proteins encoded by those genes do not contribute to the further deterioration of endothelium-dependent relaxations and the acceleration of atherosclerosis caused by the combination of a high cholesterol intake and the regeneration process (31, 33–35).

Since most of the changes in genomic expression in the regenerated cell cultures of the PUFA-rich diet group at least qualitatively mimic those observed in control regenerated endothelial cells (20), it seems unlikely that this diet significantly modified the genomic impact of the regeneration process. The protective effect of FO against endothelial dysfunction is exemplified best by the unchanged (F2RL1, MMP23, and PLA2G7) or downregulated (COX2, F2, TF, SELL) expression levels of certain genes that are overexpressed in regenerated cell cultures of the high CHL group and by the absence of downregulation of other genes (GPX3 and TFPI).

The major finding of the present study is the demonstration of differential changes in gene expression between regenerated cell cultures of the high-CHL and PUFA-rich diet groups. It is tempting to conclude that the proteins encoded by these genes may be involved, on the one hand, in the exacerbation of endothelial dysfunction and the acceleration of atherosclerotic process caused by high CHL diet or, on the other hand, may contribute to the potentiation by PUFAs of endothelium-de-
pendent relaxations and their protective effect against accelerated atherosclerosis (31, 33–35). Although the gene expression of eNOS was downregulated in regenerated cells of both diet groups, a decreased presence of eNOS protein was observed in regenerated cultures from the CHL group but not from the FO group, illustrating an important protective role of the latter diet (6). Other obvious alterations in genomic expression that may underlie the modulator effect of diet on endothelial function are those where genes change in opposite directions are observed in the two experimental groups, whether upregulation in CHL and downregulation in PUFA-rich group (ADAM17, IL1A, CSF, SELE, STAT1, and TNFAIP6) or vice versa (GADD45A, GPR158, and PDK4). Activation of an inflammatory reaction is crucial to atherosclerotic plaque development. Of the genomic changes favoring such inflammatory response, the present findings point to ADAM17 (4), IL1A (5), CSF (43), TNFAIP6, and STAT1 (25). In particular, upregulation of JAK/STAT1 activates the intracellular signaling pathway leading to atherosclerosis (25). These proinflammatory genomic changes may be counteracted in part by a negative feedback mechanism, as suggested by the upregulation of TNFAIP6 in regenerated cultures of the CHL group. This gene encodes for a protein that suppresses inflammatory tissue destruction (extracellular matrix remodeling) by enhancing the serine protease-inhibitory activity (24).

The overexpression of the gene (PLA2G7) encoding for platelet-activating factor acetylhydrolase was also reduced by PUFA-rich diet. This enzyme is associated mainly with apolipoprotein B-containing-LDL and is involved in the reactive oxygen species (ROS) producing metabolism of platelet activating factor, AA, and oxidized phospholipids (7, 18, 37). A reduced ROS production favors endothelium-dependent relaxations (6). The upregulation of MMP23 (observed in both control and hyperlipidemic cell cultures after regeneration) was absent in PUFA-rich group. Besides its potential role in morphological changes during regeneration and atherosclerosis, this protein peptidase possesses potassium channel blocking activity, which may contribute to endothelial dysfunction (16, 27), and thus a reduction of its presence may help to explain the beneficial effect of PUFA-rich diet on endothelium-depen-

Fig. 7. A: differential changes in gene expression between regenerated cell cultures of CHL (open bars) and PUFA-rich (FO, solid bars) diet groups detected by QPCR. The data are expressed as ratio of gene copies between R cells and N cells of the same heart (fold changes) in CHL or FO groups and shown as means ± SE. The fold changes were also compared between CHL and FO groups to indicate the effects of the diets after regeneration. Values >1 indicate upregulation, and those <1 downregulation. The dotted lines indicate ratios of gene copies between R cells and N cells of the same heart (fold changes) in control group. All differences between the expression in R cell cultures of the 2 groups are statistically significant ($P < 0.05$). *Statistically significant ($P < 0.05$) changes in expression compared with the N cell cultures of the same diet group. For definition of the abbreviations of gene names see Supplemental Table S1. B: protein presence of eNOS and F2 in CHL and FO groups determined by Western blotting. The asterisks indicate statistical significant differences ($^*$$P < 0.05$, ***$P < 0.001$) for the R samples compared with the corresponding N controls; $n = 3–5$. 

558 DIET AND ENDOTHELIAL DYSFUNCTION

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Table 3. Gene expression changes in regenerated cell cultures of CHL and FO diet groups measured by real time-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>CHL</th>
<th>FO</th>
</tr>
</thead>
<tbody>
<tr>
<td>FABP4</td>
<td>1.97 ± 0.21*</td>
<td>4.38 ± 1.05*</td>
</tr>
<tr>
<td>PTGS1/COX-1</td>
<td>0.80 ± 0.39</td>
<td>1.44 ± 0.62</td>
</tr>
<tr>
<td>eNOS</td>
<td>0.40 ± 0.15*</td>
<td>0.59 ± 0.30*</td>
</tr>
<tr>
<td>iNOS</td>
<td>0.40 ± 0.17*</td>
<td>1.89 ± 1.14</td>
</tr>
<tr>
<td>MMP7</td>
<td>1.81 ± 0.16*</td>
<td>4.73 ± 1.88*</td>
</tr>
<tr>
<td>SOD2 (MnSOD)</td>
<td>1.19 ± 0.72</td>
<td>0.76 ± 0.28</td>
</tr>
<tr>
<td>OLR1</td>
<td>0.33 ± 0.26*</td>
<td>0.15 ± 0.08*</td>
</tr>
<tr>
<td>TXAS1</td>
<td>1.13 ± 0.38</td>
<td>1.26 ± 0.44</td>
</tr>
</tbody>
</table>

The data are expressed as ratio of gene copies between regenerated cells and native cells of the same heart (fold change) in CHL/FO groups and shown as means ± SE. Values >1 indicate upregulation, and those <1 downregulation.

*Statistically significant (P < 0.05) changes in expression compared with native cell cultures of the same diet group. For definition of the abbreviations of gene names see Supplemental data, Table S1.

Limitations

The present study focuses on the high-throughput microarray analysis of the effects of high CHL and PUFA diets on endothelial cell regeneration. Because of the limited amount of protein available, the changes of only certain genes of interest in relation to endothelial dysfunction could be verified by real-time PCR, and only the presence of two proteins could be analyzed by Western blotting. Likewise, because of the limited amounts of coronary arteries lined with regenerated endothelium, no functional studies were performed, but the reproducibility of the endothelial dysfunction resulting from regeneration, and the impact of the two tested diets on this dysfunction has been repeatedly demonstrated in the laboratory. The effects of the tested diets on endothelial cell turnover (proliferation and migration) and on endothelial progenitor cell mobilization, homing, or accumulation to the injured vascular surface also could not be addressed in the present study.

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Fig. 8. Summary of the genomic effects of CHL and PUFA diets on the response to endothelial regeneration, based on the QPCR results. Endothelial regeneration leads to differential changes of various genes related to endothelial function resulting in abnormal endothelium-dependent vasodilator responses, increased oxidative stress and lipid oxidation, and facilitated coagulation. Supplementation with CHL (in red) and PUFA (FO, in green) diets can alter various genomic expressions after regeneration, which ultimately will affect the progression of atherosclerosis. ▼, Increased expression; ▲, decreased expression; †, inhibition; ‡, facilitation. For definition of the abbreviations of gene names see Supplemental data, Table S1.
Kong. This study was also supported by an unconditional grant from Les Laboratoires Servier [Neuilly-sur-Seine, France].

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: M.Y.L., S.-Y.L., Y.L., Y.Z., and B.B. performed experiments; M.Y.L., Y.C., and Y.W. analyzed data; M.Y.L., Y.W., and P.M.V. interpreted results of experiments; M.Y.L., Y.C., and P.M.V. prepared figures; M.Y.L., Y.W., and P.M.V. drafted manuscript; M.Y.L., Y.W., and P.M.V. edited and revised manuscript; M.Y.L., Y.W., and P.M.V. approved final version of manuscript; H.-F.T. and P.M.V. conception and design of research.

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