PBMCs express a transcriptome signature predictor of oxygen uptake responsiveness to endurance exercise training in men

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1Laboratory of Genetics and Molecular Cardiology, Heart Institute (InCor) University of São Paulo Medical School, São Paulo, Brazil; and 2Unit of Cardiac Rehabilitation and Exercise Physiology, Heart Institute (InCor) University of São Paulo Medical School, São Paulo, Brazil; 3School of Physical Education and Sport, University of São Paulo, São Paulo, Brazil; 4University of Campinas, São Paulo, Brazil; and 5São Paulo State Police Department, São Paulo, Brazil

Submitted 5 June 2014; accepted in final form 1 December 2014

Dias RG, Silva MSM, Duarte NE, Bolani W, Alves CR, Junior JRL, da Silva JL, de Oliveira PA, Alves GB, Oliveira EM, Rocha CS, Marsiglia JDC, Negrao CE, Krieger EM, Krieger JE, Pereira AC. PBMCs express a transcriptome signature predictor of oxygen uptake responsiveness to endurance exercise training in men. Physiol Genomics 47: 13–23, 2015. First published December 2, 2014; doi:10.1152/physiolgenomics.00072.2014.—Peripheral blood cells are an accessible environment in which to visualize exercise-induced alterations in global gene expression patterns. We aimed to identify a peripheral blood mononuclear cell (PBMC) signature represented by alterations in gene expression, in response to a standardized endurance exercise training protocol. In addition, we searched for molecular classifiers of the variability in oxygen uptake (V˙O2). Healthy untrained policemen recruits (n = 13, 25 ± 3 yr) were selected. Peak V˙O2 (measured by cardiopulmonary exercise testing) and total RNA from PBMCs were obtained before and after 18 wk of running endurance training (3 times/wk, 60 min). Total RNA was used for whole genome expression analysis using Affymetrix GeneChip Human Gene 1.0 ST. Data were normalized by the robust multiarray average algorithm. Principal component analysis was used to perform correlations between baseline gene expression and V˙O2peak. A set of 211 transcripts was differentially expressed (ANOVA, P < 0.05 and fold change > 1.3). Functional enrichment analysis revealed that transcripts were mainly related to immune function, cell cycle processes, development, and growth. Baseline expression of 98 and 53 transcripts was associated with the absolute and relative V˙O2peak response, respectively, with a strong correlation (r > 0.75, P < 0.01), and this panel was able to classify the 13 individuals according to their potential to improve oxygen uptake. A subset of 10 transcripts represented these signatures to a similar extent. PBMCs reveal a transcriptional signature responsive to endurance training. Additionally, a baseline transcriptional signature was associated with changes in V˙O2peak. Results might illustrate the possibility of obtaining molecular classifiers of endurance capacity changes through a minimally invasive blood sampling procedure.

peripheral blood mononuclear cell; cardiopulmonary exercise test; transcriptional signature; global gene expression

GENE EXPRESSION IS A DYNAMIC and important control feature of physiological processes. Physiological adaptations to the stress stimuli of exercise training reflect molecular alterations, at least in part, explained by alterations in gene expression. Additionally, the differential regulation of distinct cellular types generates a molecular signature, which is represented by specific transcript networks. Clarifying the biological role of genomic markers of exercise training, such as the transcriptome, may help trace links to specific physiological changes and describe an outcome phenotype, such as trainability.

Endurance training promotes systemic adaptations that result in enhancement of the aerobic metabolic pathway within muscle cells and ultimately improved oxygen uptake (V˙O2). Although being a trainable phenotype, the individual maximal V˙O2 gain has a relevant genetic contribution (~47%), and there is a wide variation in responsiveness to exercise training, even among individuals with similar phenotypic characteristics (5). Indeed, studies have reported an association between transcriptome and the exercise phenotype. Individuals with high and low V˙O2 responses to exercise training have different muscle gene expression patterns (25, 44). Peripheral blood cells also are responsive to alterations in the gene expression pattern in response to perturbations caused by exercise. The acute response to endurance training seems to consistently activate stress and inflammation, as well as growth and tissue repair responses (1, 8, 11, 39, 40, 45), and these are dependent on aspects of methodology, such as exercise intensity and the chosen array platform. Growing evidence shows that peripheral blood is an accessible environment in which to visualize the whole body surveillance scenario (50) that struggles to maintain homeostasis during various and successive types of insults. Whereas the inflammatory expression profile seemingly diminishes with the repetition of bouts of exercise (19), this chronic response has not been established.

We therefore aimed to identify a peripheral blood molecular signature represented by alterations in gene expression in response to a standardized endurance exercise training protocol. In addition, we searched for a baseline expression signature able to predict the magnitude of change in V˙O2, induced by exercise training.

METHODS

Participants

We selected 13 individuals from whom RNA was available before and after the exercise training protocol and who also completed the
training protocol. They were male, aged between 20 and 31 yr old and were recruits joining the São Paulo State Police Department, Brazil. Individuals’ self-reported being nonsmokers, not using medication, and not engaging in regular physical activity for at least six months prior to enrollment in the Police Department. Health status was confirmed by screening with a clinical examination, laboratory testing, echocardiography, and cardiopulmonary exercise testing. Caffeine and alcohol ingestion were prohibited 1 day before the cardiopulmonary exercise test, which was performed at least 3 h in the postabsorptive state. The Human Subject Protection Committees of the Heart Institute (InCor), Clinical Hospital, University of São Paulo Medical School, and the Ethics Committee of the Centre for High Studies on Security, São Paulo State Police Department approved the study protocol, and all participants provided written consent.

Measurements and Procedures

**Blood collection, RNA extraction, and microarray process.** Fasting blood samples for biochemical markers and gene expression measurements were withdrawn at baseline and after the exercise training protocol, 48 h after the last exercise session. Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) isolated with Ficoll-Paque (GE Healthcare, Fairfield, CT) using TRizol reagent (Invitrogen, Waltham, MA). RNA was purified in RNeasy columns (Qiagen, Germantown, MD) and treated with RNase-Free DNase Set (Qiagen). Purified RNA was checked using the Bioanalyzer 2100 System (Agilent, Santa Clara, CA) to distinguish ribosomal bands 28S and 18S. Samples with a minimum 100 ng of RNA underwent in vitro transcription with Ambion WT Expression kit (Life) in conjunction with the Affymetrix GeneChip WT Terminal Labeling and Control kit. cRNA quality were determined with the NanoDrop spectrophotometer (ND-1000; NanoDrop, Thermo Scientific, Wilmington, DE). Labeled samples were hybridized to Affymetrix GeneChip Human Gene 1.0 ST. Array hybridization, washing, staining, and scanning were performed following Affymetrix instructions. The chips were checked for experiment quality and expression signals were stored in.cel files for later analysis (Affymetrix GCOS). Raw data were put into the Gene Expression Omnibus database (GSE57999). Serum was isolated for the analysis of cardiovascular biomarkers. Glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride levels were measured at baseline and after 18 wk by standard methods. Commercial enzymatic methods were used for the determination of total cholesterol (Boehringer-Mannheim, Penzberg, Germany), glucose, and triglycerides (Abbot Laboratories). HDL-C was measured by the same method used for total cholesterol after chemical precipitation of apo B-containing lipoproteins. LDL-C was calculated by the Friedwald formula: LDL-C = total cholesterol – (HDL-C + VLDL-C), and very low-density lipoprotein cholesterol (VLDL-C) was calculated by dividing plasma concentrations of triglycerides by 5. All biomarker assays were processed in the Lipid Metabolism Laboratory, Heart Institute (InCor), Brazil.

**Cardiopulmonary exercise test.** VO2 was measured in a cardiopulmonary exercise test as previously described (34). Briefly, the test was carried out on a programmable treadmill (Quinton Q65 model 645; Quinton Instruments, Bothell, WA) using a ramp protocol with increments in workload until volitional exhaustion. A breath-by-breath gas exchange analyzer (Vmax 29; Sensor Medics, Buena Vista, CA) was used to measure VO2 and carbon dioxide production. **Exercise training protocol.** Exercise training has been previously described in more detail (2). It was performed under supervision and consisted of three 80 min sessions per week for 18 wk. Sessions consisted of a 5 min warm-up, followed by a 60 min run and 15 min of cool-down exercises. The running exercise intensity was individually controlled by heart rate (Polar FS1; Polar Electro, Kempele, Finland) corresponding to ventilatory anaerobic threshold and respiratory compensation point. During the first half of the training protocol, running intensity was moderate, and heart rate was kept between the ventilatory thresholds. During the second half, heart rate was kept slightly above the respiratory compensation point. There was no change in diet and daily routine, with respect to academic activities and rest hours, during the entire exercise-training period.

**Data analysis.** The raw data were preprocessed in R with affy and multtest packages from Bioconductor (37a). Quality control of individual arrays included various advanced quality metrics, diagnostic plots, and pseudo-images to certify that only excellent quality arrays were used prior to downstream statistical analysis. The robust multiarray average (RMA) algorithm was used for background correction, quantile normalization, and median polish summarization (23). Differentially regulated transcripts in response to exercise training were identified by analysis of variance (ANOVA), with and without correction for multiple testing using a false discovery rate (FDR) method. As no transcript reached a significance q value of 0.05 in the FDR test, only transcripts with P < 0.05 (ANOVA) and fold change (FC) ≥ 1.3 were retained. This final list represented the transcriptome responsive to training (TRT). To recognize patterns in the data, we generated a hierarchical cluster from the list of retained transcripts (P < 0.05, FC ≥ 1.3), clustering by samples and transcripts, using Euclidean distance and average linkage.

Our second objective was to study the relationship between the individual baseline transcription profile and the change (Δ) in maximal oxygen uptake. For this, Pearson correlation analysis was performed in R. Transcripts with correlation coefficient (r) > 0.75 and P < 0.01 were retained. It thus provided a list of candidate transcript genes that may represent oxygen uptake responsiveness, hereby called the “molecular classifier.” Principal component analysis (PCA) was performed to identify groups among the transcripts suggesting distinct biological characteristics or functions, based on baseline expression values of transcripts correlated with change in peak oxygen consumption (ΔVO2peak). Transcripts were considered as variables to compose the correlation matrix. Varimax and promax rotation methods were used for graphic representation of principal component (PC) loadings, which describe the importance of each transcript (variable) in accounting for the variability in the PC and scores of each individual for the PC. A further attempt to optimize association analysis included a linear (adjusted) regression model with the PC that accounted for most of the variation in the original variables, and the ΔVO2peak. Lastly, to confirm if a reduced number of markers could reproduce the association found for the molecular classifiers, we selected 10 transcripts with the highest loadings from the original list of transcripts correlated with ΔVO2peak.

We uploaded the TRT and the molecular classifier into MetaCore software (GeneGo, St. Joseph, MI) to identify relevant differentially expressed pathways. Pathway enrichment analysis in Metacore are based on curated literature and complex algorithms for mining quality biomedical literature(13). The main enriched signaling and metabolic canonical pathways and Gene Ontology (GO) processes were identified with a significance threshold of P < 0.05 and an FDR < 0.05. Buid Network function was used to discover interactions among transcripts pertaining to the molecular classifier and the TRT.

**RESULTS**

**Phenotypic Response to 18 Weeks of Endurance Exercise Training**

Full details of demographic and cardiopulmonary measures are presented in Table 1. Before endurance training, individuals’ mean absolute VO2peak was 3.85 ± 0.43 l/min. After training, absolute VO2peak increased to 4.12 ± 0.35 l/min (P = 0.005, 7 ± 8%) with large interindividual variation (range −4% to 26%). Relative VO2peak significantly increased from 46.5 ± 4.5 to 51.2 ± 3.2 ml·kg⁻¹·min⁻¹ (P = 0.001) (11 ± 9%, range 1–27%) more pronouncedly due to decreases in
Table 1. Demographic measures and oxygen uptake of the 13 individuals in untrained (pre) and trained (post) states

<table>
<thead>
<tr>
<th>Individuals</th>
<th>Age, yr</th>
<th>Height, m</th>
<th>BM Pre, kg</th>
<th>BM Post, kg</th>
<th>aV˙O2peak Pre, l/min</th>
<th>aV˙O2peak Post, l/min</th>
<th>ΔV˙O2peak, l/min</th>
<th>rV˙O2peak Pre, ml·kg⁻¹·min⁻¹</th>
<th>rV˙O2peak Post, ml·kg⁻¹·min⁻¹</th>
<th>ΔrV˙O2peak, ml·kg⁻¹·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>27</td>
<td>1.79</td>
<td>71.1</td>
<td>71.3</td>
<td>3.97</td>
<td>4.01</td>
<td>0.04</td>
<td>55.8</td>
<td>56.2</td>
<td>0.04</td>
</tr>
<tr>
<td>b</td>
<td>24</td>
<td>1.75</td>
<td>82.0</td>
<td>79.0</td>
<td>3.61</td>
<td>3.59</td>
<td>-0.02</td>
<td>44.0</td>
<td>45.4</td>
<td>-0.02</td>
</tr>
<tr>
<td>c</td>
<td>30</td>
<td>1.70</td>
<td>80.2</td>
<td>80.3</td>
<td>3.19</td>
<td>4.03</td>
<td>0.84</td>
<td>39.8</td>
<td>50.1</td>
<td>0.84</td>
</tr>
<tr>
<td>d</td>
<td>22</td>
<td>1.65</td>
<td>68.0</td>
<td>65.5</td>
<td>2.92</td>
<td>3.30</td>
<td>0.38</td>
<td>42.9</td>
<td>50.3</td>
<td>0.38</td>
</tr>
<tr>
<td>e</td>
<td>24</td>
<td>1.78</td>
<td>83.0</td>
<td>78.4</td>
<td>4.14</td>
<td>4.24</td>
<td>0.10</td>
<td>49.8</td>
<td>51.1</td>
<td>0.10</td>
</tr>
<tr>
<td>f</td>
<td>26</td>
<td>1.80</td>
<td>84.0</td>
<td>77.0</td>
<td>3.52</td>
<td>4.11</td>
<td>-0.18</td>
<td>48.2</td>
<td>50.6</td>
<td>-0.18</td>
</tr>
<tr>
<td>g</td>
<td>25</td>
<td>1.77</td>
<td>89.0</td>
<td>81.2</td>
<td>4.29</td>
<td>4.11</td>
<td>-0.18</td>
<td>48.2</td>
<td>50.6</td>
<td>-0.18</td>
</tr>
<tr>
<td>h</td>
<td>24</td>
<td>1.78</td>
<td>84.5</td>
<td>80.5</td>
<td>3.92</td>
<td>4.17</td>
<td>0.25</td>
<td>51.9</td>
<td>54.2</td>
<td>0.25</td>
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<tr>
<td>i</td>
<td>22</td>
<td>1.73</td>
<td>90.0</td>
<td>87.3</td>
<td>4.12</td>
<td>4.17</td>
<td>0.25</td>
<td>51.9</td>
<td>54.2</td>
<td>0.25</td>
</tr>
<tr>
<td>j</td>
<td>24</td>
<td>1.81</td>
<td>75.5</td>
<td>77.0</td>
<td>3.92</td>
<td>4.17</td>
<td>0.25</td>
<td>51.9</td>
<td>54.2</td>
<td>0.25</td>
</tr>
<tr>
<td>k</td>
<td>31</td>
<td>1.81</td>
<td>92.5</td>
<td>89.0</td>
<td>4.15</td>
<td>4.61</td>
<td>0.46</td>
<td>44.9</td>
<td>51.8</td>
<td>0.46</td>
</tr>
<tr>
<td>l</td>
<td>20</td>
<td>1.72</td>
<td>98.0</td>
<td>95.0</td>
<td>4.23</td>
<td>4.42</td>
<td>0.19</td>
<td>43.1</td>
<td>46.5</td>
<td>0.19</td>
</tr>
<tr>
<td>m</td>
<td>23</td>
<td>1.71</td>
<td>82.0</td>
<td>82.0</td>
<td>4.15</td>
<td>4.37</td>
<td>0.22</td>
<td>50.5</td>
<td>53.2</td>
<td>0.22</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>25 ± 3</td>
<td>1.75 ± 0.05</td>
<td>83 ± 8</td>
<td>80 ± 7</td>
<td>3.85 ± 0.43</td>
<td>4.12 ± 0.35</td>
<td>0.27 ± 0.28</td>
<td>46.5 ± 4.5</td>
<td>51.2 ± 3.2</td>
<td>4.7 ± 3.7</td>
</tr>
<tr>
<td>P value</td>
<td>0.003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are from paired t-tests of pre- vs. postvalues. BM, body mass; aV˙O2 peak; rV˙O2 relative oxygen uptake; Δ, absolute change; HR, resting heart rate.

PBMC Transcriptome Associated With the Training-induced Change in V˙O2peak

The baseline expression of 98 transcripts was associated with the change in absolute V˙O2peak with a strong correlation (r > 0.75, P < 0.01) (Supplementary Table S1). Except for SNORD24, which had a 40% upregulation in expression, the remaining transcripts had low or no change in expression, with an average FC of 7 ± 7% and thus were not part of the TRT, presented below. PCA used a correlation matrix among the expression values of the 98 transcripts. In this, the first PC (PC1) explained 69% of the total variance in the data. The 13 scores from the individuals obtained from the PC1 were strongly associated with the variation in absolute ΔV˙O2peak (adjusted R² = 88%, P = 1.3·10⁻⁶) following a linear distribution (Fig. 1A). The distribution matches the stratification observed in percentage of V˙O2 variation as shown in Fig. 1 (%Δ absolute V˙O2peak).

To better understand the physiological meaning of the expression-based classification panel, we performed new correlations to further investigate the relationship between baseline gene expression and V˙O2 response. Baseline V˙O2peak was negatively correlated with ΔV˙O2peak (r = -0.6) and positively correlated with posttraining V˙O2peak (r = 0.8). Furthermore, PC1 scores (representing the 98 expression values, obtained from the above analysis) were also correlated with baseline V˙O2peak (r = -0.5).

Given that the molecular classifier is strongly associated with the response variable ΔV˙O2peak, it was expected that this signature would provide a molecular classifier of individual V˙O2 responsiveness to endurance training and subsequently steer further scope targeting these markers. To further narrow down the candidate markers, we selected a set of 10 transcripts with the highest loadings for subsequent explorations with PCA. The individual scores of the PC1 were plotted against ΔV˙O2peak, and the distribution followed the similar results observed for the 98-signature (adjusted R² = 77%, P = 4.5·10⁻⁵) (Fig. 1B), confirming that the preset expression levels of these 10 markers are able to classify the 13 individuals in our sample according to their potential to improve V˙O2max (Table 2).

All the above analyses were performed using relative V˙O2peak as well. Correlation analysis resulted in 53 transcripts associated with the change in relative V˙O2peak (r > 0.75, P < 0.05) (Supplementary Table S2). Transcripts had low or no change in expression, with an average FC of 6 ± 8%, and only one transcript had a considerable change in expression after exercise training (ZNF778, FC = -52%). PC1 explained 69% of the total variance in the data. The 13 scores from the individuals obtained from the PC1 were strongly associated with the variation in relative ΔV˙O2peak (adjusted R² = 87%, P = 1.7·10⁻⁶) following a linear distribution (Fig. 1C). PCA with a selection of 10 transcripts with the highest loadings (Supplementary Table S3) confirmed that their baseline expression levels predict and classify the change in relative V˙O2 (Fig. 1D) (adjusted R² = 74%, P = 1·10⁻⁶). The correspondence between transcript lists obtained from associations with absolute and relative V˙O2peak was: 98 vs. 53 = 26 transcripts and 10 vs. 10 = 3 transcripts.

Functional Enrichment Analysis of the Transcriptome Signature Associated With ΔV˙O2peak

Functional enrichment was performed to discover pathways modulated by the 98 molecular classifiers. Only two pathways passed the threshold (P < 0.05 and FDR < 5%), leukocyte chemotaxis, and chromosome condensation in the cell cycle (Supplementary Table S4). The five transcripts retained in those pathways had a discrete downregulation (average FC = 5%). As to GO processes, 967 were enriched (P < 0.05 and FDR < 5%) in many categories, such as feeding behavior,
PBMC Transcriptome Reflecting a Differential Response to Endurance Training

Eighteen weeks of endurance exercise training induced changes in gene expression profile of PBMCs. From a total of 28,231 transcripts, ANOVA identified 1,846 that were differentially expressed.

### Table 2. Molecular classifiers of ∆VO2peak

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
<th>PC1 Scores</th>
<th>r</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4orf46</td>
<td>chromosome 4 open reading frame 46. mRNA</td>
<td>0.79</td>
<td>-0.76</td>
<td>3E-03</td>
</tr>
<tr>
<td>CCDC50</td>
<td>coiled-coil domain containing 50. transcript variant 2. mRNA</td>
<td>-3.08</td>
<td>-0.76</td>
<td>3E-03</td>
</tr>
<tr>
<td>CDK14</td>
<td>cyclin-dependent kinase 14. mRNA</td>
<td>-1.37</td>
<td>-0.75</td>
<td>3E-03</td>
</tr>
<tr>
<td>FAAH2</td>
<td>fatty acid amid hydrolase 2. mRNA</td>
<td>1.08</td>
<td>0.78</td>
<td>2E-03</td>
</tr>
<tr>
<td>HIST1H2BF</td>
<td>histone cluster 1. H2bf. mRNA</td>
<td>-2.38</td>
<td>-0.76</td>
<td>2E-03</td>
</tr>
<tr>
<td>IGLJ3</td>
<td>mRNA for scFv collagenase IV antibody. complete cDNA</td>
<td>1.60</td>
<td>0.70</td>
<td>2E-03</td>
</tr>
<tr>
<td>PIP5K1B</td>
<td>phosphatidylinositol-4-phosphate 5-kinase. type I. beta. transcript variant 2. mRNA</td>
<td>-0.32</td>
<td>-0.77</td>
<td>2E-03</td>
</tr>
<tr>
<td>SCARNA9L</td>
<td>small Cajal body-specific RNA 9-like (retrotransposed). guide RNA</td>
<td>1.60</td>
<td>-0.79</td>
<td>1E-03</td>
</tr>
<tr>
<td>SNORA48</td>
<td>ncrna: snoRNA chromosome. gene:ENSG00000212383</td>
<td>3.21</td>
<td>0.79</td>
<td>1E-03</td>
</tr>
<tr>
<td>TCF4</td>
<td>transcription factor 4. transcript variant 1. mRNA</td>
<td>-3.43</td>
<td>-0.89</td>
<td>4E-05</td>
</tr>
</tbody>
</table>

Shown are 10 transcripts whose baseline gene expression patterns classified the 13 individuals according to their ∆VO2peak, showing a strong association with the oxygen uptake response. r and P values are from correlation test. PC1 scores are scores of the 1st principal component from principal component analysis (PCA).
entially regulated ($P < 0.05$). Filtering transcripts with at least 30% of altered expression ($|H/E| > 1.3$) resulted in 211 PBMC transcripts responsive to endurance training (TRT), among which 152 were up- and 59 were downregulated (Supplementary Table S6). Both up- and downregulated transcript groups had a similar change in mean expression (8%). Among this TRT list, there were 74 noncoding RNAs (ncRNA), mostly small nuclear RNAs and small nucleolar RNA (snoRNA), and also included microRNAs (miRNA or miR) miR-21, miR-223, miR-29c, and miR-let-7f-1, miscellaneous RNAs, and pseudogenes. Transcripts with GO annotations were mainly involved in biological processes basically related to angiogenesis; cell division, differentiation, growth, and development; cell cycle; transcriptional regulation; oxidative phosphorylation; and immune response (data not shown). To have an initial look at the training-induced transcriptional response, hierarchical clustering of expression patterns by individuals and transcripts was performed and revealed interindividual differences at baseline and after exercise training (Fig. 2). Individuals were grouped into two clusters. The baseline transcription pattern was similar among all individuals. Interestingly, after exercise training, the transcriptional pattern changed and individuals were grouped in another cluster, except for a, b, and m, who did not seem to have a substantial change in their expression profile.

**Functional Enrichment Analysis of the TRT**

To associate the changes in transcriptional profile with effects in biological pathways, we used MetaCore software to perform functional enrichment analysis of the 211 TRT. The top seven associated canonical pathways are shown in Table 3. Sixteen differently regulated transcripts were mainly enriched ($P < 0.05$ and FDR < 5%) in seven pathways associated with immune response of heat shock proteins (HSP), cytokines interleukin (IL)-1 and oncostatin M signaling, initiation of mitosis in cell cycle, and development through epidermal growth factor receptor activation and growth hormone signaling. The most enriched pathway includes a set of three upregulated and three downregulated transcripts involved in HSP60 and HSP70/toll-like receptor (TLR) signaling (Fig. 3). HSP70 (HSPA1A) encodes heat shock proteins 70 kDa, which stabilizes existing proteins against aggregation and mediates the folding of newly translated proteins in the cytosol. Subsequent binding to TLRs in cell membrane initiate signaling pathways ultimately resulting in an inflammatory response and immunoregulation. Subsequent cascade reactions in cytoplasm result in downregulation of AP-1 and c-Jun that activate transcription of proinflammatory mediators (TNF-$\alpha$, IL-6) and antagonist anti-inflammatory cytokines (IL-10). Putative train-

**Table 3. Canonical pathways associated with the TRT**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>$P$ Value</th>
<th>FDR</th>
<th>Transcripts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response: HSP60 and HSP70/TLR signaling pathway</td>
<td>5E-07</td>
<td>1E-04</td>
<td>CD69, CD83, AP-1, c-Jun, MHC class I, HSP70</td>
</tr>
<tr>
<td>Immune response: IL-1 signaling pathway</td>
<td>1E-04</td>
<td>1E-02</td>
<td>AP-1, COX-2 (PTGS2), c-Jun/c-Jun, c-Jun</td>
</tr>
<tr>
<td>Cell cycle: initiation of mitosis</td>
<td>4E-04</td>
<td>3E-02</td>
<td>FOXM1, Histone H1, PLK1</td>
</tr>
<tr>
<td>Proteolysis: putative SUMO-1 pathway</td>
<td>6E-04</td>
<td>3E-02</td>
<td>c-Jun, SUMO-1, DAXX</td>
</tr>
<tr>
<td>Immune response: oncostatin M signaling via MAPK in human cells</td>
<td>1E-03</td>
<td>4E-02</td>
<td>EGR1, AP-1, c-Jun</td>
</tr>
<tr>
<td>Development: beta-adrenergic receptors transactivation of EGFR</td>
<td>1E-03</td>
<td>4E-02</td>
<td>AP-1, c-Jun, HB-EGF</td>
</tr>
<tr>
<td>Development: growth hormone signaling via PI3K/AKT and MAPK cascades</td>
<td>2E-03</td>
<td>5E-02</td>
<td>EGR1, c-Jun, C/EBP zeta</td>
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TRT, transcriptome responsive to training; FDR, false discovery rate.
ing-induced positive regulation of NF-κB transcription factor activity, also initiated by HSP70 expression, might have induced upregulation of CD83 and CD69, which contributes to maintaining an enhanced immunologic response. Activated NF-κB pathway also regulated inflammatory response of pro- and anti-inflammatory cytokines, as well as of nitric oxide. Furthermore, upregulation of HSP70 downregulates expression of major histocompatibility complex class I (MHC class I), and together with upregulated CD69 and CD83, these molecules may be responsible for differentiation, proliferation, and activation of lymphocytes and dendritic cells.

As to transcripts associated with GO cellular processes, ~350 were enriched (P < 0.05 and FDR < 5%), and the top 10 showed that the differentially regulated transcripts were enriched in categories including gene expression, RNA and ncRNA metabolic processes, defense response, regulation of T cell, and antigen processing and presentation.

We were also able to find eight direct interactions between the molecular classifiers and the TRT (Fig. 4), indicating that although they are seemingly not affected by endurance training, they may be regulating the same responsive transcriptional networks.

**DISCUSSION**

We identified molecular classifiers of the \( \dot{V}O_2 \) response to endurance training. The preset expression levels of 98 and 53 molecular markers mostly with unaltered expression after endurance training, therefore not included among the TRT, were correlated with the change in absolute and relative \( \dot{V}O_2 \), respectively. In addition, 18 wk of endurance exercise training induced a change in PBMCs’ transcription patterns of untrained individuals. This phenomenon was reflected in a global and interwoven modification of the cell’s regulatory behavior, visualized through the differential expression of 211 TRTs. We hereby identified in PBMCs transcriptional signatures reflecting endurance training-induced \( \Delta \dot{V}O_2 \). Despite the novelty of the results, these findings are exploratory and aimed to provide information for future (properly controlled and validated) investigations.
Following a similar approach adopted by Timmons et al. (44), we discovered that the baseline blood tissue (PBMCs) expression pattern enables the identification of a 98-transcript signature (molecular classifiers) that was associated with the change in absolute \( \Delta \text{V}_\text{O2peak} \) and stratified individuals according to their percentage change in \( \text{V}_\text{O2peak} \). None of the transcripts described here were among those reported in the work mentioned, which was carried out using skeletal muscle (44). Interestingly, we demonstrated the associations of the molecular classifiers with baseline and posttraining absolute \( \text{V}_\text{O2peak} \). Additionally, the negative association between baseline absolute \( \text{V}_\text{O2peak} \) and \( \Delta \text{V}_\text{O2peak} \) reflects a previous knowledge that the initial \( \text{V}_\text{O2} \) status is a predictor of the change in response to endurance training. Thus, the observed relationship between the molecular classifiers and absolute \( \text{V}_\text{O2} \) indicates that the reported baseline expression signature marks the individual punctual cardiovascular capacity and has a predictive potential of cardiovascular adaptations over endurance training. Furthermore, a smaller panel of 10 transcripts was able to represent this signature to a similar extent, and this might illustrate the possibility of obtaining molecular classifiers of endurance capacity changes through a minimally invasive blood sampling procedure.

As changes in body mass can also influence absolute \( \Delta \text{V}_\text{O2} \) we also performed correlation analysis using relative \( \text{V}_\text{O2} \). As described, the baseline expression profile enables the identification of a 53-transcript signature that was associated with the change in relative \( \text{V}_\text{O2peak} \); from which 26 transcripts were also present among the 98-transcript signature associated with the change in absolute \( \text{V}_\text{O2peak} \). Regardless of similarities among both signatures, variations in body mass stratified individuals differently than when considering absolute \( \text{V}_\text{O2peak} \) (Fig. 1, C and D), and this draws attention to factors other than exercise training influencing body mass, such as food intake. Despite the fact that food intake may alter the results of correlation analysis by influencing body mass variations and consequently \( \Delta \text{V}_\text{O2} \), in this study we did not control this phenotype. Furthermore, exercise training also promotes gene expression alterations, represented in this study by the TRT. Although body mass changes may affect gene expression as well, we were not able to quantify the variance of expression alterations represented by each phenotypic change. Indeed, some of transcripts that were differentially expressed are associated with energy metabolism, inflammation, and immune function (see Supplementary Table S6), and their alteration may have been induced by body mass changes independent of exercise training-induced changes.

The transcriptional alterations that occurred in peripheral blood strikingly replicate current assumptions about underlying mechanisms potentially controlling training-induced physiological adaptations. One extensively reviewed health improvement benefit of chronic mild-to-moderate exercise is improved immune function (36, 49). Although it still needs to be clarified whether it happens through altering immune cell function to battle or to prevent inflammatory and infectious states, there is consensus as to the change in leukocyte counts and function with exercise training in those with inflammation (32) and in athletes submitted to intensified and sustained training loads (4, 29, 48). However, these changes do not seem...
to occur in normal individuals subjected to regular exercise training (20), except in acute responses to exercise (up to 24 h), which elicits a transient change in immune cells (49). This study’s sample included only healthy individuals, and blood samples were extracted at rest with at least 48 h after the last exercise session. Nonetheless, only by performing blood cell counts would one verify if cell numbers were unchanged. Among the TRT, the most represented pathway activates protein folding and immune regulation by modulating stress-responsive proteins (HSP70) and MHC class I, C (HLA-C), and CD83 and CD69. The genes involved in MHC class I codify molecules responsible for having peptides released into the cytosol to be recognized and directed by T cells, initiating an immune response. MHC class I complex was upregulated in muscle after a training period similar to the one adopted here (25) and after one exercise session (24, 31). In our data, endurance training downregulated HLA-C and upregulated CD83 and CD69. One might generate the hypothesis that, because CD83 and CD69 are markers of T lymphocytes activation (7, 18), they could thus contribute to settling an adapted or putatively enhanced immune function, notwithstanding the diminished expression of HLA-C. Moreover, the opposite response verified elsewhere in muscle after an exercise session may account for a different activation of MHC class I, as the increased expression of this complex was associated with a local inflammatory reaction, but not with the response of circulating inflammatory markers (31). Upregulation of HSP70 is consistent with that previously reported in white blood cells after acute exercise sessions (15), although to a larger extent (FC > 1.6) (8, 11, 24, 39), and also after endurance exercise training (trained vs. untrained) (15). It seems that the repeated stimulus of exercise establishes a continuum repair of possible cellular damages provoked by stress (12, 15), which is necessary for appropriate recovery and protection from subsequent strains. Altogether, the key activated pathways seem to be targeting control and repair of immune function, cell cycle processes, development, and growth (see Table 3), commonly known to occur in the trained muscle (25, 44). In turn, previous studies reported decrease of baseline serum protein levels of hsp70 in well-trained individuals (17, 21, 42, 53). Despite some hypotheses that this might be due to HSP70 gene downregulation as an exercise-training-induced adaptation, other posttranscriptional mechanisms can interfere with protein expression (42). For example, muscle-specific miR-1 inhibits hsp70 translation and promotes mRNA degradation in a nonphysiological condition (induced muscle atrophy) (28). Nonetheless, stability of hsp70 protein levels may not be accompanied by HSP70 mRNA levels, as observed previously (15, 30). Moreover, literature is sound as to the decrease in hsp70 levels as a chronic adaptation. Nonetheless, one should pay special attention to the overall differences in our study sample compared with the one of previous works. Regardless of the postraining enhancements observed in this study, the physical capacity here observed (relative $\dot{V}_{O_2}$peak = 51.2 ± 3.2 ml·kg$^{-1}$·min$^{-1}$) is well below the described in other studies (athletes, or relative $\dot{V}_{O_2}$peak = 55 ± 0.8 ml·kg$^{-1}$·min$^{-1}$, or running = 53.3 ± 18.4 km/wk) (17, 21, 42, 53), and also the participants were subjected to substantially different training workloads. Presumably, athletes have unique oxidative stress profiles toward a decreased production of reactive oxygen species at rest (16), which indirectly influences the production of stress-responsive proteins, such as HSPs and could also explain their decreased levels elsewhere, as previously suggested (15, 17). Still, even with the increased transcriptional activation of stress-responsive HSP70 in our study, the possibility that the participants are in an acute-stress status is less likely, as the other activated pathways regulated by differentially expressed transcripts (see Table 3) seem to be heading toward the opposite direction.

Despite the fact that the mononuclear fraction of blood cells is not directly involved in oxygen transport and consumption, the pathways activated may conceivably reflect a humoral regulatory mechanism triggered by training. This hypothesis relies on several recent observations: 1) Several physiological perturbations induced during exercise stimulate the immune system (37) and change gene expression patterns of white blood cells (8, 11, 24, 39, 40); 2) exercise regulates energy metabolism- and potassium homeostasis-related genes in white blood cells (8), together with a similar transcriptional response between skeletal muscle and lymphocytes, of genes involved in oxidative metabolism, after endurance training (51), and 3) the release of other factors such as cell-free DNA (6) and miRNAs into circulation from a variety of tissues (27, 52) after strenuous exercise (10) could be exerting an endocrine effect on blood cells, and transcriptional alteration is a plausible mechanism (52). Previous works showed alterations in global PBMC expression profiles induced by isolated exercise bouts and described known pathways regulating expression patterns (1, 8, 11, 39, 40, 45). Analyses of differentially regulated genes and pathways suggested activation of an acute inflammatory response directly dependent on exercise intensity. Compared with these previous studies, the effect of successive bouts of exercise appears to attenuate the inflammation-related transcription networks, which is consistent with the known reduction in inflammatory state evidenced by biomarkers (33, 49) and downregulation of proinflammatory genes (RAGE, NADPH oxidase p47phox, inducible nitric oxide synthase, MCP-1, NF-κB p65, and TNF-α) (19). To our knowledge, a study of the chronic effect of endurance exercise on global expression patterns in blood cells vs. local working skeletal muscle tissue has not yet been attempted. These observations highlight the contribution of the present work, since although the skeletal muscle seems to be the target tissue for studying morpho-functional and molecular alterations in response to exercise training interventions, collecting biopsy samples is a major limiting factor. As such, PBMCs carry important circulatory information, serving as biological sensors of the systemic environment and important sources of genomic information.

Other aspects of the TRT are interesting and must be pointed out. ncRNAs corresponded to 37% of TRT. Although these molecules are scarcely understood, ncRNAs are transcriptional regulators involved in numerous biological processes (47), including immune response (9). As an example of the class, miRNAs have emerged as an important feature in adaptations induced by exercise training (25, 45). Here, the four differentially expressed miRNAs were upregulated: miR-21, miR-223, miR-29c, miR-let-7f-1. Although little is known about the role of miRNA in exercise-induced regulation of blood cells, recent studies have reported an increase in circulating (plasma) miR-21 (3) and miR-223 (35) after one endurance exercise session and the decrease of circulating miR-21 after endurance training (35). Although the results seem to be divergent, the
selective release of miRNAs into circulation may result in different intra- and extracellular miRNA profiles (38). miRNAs have known roles in biological processes that are important to cardiopulmonary function, e.g., miR-21 is linked to vascular inflammation (46) and skeletal and cardiac muscle function (43). Recent works have linked differentially expressed miRNAs to the control of mRNA networks in blood cells in response to acute exercise. Among the miRNAs identified are miR-21-5p (mature form of miR-21) (45) and miR-233 (41), both upregulated in our study. Pathway analysis of miRNAs regulated by the miRNAs showed the activation of similar immunity/inflammation-related responses (cell death, stress response, proliferation; ubiquitin-mediated proteolyis, Jak-STAT signaling pathway, and Hedgehog signaling pathway) and established substantial connections between the miRNAs-mRNA networks (45). It is noteworthy to mention that miR-21 has been functionally demonstrated to activate TLR-mediated NF-κB signaling, leading to increased secretion of proinflammatory cytokines IL-6 and TNF-α, and subsequent proliferation of cancer cells (14). This was the most differentia ted pathway found in our study (HSP60 and HSP70/TLR signaling pathway; Table 3, Fig. 3) and appears to be down-regulating transcript of IL-6 and TNF-α. Still, comparisons among works are difficult. Along with the acute exercise approaches, the cell populations used are two important methodological differences between these studies and the current. In addition, all snoRNAs were also upregulated, suggesting an increase in ribosomal RNA biogenesis (26). Other noncoding molecules were downregulated, pointing to their distinct biological roles. These results reinforce a recent paradigm break where the “dark matter” may conceivably exert significant control over transcriptional configuration.

Functional enrichment analysis revealed direct links between the molecular classifiers and components of the TRT (Fig. 4). In addition, only one transcript from the 98-signature is also included among the 211 differentially expressed (SNORD24). Interestingly, although expression alterations of the remaining were not captured by the statistical model, baseline expression levels were associated with the physiological response of VO₂. These observations are in concordance with previously launched hypotheses (25, 44), where transcriptional markers (in muscle) regulate physiological alterations by mechanisms other than exercise training stimuli and meanwhile activate transcriptional networks that coordinate the magnitude of adaptation. In addition, the molecular classifiers were associated with the generic pathways leukocyte chemotaxis and chromosome condensation and, not less importantly, included a number of noncoding molecules, showing that these transcripts could be connecting branches of different transcriptional networks, as illustrated by the TRT. Although no functional validation in another population has been performed in this study, the high correlation with the phenotypic change suggests that these markers may contribute to the variability in VO₂ responsiveness. Further research should focus on finding highly connected genes, as well as those that play a controlling role in regulatory networks.

In conclusion, PBMCs reveal a transcriptional signature (211 transcripts) responsive to endurance training that may be linked to control and repair of immune function, cell cycle processes, development, and growth. In addition, the baseline transcriptional signature was associated with changes in VO₂, suggesting important links between genomic interactions and phenotypic response to exercise training interventions. The molecular profile here described might aid in the search for causal relationships among global gene expression and training-induced responses. Such contributions will potentially help develop personalized interventions taking into account individual molecular patterns, obtained from a minimal amount of blood.

Study Limitations

Caution is needed in interpreting our study results. One may argue that the identified expression alterations could be confounded by other effects than exercise training. Thus, verifying the expression patterns of a control group that did not undergo exercise training would be a way of clarifying this issue. Unfortunately, we did not perform this and our results should be interpreted in light of the lack of this control group. Another limitation is the lack of women participating in the study. It is still necessary to replicate the molecular classifier signature in another sample for validation. Given our small sample size, it was not possible to develop segmented analyses by magnitude of response, though we hope to perform this in the near future. Current approaches were unable to link ncRNAs to pathways regulated by endurance training. Although it is a common practice to control the FDR in hypothesis testing of gene expression studies, this study did not include this criterion.

ACKNOWLEDGMENTS

We thank the reviewers and the Journal’s peer review team for valuable contributions and kind support throughout the revision of this work. We thank the participants from São Paulo State Police Department for absolute cooperation and diligence.

GRANTS

This work was supported by grants from São Paulo Research Foundation (FAPESP #2005/59740-7) and, in part, by the Zerbinini Foundation. R. G. Dias was supported by National Council for Scientific and Technological Development Grants CNPq #482863/2011-0, #483509/2012-4. Funding sources had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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