Circulating inflammatory miRNA signature in response to different doses of aerobic exercise

David de Gonzalo-Calvo,1,2 Alberto Dávalos,3 Ana Montero,4 Ángela García-González,4 Iryna Tyshkovska,3 Antonio González-Medina,4 Sara M. A. Soares,3 Pablo Martínez-Cambor,5,6 Patricia Casas-Agustench,3 Manuel Rabadán,7 Ángel E. Díaz-Martínez,7 Natalia Úbeda,4 and Eduardo Iglesias-Gutiérrez2,4

1Lipids and Cardiovascular Pathology Group, Research Institute of the Sant Pau Hospital (IIB Sant Pau), Barcelona, Spain; 2Department of Functional Biology (Physiology), University of Oviedo, Oviedo, Spain; 3Laboratory of Disorders of Lipid Metabolism and Molecular Nutrition, Madrid Institute for Advanced Studies-Food, Campus de Excelencia Internacional Autonómica de Madrid, Madrid, Spain; 4Department of Pharmaceutical and Health Sciences, Centro de Estudios Universitarios San Pablo University, Madrid, Spain; 5Hospital Universitario Central de Asturias, Hospital Universitario Central de Asturias, Asturias, Spain; 6Universidad Autónoma de Chile, Santiago, Chile; and 7Sports Medicine Center, Clinical Laboratory, Higher Council for Sports, Madrid, Spain

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ACUTE EXERCISE INDUCES A NUMBER OF physiological adaptations in virtually all body systems (24). One type of physiological responses that has attracted much attention in the last decade is the exercise-induced inflammatory cascade. In vitro, in vivo, and observational evidence suggests that moderate acute exercise activates strong anti-inflammatory mechanisms, which are implicated in training adaptation and the health benefits of physical activity (23, 33, 55, 57). However, strenuous exercise is also clearly linked to deleterious inflammatory perturbations that can cause tissue damage and physiological malfunction (28, 52). It is fundamental to elucidate the molecular pathways that control the initiation, propagation, magnitude, and resolution of the exercise-induced inflammatory cascade (48). Alterations in the number and functionality of circulating immune cells and the secretion profile of inflammatory mediators released from tissues implicated in the exercise-induced inflammatory response have been extensively studied (11, 20, 45, 61, 62, 80). However, the role of novel regulators, such as microRNAs (miRs), in this inflammatory response remains poorly understood.

miRs are small noncoding RNAs that control gene expression at the posttranscriptional level by degrading or inhibiting mRNA translation. miRs play a critical role in normal development, homeostasis, and the cellular response to physiologic and pathophysiologic stress (17, 39). Indeed, they have been described as fine-tuning regulators of many biological processes in both health and disease, including exercise and inflammation. Both acute and chronic exercise alters the intracellular profile of miRs in skeletal muscle and in the immune and cardiovascular systems (14, 38, 69). Previous studies have highlighted the role of miRs in exercise-induced adaptation, including skeletal muscle hypertrophy, remodeling and metabolism (15, 66), angiogenesis (34) and cardiac structural alterations (16). A reciprocal association between inflammation and miRs has been reported. In vitro and in vivo investigations have demonstrated that inflammatory stimuli activate miRNA biogenesis (73). Genetic ablation of miRNA biogenesis induces inflammatory perturbations (65). Given their dynamic nature, miRs have been proposed to participate in the optimization of the inflammatory response and the maintenance of homeostasis (36). The cellular pathways altered by inflamma-
tion-related miRs range from cytokine synthesis to leukocyte activation (48). Interestingly, evidence indicates a miR-mediated link between exercise and inflammation; brief bouts of intense exercise alter the miR profile of mononuclear leukocytes, many of which are involved in inflammatory mechanisms (59).

Relatively recent studies have demonstrated that miRs can be detected in body fluids (41). miRs are actively secreted in extracellular vesicles (81) or associated with RNA-binding proteins (3) or lipoproteins (76). Importantly, recently reported data point to extracellular miRs as key regulatory signals in cell-cell communication (25). Cells selectively release specific miRs, which, like other soluble factors such as cytokines or hormones, function as autocrine, paracrine, or endocrine factors by mediating intercellular gene regulation and phenotypic control (9).

Altered c-miR profiles have been reported after both acute endurance and resistance exercise and chronic exercise interventions (4, 67). However, most studies have analyzed a limited selection of miRs previously described as mediators of physiological processes linked to exercise adaptations in skeletal muscle, heart, or vasculature (2, 4–6, 21, 43, 75). Given the physiological importance of the exercise-induced inflammatory response in health and training adaptation, the roles of miRs as intercellular communicators and regulators of physiological responses, and their potential as biomarkers, more detailed investigation is warranted. To date no studies have specifically examined the global response of circulating inflammation-related miRs (c-inflammamiRs) to acute exercise. Moreover, any analysis of exercise-induced changes in c-inflammamiRs should consider the magnitude of the inflammatory response to acute exercise. The goal of the present study was to analyze the response of a panel of c-inflammamiRs to different doses of acute aerobic exercise to further our understanding of the role of c-inflammamiRs in exercise-induced inflammation.

MATERIALS AND METHODS

Ethics statement. All experimental procedures were approved by the respective Research Ethics Committees of the Centro de Estudios Universitarios San Pablo University and the University of Oviedo, in accordance with the Declaration of Helsinki. All participants provided written informed consent.

Experimental design. An observational experimental design was used. Participants completed three races: a 10-km race (10-km), a half-marathon (HM), and a marathon (M), each separated by 1 mo. Although all trials involved the same type of exercise (endurance running), they differed in terms of duration, intensity, and energy demands and thus represent distinct exercise doses (8, 12, 40, 68).

Participants. Volunteers were recruited from the members of MAPOMA Sports Association, a sports club open to amateur and professional runners who wish to participate in the popular Madrid marathon. Figure 1 shows a schematic overview of participant recruitment. All runners in the amateur training group (n = 35) were invited to an informative briefing. A member of the research team presented the aims and methodology of the study and answered the questions of the potential participants, of whom 18 (51%) agreed to participate. The remainder (n = 17) showed interest in the study but were unable to participate for various reasons (family and professional commitments, travel, or injuries). Before participation, each volunteer underwent a thorough medical screening to determine eligibility. A number of inclusion and exclusion criteria were established. The inclusion criteria were as follows: 1) men over 18 yr (only men were selected for their more stable hormonal status); 2) no smokers or frequent passive smokers; 3) officially registered for the Madrid marathon; 4) regular training (at least 50 km/wk); 5) previous participation in at least two marathons; and 6) written informed consent. The exclusion criteria were as follows: 1) any chronic disease; 2) body mass index (BMI) >30 kg/m²; and 3) any dietary or pharmacological treatment during the course of the study. Although 14 volunteers fulfilled these criteria and initially participated in the study, only 9 participants completed the study. The remaining five participants were unable to finish at least one of the races or missed one of the blood extractions and were excluded.

Assessment of body composition and aerobic capacity. Two weeks before the first race, the participants had their body composition and maximal oxygen uptake (V̇O₂max) assessed at the Physiology of Effort Unit. Two ISAK Level III-certified anthropometrists measured height and body mass using a combined medical scale (model 778; Seca, Hamburg, Germany; precision: 0.1 cm for height and 0.1 kg for weight). BMI was then calculated from these measurements. The equation of Kyle et al. (32) was used to estimate percent body fat (%BF) based on the information obtained using a multifrequency bioimpedance device (Total Body Scan; Bio-Logic). This equation is considered the most appropriate by the Spanish Group of Kinanthropometry (1).

V̇O₂max was determined by indirect calorimetry (Oxycon Pro; Jaeger) using an incremental protocol on a treadmill (LE-600 C; Jaeger-HP Cosmos).

Training schedule and training history. During their visit to the laboratory, the volunteers were interviewed about their training volume over the previous 3 mo and their training history. Participants were asked not to alter their usual training schedule, and all performed low-intensity training the day before each race.
Blood sampling. Four blood samples were collected for each race: one before and three at various stages after the end of the exercise bout. Identical sampling time points were used for all races. Samples were collected by experienced technical staff, using standardized techniques and materials. The first blood sample (Basal) was collected from fasting participants ~1.5 h before the race before warm-up. Participants then ate their breakfast, performed their warm-up exercises, and began the race. A second blood sample (Post0) was drawn within 10 min of completing the race. In both cases, samples were collected and processed in a field laboratory near the race start and finish lines.

The following morning (Post24) and 3 days after the race (Post72), participants reported to the Clinical Laboratory at the Center for Sports Medicine after overnight fasting for collection of additional samples. These samples were taken at the same time of day as basal sample. The total volume of blood taken per race was <50 ml.

For complete blood counts (total leukocytes, neutrophils, lymphocytes, monocytes, and eosinophils) and lymphocyte subsets analysis, blood was collected in EDTA vacuum blood tubes (Becton Dickinson, Franklin Lakes, NY), stored at room temperature (20–25°C), and analyzed within 4 h. For serum separation, blood samples were collected in vacutainers [No Additive (Z); Becton Dickinson, Franklin Lakes, NJ]. Samples were stored at room temperature for at least 15 min to allow clot formation and then immediately centrifuged for 15 min at 4,000 rpm and 10°C. Serum samples were then aliquoted and stored at −80°C for later analysis.

Dietary control. Participants were asked to keep a food diary for 5 consecutive days: 2 days before the first race, the race day, and the 2 following days. They were specifically asked not to alter their usual dietary pattern during the recording periods but were asked to abstain from caffeine and alcohol consumption for at least 24 h before race. No limitations on the type or amount of food or beverages consumed were imposed at any time during these 5 days of food recording. All foods and beverages consumed were recorded using standard culinary measures (44), and food labels were collected for information on food supplements, snacks, and packaged foods. No participants reported using nutritional supplements. Volunteers received specific oral guidelines and detailed written instructions on how to put this method into practice. A telephone number was made available to answer any queries about the dietary recording process.

To minimize the impact of food intake on results, volunteers were asked to apply the same dietary regimen on the days before and after the other races and to keep a food diary, following the same instructions given for the first race.

Food records were carefully reviewed immediately after completion, and participants were contacted to clarify any ambiguities. Dietary records were analyzed using nutrient analysis software (DIAL: Alce Ingenieria).

Leukocyte counts and biochemical determinations. Complete blood counts were determined by standardized clinical laboratory procedures using an automated hematolgy analyzer (ADVIA 120; Siemens Healthcare Diagnostics). Whole blood samples (100 μl) were incubated in darkness at room temperature (20–25°C) for 15 min with 10 μl of specific fluorescent-conjugated monoclonal antibodies (Becton Dickinson Biosciences) for subsequent evaluation of the CD16+CD56+ natural killer (NK) subset by flow cytometry. Next, 2 ml of lysing solution (Becton Dickinson Biosciences) were added to lyse red blood cells and the samples were mixed gently and incubated for 20 min in darkness at room temperature. Immediately after incubation, tubes were centrifuged for 5 min at 330 g and 4°C and the supernatant was removed. Samples were washed twice with phosphate-buffered saline (PBS) and centrifuged for 5 min (300 g), and the supernatant was removed at the end of each step. Finally, 500 μl of PBS were added to each tube. The samples were also incubated with the corresponding control. Fluorescence was analyzed using a FACSCALIBUR cytometer (Becton Dickinson).

High-sensitivity C-reactive protein (hs-CRP) and creatine kinase (CK) were analyzed in serum samples using an AU400 clinical analyzer (Beckman Coulter) and Beckman reagents. Cytokines (IL-1β, IL-6, IL-8, IL-10, and TNF-α) were assayed using an IMMULITE ONE analyzer (Siemens Healthcare Diagnostics) and proprietary cytokine kits.

RNA isolation and quantitative RT-PCR. Total circulating RNA was isolated from 200 μl of serum using the miRCURY RNA isolation kit (Exiqon), following the manufacturer’s instructions. RNA spike-in kit (Exiqon) was used in all extractions. For RNA extraction, samples were supplemented with 5 fmol of Caenorhabditis elegans mir-39-3p (cel-miR-39-3p) for normalization of the data. For miRNA quantification, cDNA was synthesized using the universal cDNA synthesis kit II (Exiqon) and miRNAs were quantified by quantitative real-time PCR (qRT-PCR) using the ExiLENT SYBR green master mix (Exiqon) and a 7900HT Fast Real-Time PCR System (Applied Biosystems).

To rule out hemolysis or the presence of nucleases or inhibitors, the miRCURY miRNA Quality Control Panel (Exiqon) was used before miRNA analysis. This panel (Exiqon) includes specific primers to analyze miR-23a and miR-451, which are normally found in plasma and serum and serve as markers of hemolysis.

Quantification of circulating miRNAs. miRNA quantitative analysis was restricted to a panel of 106 circulating miRs whose roles in inflammatory processes have been previously demonstrated. Those miRs were chosen after an extensive review of the literature. Specifically, we chose miRs implicated in the regulation of cytokine synthesis and inflammatory pathways and in leukocyte proliferation, differentiation, and activation (36–38, 48, 49, 79). Candidates were also selected based on previous studies of extracellular inflammation-related miRs in pathological conditions (9, 19, 22, 26, 30, 50). Some of those miRs (miR-126, -143, -146a, -155, -21, -221, and -223) have been previously studied in exercise-related studies (4 – 6, 43, 46, 75). To avoid potential bias due to controversy in miR nomenclature, we evaluated -3p and -5p chains. Expression of circulating miRNA was normalized to that of cel-miR-39-3p and relative expression was performed using GenEx software (MultiD Analyses AB).

Functional in silico analysis. In silico identification of molecular pathways potentially affected by the observed alterations in c-inflammation levels was performed using the web-based computational tool DIANA-miRPath v2.0 (77). DIANA-miRPath v2.0 utilizes experimentally verified miRNA targets from TarBase v6 and predicted miRNA targets from the DIANA-microT-CDS algorithm and combines the results with the pathway tool KEGG (Kyoto Encyclopedia of Genes and Genomes) to identify possible targets. The level of significance was set at P < 0.05.

Statistical analysis. Descriptive statistics were used to characterize study populations and to analyze the studied parameters. Normality was determined using the Kolmogorov-Smirnov test. One-way ANOVA for repeated measures with Bonferroni correction was used to compare inflammatory parameters between races (10-km, HM, and M) and sampling time points (Basal, Post0, Post24, and Post72). Fold change with respect to the Basal sample was determined to quantify the magnitude of the inflammatory response. Data are presented as the means ± SE. Correlations between variables were analyzed using Pearson’s correlation analysis, and the results are presented using Pearson’s correlation coefficient and 95% confidence interval [p (95% CI)]. Some samples showed concentrations that were below the limit of detection (cytokines IL-6, IL-8, IL-10, and TNF-α in Basal, Post24, and Post72). The limit of detection was used for statistical purposes. All statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Chicago, IL). Differences were considered statistically significant at P < 0.05.

For miRNA analysis, each detected miRNA was analyzed using the following test: H_0,0:i:0=\mu_{i} versus \mu_{24h,i} and \mu_{i} versus \mu_{24h,i} are the means of the i-th miRNA (1 ≤ i ≤ 81) at Post0 and Post24. Data were analyzed using repeated measures...
Table 1. Physical characteristics and training profile of participants

<table>
<thead>
<tr>
<th>Anthropometric Data/Training Profile</th>
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<tr>
<td>Physical characteristics</td>
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<tr>
<td>Age, yr</td>
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<tr>
<td>Height, cm</td>
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<tr>
<td>Weight, kg</td>
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<tr>
<td>BMI, kg/m²</td>
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<tr>
<td>%BF*</td>
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<tr>
<td>VO₂max, ml·kg⁻¹·min⁻¹</td>
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| Training habits                       |
| Training history, yr                  | 6.6 ± 1.3 |
| Training volume, km/wk                | 69.7 ± 5.0 |

Data represent means ± SE. BMI, body mass index; %BF, percent body fat; VO₂max, maximal oxygen uptake. *The equation of Kyle et al. (32) was used to estimate percent body fat (%BF) based on the information obtained using a multifrequency bioimpedance device.

ANOVA and P values were adjusted using the false discovery rate (FDR) criterion, applying the Benjamini-Hochberg method. For miRNAs with a FDR < 0.05, we analyzed the difference between each pair; these differences were considered relevant if they fulfilled the following criteria: 1) P < 0.050 (paired Student’s t-test) and 2) a difference between means of >1.5. A customized R (www.r-project.org) function was used for all analyses.

RESULTS

Physical characteristics, training habits, and dietary control. Anthropometric data and the training practices and training history of the participants are presented in Table 1. The overall profile of the participants is one of middle-aged, slightly overweight, experienced amateur runners with moderately high workloads. No differences in the daily energy and macronutrient intake of participants were observed over 5 consecutive days before, during, and after each race (data not shown).

Classical inflammatory response. Based on their demonstrated role in exercise-induced inflammatory responses, a panel of cellular and soluble inflammatory markers was chosen for comparative analysis. Total leukocyte and neutrophil counts increased immediately after all races, as expected (Fig. 2, A and B). Twenty-four hours after the 10-km and HM races, leukocyte and neutrophil counts in samples had returned to preexercise levels. After the marathon, leukocyte and neutrophil counts returned to basal levels in less than 24 and 72 h, respectively. Lymphocyte and monocyte counts increased significantly immediately after 10-km and M races, respectively. Both parameters returned to basal levels in less than 24 h (Fig. 2, C and D). Significant perturbations in eosiophils [Basal vs. Post0, 10-km: 160 ± 3 vs. 110 ± 3 (P < 0.010); HM: 190 ± 3 vs. 100 ± 3 (P < 0.050); M: 120 ± 2 vs. 60 ± 1 (P < 0.050)] and NK counts [Basal vs. Post0, 10-km: 433.62 ± 59.30 vs. 801.15 ± 78.18 (P < 0.010)] were also observed.

Levels of the soluble inflammatory mediators IL-6, IL-8, and IL-10 increased immediately after the M race (Fig. 2, E–G). IL-6 and IL-10 levels returned to basal levels within 24 h. By contrast, IL-8 levels remained elevated at 24 h and had returned to basal levels by 72 h. In HM samples levels of IL-6 and IL-10, but not IL-8, were increased immediately after the race, and returned to preexercise levels within 24 h (Fig. 2, F and G). hs-CRP levels were increased in HM and M samples at 24 and 72 h (Fig. 2H). Soluble inflammatory mediators showed no significant changes at any time point after the 10-km race. No differences in circulating TNF-α levels were observed for any race (data not shown). IL-1β levels were below the limit of detection for all races and time points.

Based on the kinetics observed and to further characterize the magnitude of the inflammatory response for each race, we compared the Post0-Basal fold change between the three races. Significantly greater fold changes in leukocyte, neutrophil, and monocyte counts and IL-8 levels were observed in M samples compared with both 10-km and HM samples (Table 2). Significant differences were also observed for IL-6 and IL-10 in M vs. 10-km samples; IL-6, IL-8, and IL-10 in HM vs. 10-km samples; and lymphocyte count in 10-km vs. M samples (Table 2). As expected, hs-CRP showed delayed kinetics with respect to other inflammatory parameters. Analysis of the Post24-Basal fold change for this parameter revealed a significant increase in M samples compared with both 10-km and HM samples and in HM vs. 10-km samples.

Previous studies have demonstrated a close and direct association between muscle damage and exacerbated exercise-induced inflammatory responses (31, 53). We thus analyzed levels of the muscular damage marker CK in all three races. For HM and M races, circulating CK levels were increased at Post0, Post24, and Post72 with respect to basal levels (Table 3). CK increases were also observed at Post0 and Post24 after the 10-km race (Table 3). For all races the greatest increases were detected at Post24: significant increases were observed for M vs. both 10-km and HM and for HM vs. 10-km.

Circulating inflammatory miRN response. Once we characterized the inflammatory response for each of the different races we evaluated the profile of a panel of circulating inflammation-related miRs. To avoid the confounding influence of erythrocyte miRNAs released due to hemolysis we first analyzed miR-23a and miR-451. In all samples, the ΔCt values for these two miRNAs were far below 7 (data not shown), ruling out the possibility of hemolysis. c-inflammamiR profiles were determined for the 10-km (low inflammatory response) and M (high inflammatory response) races by analyzing Basal, Post0, and Post24 samples. Due to the possible confounding effects of factors such as diet, lifestyle, training, and recovery on c-miR levels we did not analyze Post72 samples. c-miR-150 levels were significantly increased (2.7-fold) after the 10-km race (Fig. 3A). Significant increases in the levels of c-let-7d-3p, -7f-2-3p, miR-125b-5p, -132-3p, -143-3p, -148a-3p, -223-3p, -223-5p, -29a-3p, 34a-5p, -424-3p, and -424-5p were observed after the M race (Fig. 3B). c-miR-223-5p showed the greatest increase (4.7-fold). All c-miRs returned to basal levels by 24 h postrace. Significant correlations were detected between c-inflammamiRs whose expression was significantly increased after the M race, except for c-miR-424-3p (data not shown). A total of 80 and 69 c-inflammamiRs showed no differences in expression after the 10-km and M races, respectively, with respect to corresponding basal levels (Table 4). Twenty-five c-inflammamiRs with Ct values >35 or whose replicates showed inconsistent results were excluded from further analysis (Table 4).

Based on our findings of increased muscle damage in response to exercise and to further characterize the c-inflammamiR response to exercise, we analyzed levels of four circulating myomiRs: c-miR-1, -133a, -133b, and -206. Results re-
Correlations between inflammatory parameters and c-inflammamiRs. To further explore the association between classical inflammatory response induced by exercise and the observed c-inflammamiR alterations, we correlated alterations in the levels of inflammatory variables with those in c-inflammamiR expression. Increases in miR-150-5p expression immediately after the 10-km race were directly correlated with increased leukocyte count \[0.715 (0.100 – 0.930)\] and neutrophil count \[0.747 (0.170 – 0.940)\] during the race. Increased let-7f-2-3p expression immediately after the M race was directly correlated with increased hs-CRP levels during Post24-Post0 \[0.704 (0.080 – 0.930)\]. The observed increase in miR-29-3p expression after the M race was inversely correlated with the fold change in IL-10 during the Post24-Post0 recovery period \[-0.682 (−0.930; −0.030)\]. A weak association was observed between the increases in miR-125b-5p and miR-34-5p expression during the M race and the decreases in hs-CRP \[0.661 (−0.010; 0.920)\] and IL-6 \[0.676 (−0.060; 0.930)\], respectively, during the recovery phase.

Pathways targeted by c-inflammamiRs. In silico analysis was performed to identify the biological mechanisms, pathways, and functions most closely linked with the observed c-inflammamiR profile. The DIANA-miRPath v.2.0 computational tool was used to analyze verified and predicted molecular pathways that may have been altered in response to the c-inflammamiR profile observed after the M race (77). Let-7d-3p, let-7f-2-3p, and miR-223-5p were not experimentally verified to target any gene, while miR-424-3p was verified to target gene X.
Characterization of the inflammatory response in each of the different races (10-km, HM, and M) showed that, as expected (78), aerobic exercise of sufficient duration and intensity induces an inflammatory response, followed by a recovery period over the next 24 h during which most inflammatory parameters return to basal levels. The magnitude of the inflammatory response differed between races. We observed significant alterations in leukocyte subsets and in the levels of soluble inflammatory mediators (IL-6, IL-8, IL-10, and hs-CRP) after the M race, in line with previous studies that have analyzed the inflammatory response after prolonged or exacerbated acute exercise (7, 31, 51). These increases were significantly higher than those seen after 10-km and HM races. Samples taken after the HM race revealed a greater inflammatory response than seen after the 10-km race, particularly in the levels of soluble inflammatory mediators. Taken together, our results indicate a clear dose-dependent effect of aerobic exercise on systemic inflammation.

After analyzing the exercise-induced inflammatory response, we next characterized the c-inflammamiR profile after the races with lowest (10-km) and highest (M) inflammatory responses. Interestingly, we detected specific exercise dose-dependent c-inflammamiR responses. miR-150-5p was the only miR from the c-inflammamiR panel for which significant changes in expression were found after the 10-km race. This response differed substantially to that observed after the M race, which induced alterations in 12 c-inflammamiRs (let-7d-3p, -7f-2-3p, miR-125b-5p, -132-3p, -143-3p, -148a-3p, -223-3p, -223-5p, -29a-3p, -34a-5p, -424-3p, and -424-5p) (P < 0.050). Most pathways were related to cancer, immune system disease, and inflammation. The let-7d-3p was predicted to target three genes and thus was not considered for further analysis. Fifty-six KEGG pathways were enriched with the predicted targets of c-inflammamiRs miR-125b-5p, -132-3p, -143-3p, -148a-3p, -223-3p, -29a-3p, -34a-5p, and -424-5p (P < 0.050). Most pathways were related to cancer, immune system disease, and inflammation. The let-7d-3p was predicted to target three genes and thus was not considered for further analysis. Fifty-six KEGG pathways were enriched with the predicted targets of c-inflammamiRs let-7f-2-3p, miR-125b-5p, -132-3p, -143-3p, -148a-3p, -223-3p, -223-5p, -29a-3p, -34a-5p, -424-3p, and -424-5p (P < 0.050). A number of inflammation-related pathways were identified, including the P38K-Akt signaling pathway, the TGF-β signaling pathway, the Wnt signaling pathway, the MAPK signaling pathway, the adipocytokine signaling pathway, the mammalian target of rapamycin signaling pathway, the B-cell receptor signaling pathway, the p53 signaling pathway, the hypoxia-inducible factor-1 signaling pathway, the T-cell receptor signaling pathway, the VEGF signaling pathway, and the Hedgehog signaling pathway.

**DISCUSSION**

The close association between inflammation and physical activity has important consequences for training adaptations and health. However, limited information is currently available regarding the c-inflammamiR response to acute exercise. Given the key role proposed for c-miRs in cell-cell communication and their potential as biomarkers, we analyzed the effects of different doses of acute aerobic exercise on a panel of c-inflammamiRs. We compared the profiles of c-inflammamiRs before, immediately after, and 24 h after participants ran 10-km and M races. Our finding revealed that acute aerobic exercise induces a c-inflammamiR response and that this response varies with exercise dose.

**Table 2.** Post0-Basal fold change for inflammatory parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>10-km</th>
<th>HM</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte count</td>
<td>1.38 ± 0.07</td>
<td>1.44 ± 0.06</td>
<td>3.24 ± 0.20*</td>
</tr>
<tr>
<td>Neutrophil count</td>
<td>1.47 ± 0.07</td>
<td>1.87 ± 0.15</td>
<td>5.13 ± 0.39*</td>
</tr>
<tr>
<td>Monocyte count</td>
<td>1.09 ± 0.05</td>
<td>0.90 ± 0.11</td>
<td>2.02 ± 0.14*</td>
</tr>
<tr>
<td>Lymphocyte count</td>
<td>1.33 ± 0.08</td>
<td>1.12 ± 0.14</td>
<td>0.89 ± 0.07*</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.94 ± 0.28</td>
<td>8.30 ± 1.34b</td>
<td>19.95 ± 5.03*</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.12 ± 0.10</td>
<td>1.37 ± 0.11*</td>
<td>7.07 ± 1.75*</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.11 ± 0.08</td>
<td>3.44 ± 0.55*</td>
<td>11.59 ± 3.40*</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>1.88 ± 0.31</td>
<td>4.69 ± 0.88*</td>
<td>21.34 ± 4.84*</td>
</tr>
</tbody>
</table>

Data represent means ± SE. Basal, 1.5 h before race; Post0, 10 min after race; Post24, 24 h after race; HM, half-marathon; M, marathon. For hs-CRP, high-sensitivity C-reactive protein hs-CRP: Post24-Basal fold change. 10-km vs. HM: *P < 0.050; 10-km vs. M: *P < 0.010, *P < 0.001; HM vs. M: *P < 0.050, *P < 0.001.

Table 3. *Circulating creatine kinase levels in 10-km, half-marathon, and marathon races*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Basal</th>
<th>Post0</th>
<th>Post24</th>
<th>Post72</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-km</td>
<td>181.67 ± 41.39</td>
<td>246.22 ± 57.35*</td>
<td>264.00 ± 48.66a</td>
<td>178.78 ± 36.09</td>
</tr>
<tr>
<td>HM</td>
<td>182.67 ± 50.27</td>
<td>273.89 ± 66.66b</td>
<td>416.89 ± 113.59a,b</td>
<td>232.67 ± 53.72a</td>
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<tr>
<td>M</td>
<td>136.22 ± 23.55</td>
<td>298.89 ± 42.92c</td>
<td>578.67 ± 67.66c,d</td>
<td>261.00 ± 41.32c</td>
</tr>
</tbody>
</table>

Data represent means ± SE. CK, creatine kinase. Post72, 72 h after race. Basal vs. Post0, Post24, and Post72: *P < 0.050, *P < 0.010, *P < 0.001. Post24-Post0 fold change: 10-km vs. HM: *P < 0.050; 10-km vs. M: *P < 0.001; HM vs. M: *P < 0.001.

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Fig. 3. A: circulating inflammatory microRNAs (c-inflammamiRs) response for 10-km race. miR-150-5p was upregulated immediately after exercise (Post0), returning to basal levels in <24 h (Post24). B: c-inflammamiR response for marathon race. Twelve miRNAs were upregulated immediately after exercise (Post0), returning to basal levels in <24 h (Post24). Data were analyzed by one-way ANOVA for repeated measures and $P$ values adjusted using the false discovery rate criterion. Results represent the mean relative expression with respect to Basal sample. AU, arbitrary units. * $P < 0.050$ and difference between means $>1.5$.
exercise dose and is related to the classical inflammatory response.

miRs have been proposed as fine-tuners of the inflammatory response, controlling the switch from the initial proinflammatory response to the resolution phase (49). In our study, c-inflammamiR levels increased immediately after exercise and decreased to basal levels within 24 h of running the 10-km and M races, indicating a similar kinetic profile to that seen for inflammatory parameters. The magnitude of the inflammatory response after each race correlated with that of the c-inflammamiR response. In addition, as seen for classical inflammatory mediators such as cytokines, soluble receptors, or acute phase proteins, alterations in c-inflammamiR levels were highly correlated. We thus hypothesized that the exercise-induced inflammatory response is mediated not only by classical inflammatory mediators but also by c-inflammamiRs. Due to the experimental design used, causal relations could not be drawn from our data. However, previous data about the mechanisms in which our altered c-inflammamiRs participate, together with correlation analysis, support a role for these mediators in the exercise-induced inflammatory response. Intracellular expression of the let-7 family members miR-125b, -132, -143, -150, -223, -29a, and -34a is a highly regulated process induced by inflammatory stimuli and signals (35, 48, 49, 79), including acute exercise (59, 60). c-InflammamiRs for which significant alterations were observed are implicated in the tight regulation of the inflammatory response at different levels. Intracellular let-7 family members miR-125b, -132, -223, and -29a have been reported to target the expression of receptors [Toll-like receptor (TLR)3, TLR4, and TLR8], transcription factors (p300), signaling molecules (IKKα), and molecular regulators (ACHE, TNFAIP3) of TLR signaling pathways, which play key roles in the initiation and development of inflammatory responses (49, 50). Our findings could explain the amount of data showing a reduction in TLR-related response after strenuous exercise (33). Let-7 family members miR-125b, -148, -150, and -34a are predicted to target mRNAs involved in proinflammatory mediator expression, including the cytokine IL-6 (27, 35). miR-223 and -424 are fine-tuners of neutrophil and monocyte proliferation and activation (29, 64). In silico analysis corroborates this notion. We observed a close association between the pattern of altered c-inflammamiR expression and cellular pathways intimately involved in the inflammatory process. Finally, while the literature on extracellular inflammation-related miRs is scarce, previous studies support a role of c-inflammamiRs in the exercise-induced inflammatory cascade. Exosomes from dendritic cells containing miR-34a, -125b-5p, and miR-148, as well as other short noncoding RNAs, are proposed to regulate several physiological responses, including cytokine synthesis (42). miR-150 secreted from monocyte/macrophage has been described as a class of inflammatory factor (82). A recent study reported that HDL-miR-223 delivery to endothelial cells induces the translational repression of the intercellular adhesion molecule (ICAM-1), which is implicated in the recruitment of inflammatory leukocytes (72). The coordinated alteration in the c-inflammamiR profile, in concert with other mechanisms, may thus modulate the inflammatory cascade during acute exercise. Our results point to a role of c-miRs as regulators of the body’s response to acute exercise, in line with previous findings (4, 6, 43, 67).

The contribution of c-inflammamiRs to the exercise-induced inflammatory cascade and the role of extracellular miRs in cell-cell communication, including inflammatory processes (26), opens the door to speculation as to their pro- or anti-inflammatory functions. Strenuous exercise, such as marathon running and ultrarunning, has been associated with a deleterious proinflammatory response mainly related to cell damage (13, 78). It is thus reasonable to hypothesize that the c-inflammamiR profile observed after running the M race is associated with a proinflammatory response. Nevertheless, as stated above, the c-inflammamiR pattern observed after the M race has predominantly anti-inflammatory effects. These results support previous findings linking alterations in the levels of inflammatory-related miRs during pathological processes with the appropriate inhibition and termination of the proinflammatory response (49). The c-inflammamiR response should be interpreted as a compensatory anti-inflammatory mechanism to control the development, magnitude, and resolution of the response rather than a proinflammatory response to acute exercise. This situation is highly related to the anti-inflammatory nature of the classical inflammatory response induced by exercise. IL-6 is the first cytokine released into circulation during exercise, mainly from skeletal muscle. The circulating concentration of this inflammatory mediator increases exponentially during exercise and decreases after exercise (54). IL-6 is traditionally described as a proinflammatory cytokine. However, in the context of physical activity, IL-6 has a strong anti-inflammatory component (56). The infusion of recombinant IL-6 in young healthy volunteers, simulating acute exercise, induces an anti-inflammatory rather than a proinflammatory response (71). Indeed, secretion of this cytokine during acute exercise stimulates the secretion of anti-inflammatory mediators such as IL-10, IL-1 receptor antagonist (IL-1ra), and the soluble TNF receptors (sTNF-R) (18, 51, 71). Furthermore, the release of IL-6 in response to muscle contraction appears to regulate the biological activity of the proinflammatory cytokines TNF-α and IL-1β (70).

As anticipated based on previous findings (31, 53), we observed an increase in CK levels 24 h after the 10-km and M races, with the greatest increase detected after the M race. We thus investigated whether exercise-induced damage to skeletal muscle may trigger the nonspecific release of c-inflammamiRs into circulation. Although the intracellular muscle-specific miRs miR-1 and miR-133a are upregulated by endurance
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


