J Appl Physiol 104: 236–243, 2008.
First published November 15, 2007; doi:10.1152/japplphysiol.00872.2007.

Effects of 30 min of aerobic exercise on gene expression in human neutrophils

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Submitted 14 August 2007; accepted in final form 9 November 2007

Radom-Aizik S, Zaldivar F Jr, Leu S-Y, Galassetti P, Cooper DM. Effects of 30 min of aerobic exercise on gene expression in human neutrophils. *J Appl Physiol* 104: 236–243, 2008. First published November 15, 2007; doi:10.1152/japplphysiol.00872.2007.—Relatively brief bouts of exercise alter gene expression in peripheral blood mononuclear cells (PBMCs), but whether exercise changes gene expression in circulating neutrophils (whose numbers, like PBMCs, increase) is not known. We hypothesized that exercise would activate neutrophil genes involved in apoptosis, inflammation, and cell growth and repair, since these functions in leukocytes are known to be influenced by exercise. Blood was sampled before and immediately after 30 min of constant, heavy (~80% peak O_2 uptake) cycle ergometer exercise in 12 healthy men (19–29 yr old) of average fitness. Neutrophils were isolated using density gradients; RNA was hybridized to Affymetrix U133+2 Genechip arrays. With false discovery rate (FDR) <0.05 with 95% confidence, a total of 526 genes were differentially expressed between before and after exercise. Three hundred and sixteen genes had higher expression after exercise. The Jak/STAT pathway, known to inhibit apoptosis, was significantly activated (EASE score, $P < 0.005$), but 14 genes were altered in a way likely to accelerate apoptosis as well. Similarly, both proinflammatory (e.g., IL-32, TNFSF8, and CCR5) and anti-inflammatory (e.g., ANXA1) were affected. Growth and repair genes like AREG and FGF2 receptor genes (involved in angiogenesis) were also activated. Finally, a number of neutrophil genes known to be involved in pathological conditions like asthma and arthritis were altered by exercise, suggesting novel links between physical activity and disease or its prevention. In summary, brief heavy exercise leads to a previously unknown substantial and significant alteration in neutrophil gene expression.

microarrays; inflammation; polymorphonuclear neutrophils; growth; repair

WE NOW KNOW from work done in this and other laboratories that exercise induces a robust increase in circulating peripheral blood mononuclear cells (PBMCs; lymphocytes, monocytes, and natural killer) and neutrophils (6, 27, 35) as well as changes in leukocyte function (28). The response of immune cells to physical activity is of growing interest because some of the key health effects of exercise, both beneficial (e.g., reduction of cardiovascular disease risk) and detrimental (e.g., exercise-induced asthma), may well be mediated through the activity of circulating leukocytes (11).

It is not surprising that exercise could influence immune cells so profoundly. The physiological perturbation induced by even brief bouts of physical activity includes thermodynamic [increased temperature (38)], physiochemical [lactic acidosis (23), hypoxia (34)], hormone [catecholamines (22)], cytokine (2), and physical [turbulence and dynamic shear forces (1)] factors, all of which can alter leukocyte function. In the case of PBMCs, it is now known that relatively brief exercise leads to substantial changes in cellular gene expression (9). Consequently, we hypothesized that exercise would also influence the gene expression profile in circulating neutrophils, a class of cells that until relatively recently had been (incorrectly) classified as terminally differentiated, incapable of altering gene regulation in response to environmental stresses (18).

Unlike the PBMCs that decrease rapidly from the circulation at the cessation of an exercise bout, neutrophils remain elevated or may even continue to increase during the postexercise recovery period (42). In humans, relatively brief episodes of exercise can activate neutrophils (25, 32, 40), but the ultimate impact of exercise on neutrophil function varies and depends on the duration and intensity of the exercise challenge (45–47). An intriguing emerging aspect of neutrophil biology is the mounting data that these cells play a role in tissue repair, remodeling, and growth (16, 43). Neutrophils act as an elegant paracrine type of hormonal system—the cells are attracted from the central circulation to a specific tissue (e.g., a wound or injured muscle) and then are induced to secrete mediators that impact tissue growth (16).

We reasoned that genes involved in cellular activation, inflammation, and growth and repair would likely be affected by a single bout of heavy exercise. Since these pathways involve a variety of genes, we used microarray technology along with verification by RT-PCR of selected genes. We chose to focus on neutrophil gene responses initially rather than on the complex ensuing physiological or functional changes. Conceptually, we wanted to test hypotheses on the mechanisms responsible for the early control of neutrophil function, and this would be best accomplished by examining gene regulation. By first identifying a discrete set of genes in circulating neutrophils that were altered by exercise, we could subsequently design more efficient experiments targeting specific proteins and physiological responses that were likely controlled by the identified genes.

MATERIALS AND METHODS

Participants

Twelve healthy men (19–29 yr old) participated in this study (see Table 1). The decision to include only men in these initial investigations was made because it is now known that a variety of metabolic and stress/inflammatory responses are sex dependent and likely to be influenced in women by the individual phase of the menstrual cycle

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(13). Our plan was to first determine whether key neutrophil genetic pathways were altered by exercise before determining the effect of sex on these putative responses. Individuals participating in competitive sports and with a history of any chronic medical conditions or use of any medications were excluded from participation. The Institutional Review Board at the University of California, Irvine, approved the study, and written informed consent was obtained from all participants upon enrollment.

Anthropometric Measurements

Standard, calibrated scales and stadiometers were used to determine height and body mass.

Measurement of Fitness

Each subject performed a ramp-type progressive cycle ergometer using the SensorMedics metabolic system (Ergoline 800S, Yorba Linda, CA). After resting on the cycle ergometer for 3 min and 1 min of unloaded pedaling, the work rate (WR) was incremented at a rate of 20–30 W/min to the limit of the subject's tolerance. Subjects were vigorously encouraged during the high-intensity phases of the exercise protocol. Gas exchange was measured breath-by-breath and the anaerobic (lactate) threshold and peak $\dot{V}O_2$ were calculated using standard methods (10).

Exercise Protocol

At least 48 h, but not exceeding 7 days, following the completion of the ramp test, each subject performed 30 min of constant work rate cycle ergometry at a work rate equivalent to ~50% of the difference between the lactate threshold and the peak $\dot{V}O_2$. On average, this work rate represented ~80% of the participants' peak $\dot{V}O_2$.

Blood Sampling and Analysis

An indwelling catheter was inserted into the antecubital vein. A baseline sample was taken 30 min after the placement of the catheter and before the onset of exercise (Before). We waited 30 min to ensure that measurable physiological parameters of stress (e.g., heart rate and blood pressure) were at baseline levels. Subjects then completed the 30-min exercise bout, and additional blood samples were obtained immediately after exercise (After). Complete blood counts (CBC) for white blood cell analysis were obtained by standard methods from the clinical hematology laboratory.

Neutrophil Isolation

Neutrophils were isolated from EDTA-treated peripheral blood using OptiPrep Density Gradient Medium (SIGMA). The duration from blood draw to stabilization of RNA never exceeded 60 min. Using hematoxylin staining, we determined that this approach to neutrophil isolation consistently yielded ≥98% purification.

RNA Extraction

Total RNA was extracted using TRIzol (GIBCO BRL Life Technologies, Rockville, MD) reagent and purified using the RNeasy Midi columns method (Qiagen, Valencia, CA). RNA pellets were resuspended in diethyl pyrocarbonate-treated water. RNA integrity was assessed (prior to beginning target processing) by running out a small amount of each sample (typically 25–250 ng/well) onto a RNA Lab-On-A-Chip (Caliper Technologies,Mountain View, CA) that was evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

Preparation of Labeled cRNA

The detailed protocol for preparation and microarray processing was performed as recommended by the manufacturer and is available in the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Briefly, 2 μg total RNA was used as a template for double-stranded cDNA synthesis. Single-stranded then double-stranded cDNA was synthesized from the poly A spike-in controls, and mRNA present in the isolated total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) and a T-7-oligo(dT) primer (Integrated DNA Technologies, Coralville, IA) that contains a T7 RNA polymerase promoter site added to it's 3′ end. A portion of the resulting double-stranded cDNA was used as a template to generate biotin-tagged cRNA from an in vitro transcription reaction (IVT) using the Affymetrix GeneChip IVT Labeling Kit.

Hybridization to Microarray

A total of 15 μg of the resulting biotin-tagged cRNA was fragmented to an average strand length of 100 bases (range 35–200 bases) following prescribed protocols (Affymetrix GeneChip Expression Analysis Technical Manual). Subsequently, 10 μg of this fragmented target cRNA was hybridized at 45°C with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 640) to probe sets present on an Affymetrix U133+2 arrays. The GeneChip arrays were washed and then stained (SAPE, streptavidin-phycoerythrin) on an Affymetrix Fluidics Station 450, followed by scanning on a GeneChip Scanner 3000.

Data Analysis

Microarray analysis. The results were quantified and analyzed using GCOS 1.4 software (Affymetrix) using default values (Scaling Target Signal Intensity = 500). The microarray data were analyzed using ArrayAssist version 4.0.3 (STRATAGENE). We normalized the data using GC-RMA. Only probe sets that reached a signal value ≥20 in at least one array and a present call by MAS5 criteria in at least 18 arrays were selected for further analysis. Overall, 16,060 of 54,675 probe sets represented on the array met these criteria. The microarray cell files and GC-RMA normalized data have been deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/; series accession number = GSE8668). We further applied BRB-ArrayTools software Version 3.4.1 (http//linus.nci.nih.gov/~brb/tool.htm) to determine significantly changed probe sets from before to after exercise. Traditional Student's paired *t*-test was first applied to each probe set and then significantly changed probe sets were decided with permutation tests (41). With 95% confidence, the final list of significantly changed probe sets has <5% FDR. The change from before to after exercise is presented with fold change, which is defined as the geometric mean of the ratio of after-exercise expression levels over before-exercise expression levels. [Note: The logarithm (base 2) of the geometric mean of X is equivalent to the arithmetic mean of $\log_2(X)$.]

Gene annotation. The final list of significantly changed probe sets was then additionally analyzed using the functional annotation tools provided by DAVID [Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov)] to classify the genes into functional groups and, when possible, into pathways using the KEGG database. Only functional groups with EASE score <0.05 are presented in this analysis. EASE score is a modified Fisher Exact *P* value in DAVID system used for gene-enrichment analysis. EASE score *P* value = 0 represents perfect enrichment. *P* value ≤0.05 is considered as gene enrichment in a specific annotation category (http://david.abcc.ncifcrf.gov/helps/functional_annotation.html#summary).

Physiological data. The physiological data were presented as mean and SE. The two-sided paired *t*-test was applied for testing changes from before to after the exercise and the significance level was set at 0.05. All analyses were done using SAS 9 (Cary, NC).

Quantitative Real-Time Polymerase Chain Reaction

For confirmation of microarray gene expression findings, quantitative real-time polymerase chain reaction (qPCR) was carried out on six genes that we felt were of particular physiological significance (five of which had higher expression after exercise). One microgram

of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Reagents kit (Applied Biosystems) according to the manufacturer's instructions, using random primers in a 100 μl reaction. The qPCR analysis was performed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) by using TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression probes (Applied Biosystems; HSPA1B: Assay ID: Hs00271244_s1, GZMB: Assay ID: Hs00188051_m1, STAT4: Assay ID: Hs00231372_m1, IL10RA: Assay ID: Hs00155485_m1, PRF1: Assay ID: Hs00169473_m1, EGR1: Assay ID: Hs00152928_m1). Actin beta was used as an endogenous control. We used Fisher Exact Test to evaluate the association between qPCR and microarray on their directional change of gene expression level due to exercise effect.

RESULTS

Anthropometric and Physiological Characteristics

The anthropometric and physiological characteristics of the 12 subjects appear in Table 1. The average BMI was within normal limits. The subjects were of relatively normal fitness (88.7 ± 3.7 of the subjects' predicted $\dot{V}O_{2\,max}$).

Serum Lactate

The exercise bout caused a mean increase of 9.0 ± 0.84 mM in serum lactate levels [Before (1.8 ± 0.07 mM) vs. After (11.0 ± 0.84 mM), $P < 0.0001$].

Leukocyte Response to Exercise

As shown in Table 2, the number of total white blood cells, lymphocytes, monocytes, and neutrophils was significantly elevated at peak exercise ($P < 0.003$).

The Effects of Exercise on Neutrophil Gene Expression

Using FDR < 0.05 with 95% confidence, a total of 526 genes were differentially expressed between before and after exercise. Three hundred and sixteen genes had higher expression after exercise and 210 genes had lower expression after exercise (see supplementary table contained in the online version of this article). Given this criterion, the paired test *P* value for these genes was <0.0005.

We attempted to characterize the genes that were affected by exercise in several ways. First we used the Gene Ontology (GO) database provided by DAVID that allows us to annotate genes and classify them into functional categories. Figure 1 presents six groups of genes that are particularly relevant to current knowledge of the ways in which the neutrophil impacts health. As can be seen, many genes that could be classified by GO are linked to regulation of cellular physiological process, immune response, defense response, response to stress, apoptosis, and signal transduction. In Table 3, we show genes associated with apoptosis, and in Table 4, we present genes associated with growth, tissue repair, and inflammation.

Table 1. *Anthropometric characteristics and exercise responses of the 12 subjects*

Characteristics/Responses	
Age, yr	23.3±1.0
Height, cm	178.2±3.2
Body mass, kg	76.5±3.9
BMI, kg/m^2	24.0±0.8
Peak $\dot{V}O_2$, ml·kg^{-1}·min^{-1}	39.1±1.6
Average $\dot{V}O_2$ during the constant work rate as percent of peak $\dot{V}O_2$	76.5±2.4

Values are means ± SE. BMI, body mass index; peak $\dot{V}O_2$, peak oxygen uptake.

Table 2. *Leukocyte response to exercise*

	Before Exercise	After Exercise	%Change
WBC	5,425.0±335.3	9,383.3±548.0*	78.6±13.3
Lymphocytes	1,967.1±191.6	4,339.2±447.2*	144.3±28.0
Monocytes	412.8±42.9	710.1±78.4*	111.8±52.5
Neutrophils	2,871.6±210.0	4,001.9±231.1*	43.8±9.2

Values are means ± SE (cells/μl). WBC, white blood cell. *Significantly greater After Exercise than Before Exercise ($P < 0.003$).

RT-PCR Verification of Specific Gene

We verified the change from before to after the exercise of 6 genes in 11 of 12 subjects using RT-PCR (note, one of the subjects did not have sufficient RNA for this verification). In Table 5, we show that the direction of exercise effects are highly associated (Fisher Exact Test *P* value <0.0001). In HSPA1B and IL10RA, the directional change was different between the two techniques in only one subject, respectively.

DISCUSSION

We found a substantial alteration of 526 genes in circulating neutrophils immediately following 30 min of heavy exercise (a work rate that elicited a large and significant increase in circulating lactate) in healthy young men with average fitness. We used stringent statistical approaches in analyzing the microarray data to limit the possibility of false-positive results. Moreover, we found that a different technique, RT-PCR in a small group of genes, corroborated the microarray data. Consistent with our hypothesis, many of the affected genes were linked to processes associated with neutrophil activation such as microbial killing (e.g., GZMA, GNLY, SPON2; see Table

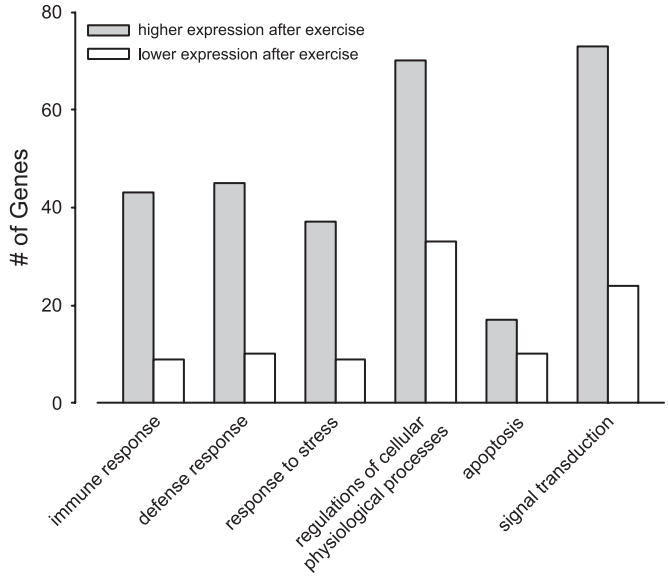

Fig. 1. Groups of genes that were altered by exercise [classified by Gene Ontology (EASE score <0.0004)].

Table 3. *Representative genes known to regulate apoptosis*

Gene Name	Gene Symbol	Fold Change	Effect of Gene Change on Apoptosis
BCL2-associated transcription factor 1	BCLAF1	0.6	↑
Growth arrest and DNA-damage-inducible, beta	GADD45B	0.7	↑
BCL2-associated athanogene 5	BAG5	0.8	↑
Tumor necrosis factor (ligand) superfamily, member 8	TNFSF8	1.3	↑
Peptidyl-tRNA hydrolase 2	PTRH2	1.3	↑
Ras homolog gene family, member B	RHOB	1.5	↑
Annexin A1	ANXA1	1.5	↑
Fem-1 homolog b (*C. elegans*)	FEM1B	1.5	↑
PreB-cell colony enhancing factor 1	PBEF1	0.7	↑
Phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, *Drosophila*)	PDE4B	1.5	↑
Granzyme a (granzyme 1, cytotoxic t-lymphocyte-associated serine esterase 3)	GZMA	2.1	↑
Granzyme h (cathepsin g-like 2, protein h-ccpx)	GZMH	2.8	↑
Perforin 1 (poreforming protein)	PRF1	3.1	↑
Granzyme b	GZMB	3.9	↑
CD27-binding (Siva) protein	SIVA	0.6	↓
Signal transducer and activator of transcription 1, 91 kDa	STAT1	0.6	↓
Interferon induced with helicase C domain 1	IFIH1	0.7	↓
B-cell CLL/lymphoma 10	BCL10	0.7	↓
Purinergic receptor P2X, ligand-gated ion channel, 1	P2RX1	0.7	↓
Tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	0.8	↓
Heat shock 70 kDa protein 1B	HSPA1B	1.9	↓

Fold change is defined as the geometric mean of expression levels of After/Before. A fold change of 0.6 indicates the expression level after exercise is ~60% of the expression level before the exercise. A fold change of 1.3 indicates the expression level after the exercise is ~130% of the expression level before the exercise.

4), viability (e.g., HSPA1B, BCLAF1, ANXA1, STAT1; see Table 3), and the regulation of growth mediators (e.g., EGR1, KSP37, GADD45B; see Table 4).

To the best of our knowledge, this is the first report demonstrating an acute effect of exercise on gene expression in circulating neutrophils. Other investigators have studied the effects of exercise on gene expression in whole blood (e.g., Refs. 7, 29, 50). Comparing the results of these earlier studies with those of the present are, however, problematic. First, because it has been known since the 1980s that neutrophils produce low amounts of RNA (Ref. 4; this is corroborated in our own experience having extracted RNA from both neutrophils and PBMCs in many subjects), and, as a consequence the contribution of putative changes in gene expression among the various leukocytes is virtually impossible to determine unless the cells are isolated. In addition, the type of exercise and its duration differs greatly among the previous studies. We showed earlier that the same exercise protocol used in the present study in a different group of participants altered PBMC gene expression in 311 genes (9). Interestingly, only 16% of the exercise-altered genes in neutrophils were similarly affected by exercise in the PBMCs. Among these similar genes were heat shock protein, granzyme, and cathepsin, all known to be involved in innate immune activation. The very small overlap in gene response suggests that neutrophils respond differently to exercise than do PBMCs.

We did not design this investigation as a crossover protocol in which one arm of the study would consist of observing changes in leukocyte gene expression over a 30-min period of rest. Although not much data exist on changes in neutrophil gene expression over short intervals, the existing literature indicates that there is very little variability in leukocyte gene expression over intervals of time in the range of 30 min. For example, Campbell and coworkers (8) studied gene expression in peripheral blood leukocytes in several subjects in whom blood was drawn at varying intervals throughout the day. They found that ". . . the peripheral blood gene expression of a broad spectrum of known function genes . . . exhibit little normal variability related to the time of sample collection." In our own studies, we have never found changes in the numbers of circulating leukocytes under resting conditions over a 30-min interval. Thus, given the cost of microarray analysis and the added complexity of a crossover design in this context, we feel that it was reasonable, although perhaps not ideal, to focus on the simpler study design.

As noted, activation of neutrophils is often accompanied by inhibition of apoptosis and prolonged neutrophil viability, but the genomic response to exercise, while involving cell viability genes, indicated a mixed response regarding the genomic regulation of apoptosis. Of the genes known to be related to apoptosis (Table 3), the exercise-associated changes in nine were consistent with instigation of apoptosis, while the changes in seven likely inhibited apoptosis. Nonetheless, subsequent pathway mapping of our results using DAVID showed that exercise activated the Jak/STAT pathway (EASE score <0.05) through alteration of nine of the pathway genes [CCND2 (fold change 1.8), IFNAR2 (1.3), IL10RA (1.6), IL2RB (2.2), PIK3R1 (1.7), STAT1 (0.6), STAT4 (2.6), SOCS1 (1.6), SOCS2 (2.0)]. Jak/STAT pathway genes are involved in many cellular functions (26), but Fortin et al. (12) recently demonstrated that Jak/STAT pathways activation rescued neutrophils from spontaneous apoptosis.

The impact of exercise on neutrophil apoptosis has yet to be completely elucidated. In rats, Lagranha et al. (19) demonstrated increased apoptotic markers (like annexin V) in neutrophils immediately following a 1-h bout of strenuous exercise. By contrast, in humans, Hsu et al. (14) showed no immediate effect of 30 min of heavy exercise on neutrophil apoptosis (using mitochondrial membrane potential), but did show accelerated neutrophil apoptosis the day after the exer-

Table 4. *Representative genes related to growth, tissue repair, and immune response*

Gene Name/Category	Gene Symbol	Fold Change
Growth and Tissue Repair		
Amphiregulin (schwannoma-derived growth factor)	AREG	3.4
FYN oncogene related to SRC, FGR, YES	FYN	3.0
Platelet derived growth factor D	PDGFD	2.8
Ksp37 protein	KSP37	2.9
Spondin 2, extracellular matrix protein	SPON2	2.9
Signal transducer and activator of transcription 4	STAT4	2.6
Transforming growth factor, beta receptor III (betaglycan, 300 kDa)	TGFBR3	2.4
Low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	2.3
Suppressor of cytokine signaling 2	SOCS2	2.0
Runt-related transcription factor 3	RUNX3	1.9
Lipin 1	LPIN1	1.5
Inhibitor of growth family, member 3	ING3	0.7
Serine/threonine kinase receptor associated protein	STRAP	0.7
G protein-coupled receptor 43	GPR43	0.7
Early growth response 1	EGR1	0.2
Growth arrest and DNA-damage-inducible, beta	GADD45B	0.7
Signal transducer and activator of transcription 1, 91 kDa	STAT1	0.6
Tropomyosin 3	TPM3	0.7
Immune response		
Interferon induced with helicase c domain 1	IFIH1	0.7
fc fragment of igg, low affinity iia, receptor (cd32)	FCGR2A	0.7
Toll-like receptor 10	TLR10	0.7
Interleukin 18 receptor accessory protein	IL18RAP	1.4
Annexin a1	ANXA1	1.5
Single immunoglobulin and toll-interleukin 1 receptor (tir) domain	SIGIRR	1.5
Chemokine (c-c motif) receptor 5	CCR5	1.6
Interleukin 32	IL32	1.7
Ras homolog gene family, member h	RHOH	1.7
Microtubule-associated protein, rp/eb family, member 2	MAPRE2	1.7
Killer cell lectin-like receptor subfamily c, member 4	KLRC4	2.0
Zeta-chain (tcr) associated protein kinase 70 kDa	ZAP70	2.1
granzyme a (granzyme 1, cytotoxic t-lymphocyte-associated serine esterase 3)	GZMA	2.1
Slam family member 7	SLAMF7	2.1
Cathepsin w (lymphopain)	CTSW	2.4
Granulysin	GNLY	2.5
Spondin 2, extracellular matrix protein	SPON2	2.9
Perforin 1 (pore forming protein)	PRF1	3.1
killer cell immunoglobulin-like receptor, 3 domains, long cytoplasmic tail, 2	KIR3DL2	3.7

Fold change is defined as the geometric mean of expression levels of After/Before. A fold change of 0.6 indicates the expression level after exercise is ~60% of the expression level before the exercise. A fold change of 1.3 indicates the expression level after the exercise is ~130% of the expression level before the exercise.

cise protocol. The data from the current study have identified a discrete number of neutrophil cell viability genes that are responsive to exercise. Clearly, alterations in gene expression associated with apoptosis in neutrophils can have profound physiological effects, but without specific studies of cell viability, the ultimate physiological consequences of the changes in gene expression that we observed remain unknown.

We also examined the impact of exercise on genes related to inflammation. Using DAVID, we found 52 genes that were significantly affected by exercise and linked to the immune response (partial list is shown in Table 4). In some cases, the effect of exercise was proinflammatory, for example granzyme A and perforin had higher expression (by 2.1- and 3.1-fold, respectively) and these genes control proteins that lyse cell membranes permitting the neutrophil to kill or damage invading cells or organisms. In contrast, we also found that exercise stimulated anti-inflammatory genes. For example, annexin 1 was upregulated. This protein is known to be expressed at high levels in neutrophils and acts to inhibit the release of oxygen radicals and other potentially toxic mediators from activated cells (36). The idea that a brief exercise bout could stimulate both pro- and anti-inflammatory responses in healthy people was suggested earlier by Ostrowski et al. (33). This notion has now been corroborated in a number of studies, including in our own recent examination on the effect of brief exercise on gene expression in circulating PBMCs (9). Apparently, exercise elicits an initial pro- and anti-inflammatory gene regulatory paradigm in circulating neutrophils as well.

We found that several genes involved in growth and tissue repair were influenced by exercise (Table 4). Amphiregulin (AREG), for example, a member of the epidermal growth factor (EGF) family of genes, increased by 3.4-fold [note, in an earlier studies we found that the epiregulin gene, structurally similar to amphiregulin and also in the EGF family of genes, was increased by exercise in PBMCs (9)]. EGF genes have been found to play a role in an increasingly large array of growth activities ranging from the regulation of body composition to bone growth early in life (48). Amphiregulin gene expression in neutrophils had not been observed previously.

A related observation was the impact of brief exercise on neutrophil genes associated with new blood vessel formation. Among these were early-growth response-1 (which was substantially downregulated), and upregulation of platelet derived growth factor-D (PDGF-D) and fibroblast growth factor receptor-2 (FGF-2). Interestingly, when EGR1 is upregulated chronically, angiogenesis is inhibited (21), whereas upregulation of PDGF-D (20) and FGF2 receptor genes (37) stimulate angiogenesis. This pattern of growth regulatory gene expression in response to exercise suggests a possible role for neutrophils in exercise-associated angiogenesis, one of the known mechanisms of improved physical activity performance that occurs in response to training (5).

Brief exercise represents a profound and complex perturbation to cellular homeostasis, and it is not surprising, given the reactive nature of the innate immune system, that we did observe changes in gene expression of the neutrophil, one of the primary cells of innate immunity. Innate immune responses are typically characterized as stereotypic or nonspecific, particularly compared with adaptive immunity (17). Consequently, to determine how similar were the neutrophil gene

Table 5. *Fold change from before to after exercise of 6 genes for 11 subjects measured by microarrays and RT-PCR*

	Microarray		
	Up	Down	Total
RT-PCR			
Up	51	1	52
Down	1	12	13
total	52	13	65

Fisher exact test for directional change: P value <0.0001.

expression responses following exercise to responses caused by other stimuli, we compared the changes in neutrophil gene expression from the present study with those observed by Zhang et al. (49) using an in vitro stimulation of neutrophils with live, opsonized *Escherichia coli* or LPS or chemoattractant formyl-methionyl-leucylphenylalanine (Table 6). We did find a set of common genes in neutrophils that responded to both the in vivo exercise and in vitro immunologic stimuli. However, as shown in Table 6, the response pattern in this common set of genes, i.e., whether the genes were up- or downregulated, differed substantially.

As noted above, even brief exercise is accompanied by substantial perturbations in temperature, pH, oxygen availability, and cytokines, each of which is known to influence neutrophil function (3, 23, 24, 39, 44). Remarkably, we found changes in neutrophil gene expression of proteins that could provide mechanistic insight, in part, to each of these previously observed changes in neutrophil function (e.g., HSPA8, HSPA1B). Collectively, these observations suggest that the neutrophil gene expression accompanying exercise is more than just a stereotypic stress response and likely represents an integrated or summed response to the various physiological perturbations associated with physical activity. Exactly how these changes in gene expression translate into actual changes in protein production with physiological consequences has yet to be determined.

Two alternative mechanisms might explain the robust effect of exercise on neutrophil gene pathways: the first, a direct effect of exercise on gene expression within the population of circulating neutrophils, and the second, an indirect effect, the mobilization into the circulation of neutrophils that were expressing genes differently in their marginal pools (medullary or extramedullary) than those neutrophils already in the circulating blood. In human studies, it would not be feasible to sample gene expression of marginal pools of neutrophils. Nonetheless, our data do permit us to draw some inferences concerning possible mechanisms.

Consider first, for example, the gene with the highest increase in expression-endothelial differentiation, sphingolipid G protein-coupled receptor 8 (EDG80). The 4.2-fold increase that we observed for this gene could occur, however, only if marginal neutrophils that entered the circulation during exercise (note, we observed a 44% increase) were expressing EDG80 at levels ~11-fold greater than the circulating neutrophils. The decrease in expression for another gene, EGR-1 which had a fold change of 0.2, is even harder to model. If, in the extreme case, the marginal neutrophils had no detectable expression of this gene, the lowest possible postexercise ratio would be 0.69. Thus it is reasonable to speculate from the current data that exercise has some effect on gene expression even in the circulating population of neutrophils.

Although brief exercise is usually tolerated well, exercise can elicit an immunologic "danger" type of stress and inflammatory response that, on occasion, becomes dysregulated and detrimental to health (11). In many cases, such as exercise-induced asthma, anaphylaxis, and chronic diseases like arthritis, there is mounting evidence that neutrophils play a pathologic role. Consequently, we found in our data a subset of neutrophil genes that were influenced by brief exercise and that also have been identified by other investigators to be influenced by asthma (Ref. 31; Table 7) or arthritis (15) (Table 8). Several

Table 6. *Comparison between the changes in gene expression from 1) stimulated neutrophils in vitro (49) and 2) circulating neutrophils following exercise in vivo (current study). Although there is a set of genes in common, the pattern in response to the two stimuli differed*

Gene Name	Gene Symbol	In Vitro	In Vivo
Similar effects			
Histone 1, H2ac	HIST1H2AC	↓	↓
Signal transducer and activator of transcription 1, 91 kDa	STAT1	↓	↓
TGFB-induced factor (TALE family homeobox)	TGIF	↑	↑
Runt-related transcription factor 3	RUNX3	↑	↑
cAMP responsive element modulator	CREM	↑	↑
SMAD, mothers against DPP homolog 7 (*Drosophila*)	MADH7	↑	↑
Opposite effects			
jun D proto-oncogene	JUND	↓	↑
H2.0-like homeo box 1 (*Drosophila*)	HLX1	↓	↑
LIM domain only 4	LMO4	↓	↑
Zinc finger protein 36, C3H type-like 2	ZFP36L2	↓	↑
Early growth response 1	EGR1	↑	↓
Topoisomerase (DNA) I	TOP1	↑	↓

Table 7. *Five genes whose expression in neutrophils was influenced by 30 min of exercise in healthy adults (current study) and were also linked to asthma[data from Ober and Hoffjan et al. (31)]*

Gene Name	Gene Symbol	Fold Change	*P* Value (Paired *t*-Test)
Signal transducer and activator of transcription 4	STAT4	2.6	7.6E-06
T-box 21	TBX21	2.5	6.0E-07
GATA binding protein 3	GATA3	1.7	2.3E-05
Chemokine (C-C motif) receptor 5	CCR5	1.6	4.2E-05
Toll-like receptor 10	TLR10	0.7	4.5E-04

Fold change is defined as the geometric mean of expression levels of After/Before. A fold change of 0.7 indicates the expression level after exercise is ~70% of the expression level before the exercise. A fold change of 1.7 indicates the expression level after the exercise is ~170% of the expression level before the exercise.

Table 8. *Five genes whose expression in neutrophils was influenced by 30-min of exercise in healthy adults (current study) and were also found to be downregulated in neutrophils in arthritis patients [data from Jarvis et al. (15)]*

Gene Name	Gene Symbol	Fold Change	*P* Value (Paired *t*-Test)
Granulysin	GNLY	2.5	9.8E-06
Dual specificity phosphatase 2	DUSP2	2.2	4.5E-05
Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	GZMA	2.1	7.5E-06
Suppressor of cytokine signaling 1, SSI-1	SOCS1	1.6	1.2E-04
Growth arrest and DNA-damage-inducible, beta	GADD45B	0.7	1.8E-05

Fold change is defined as the geometric mean of expression levels of After/Before. A fold change of 0.7 indicates the expression level after exercise is ~70% of the expression level before the exercise. A fold change of 1.6 indicates the expression level after the exercise is ~160% of the expression level before the exercise.

studies (2–5) had shown that TBX21 and Gata 3 have been associated with an asthma or atopy phenotype. This subset of neutrophil genes that are both influenced by exercise and altered in disease states may prove as useful targets for further investigations into how physical activity acts to prevent or exacerbate disease.

In summary, relatively brief but strenuous exercise caused a substantial, specific, and seemingly paradoxical, change in gene expression in the circulating neutrophils. For example, both pro- and antiapoptotic genes were altered, as well as genes responsible for inflammation, on the one hand, and growth and repair, on the other, suggesting that the genomic response in the neutrophil immediately following the perturbation of exercise might be characterized as a cellular "wake-up" call. It is then the influence of subsequent physiological stimuli that ultimately determines which cellular pathway is actually activated or inhibited leading, in some cases, to change in neutrophil function. It well may be that the balanced response observed with this protocol could be upset when exercise is of longer duration and/or greater intensity. Nor did our data permit us to determine the impact of exercise on neutrophil gene expression beyond the end-exercise (30 min) time point that we chose to sample. Our study demonstrated that the neutrophil genomic response is dynamic not only under in vitro conditions shown in previous studies in which cultured cells are stimulated by a variety of factors ranging from cytokines to LPS (30, 49), but also under physiological conditions in vivo, like exercise, that occur frequently in the daily lives of human beings.

GRANTS

This work was supported in part by National Institutes of Health Grants RO1-HL-080947 and P01HD-048721 and the UCI Satellite GCRC:MO1 RR00827.

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