Mouse skeletal muscles infected with *Plasmodium berghei* and *Plasmodium chabaudi* reveal a crosstalk between lipid mediators and gene expression

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Abstract

Background

Malaria is one of the most prevalent infectious disease in the world with 3.2 billion humans at risk. Malaria causes splenomegaly and damage in other organs including skeletal muscles. Skeletal muscles comprise nearly 50% of the human body and are largely responsible for the regulation and modulation of overall metabolism. It is essential to understand how malaria damages muscles in order to develop effective preventive measures and/or treatments. Using a pre-clinical animal model, we investigated the potential molecular mechanisms of plasmodia infection affecting skeletal muscles of mice.

Methods

We employed the Mouse Signal Transduction Pathway Finder PCR Array to monitor gene expression changes of 10 essential signaling pathways in skeletal muscles from mice infected with Plasmodium berghei and P. chabaudi. We then applied our new targeted-lipidomic approach using liquid chromatography with tandem mass spectrometry to profile 158 lipid signaling mediators (LMs), mostly arachidonoids, eicosanoids and docosanoids. We further quantified 16 key LMs directly associated with inflammation, oxidative stress, and tissue healing in skeletal muscles.

Results

Our results showed that the expression of key genes altered by malaria infection associate with inflammation, oxidative stress, and atrophy. In support to our gene profiling, lipidomics revealed higher concentrations of LMs in skeletal muscles directly related to inflammatory responses, while on the levels of LMs crucial in resolving inflammation and tissue repair reduced significantly.

Conclusion

Our results provide new insights into the molecular mechanisms of malaria-induced muscle damage and revealed a potential mechanism modulating inflammation in malarial muscles. These pre-clinical studies should help with future clinical studies in humans aimed at monitoring of disease progression and development of specific interventions for the prevention and mitigation of long-term chronic effects on skeletal muscle function.
Background
Malaria remains as the most significant human infectious diseases in the World with 3.2 billion humans at risk [1]. The human malaria is caused by *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium knowlesi*, *Plasmodium malariae* and *Plasmodium ovale*. The disease continues to spread and curb the development of various African countries, causing a huge economic and social cost [2]. Malaria pathogenesis is a process by which malaria parasites cause illness, abnormal function and damage in the hosts. The malaria pathogens have been related to cause detrimental effects on skeletal muscles of animals and humans [3-9]. In addition of affecting skeletal muscles, severe malaria may cause damage on cardiac muscles [10-12]. Circulation levels of cardiac proteins increases with the severity of malaria, indicating myocardial impairment in complicated falciparum malaria [13,14].

The skeletal muscle microvascular function and its oxygen consumption is proportionally impaired to the disease degree. Strikingly, oxygen consumption in severe malaria reduces similarly as in sepsis patients [15]. RNA and protein contents in much lower levels can be found in malaria infected skeletal muscles compared with non-infected controls [16]. We had previous demonstrated that in mice infected with *Plasmodium berghei* parasite, both glycolitic and oxidative hind limb skeletal muscles produced half of the normal contractile force, fatigued significantly more, and recovered significantly less [7]. These effects were associated with a reduced content of key contractile proteins, such as troponins and myosin [7]. Although these results could help to explain many of the symptoms in humans infected with malaria, they do not explain the molecular and cellular pathway mechanism of direct damage to the contractile machinery. The investigation of genes expression changes and of other bio molecules involved on the physiological effects of the malaria pathogen on muscles becomes crucial to understand potential mechanisms underlying this musculoskeletal disease.

Involved in many physiological processes, the lipid signaling mediators (LMs), a class of bioactive metabolites of the essential polyunsaturated fatty acids (PUFA) [17-22], have different major functions in living systems, such as being major constituents of biological membranes, efficient energy sources, modifiers of proteins, and signaling molecules [18]. These signaling molecules
generate locally through specific biosynthetic enzymes/receptors in response to extracellular stimuli, and play an important role through their signaling pathways on the regulation of pathophysiological states such as inflammation, metabolic syndrome, and cancer [18,23,24]. Thus, some LMs could serve as biomarkers in therapy or diagnosis of diseases, as well as potential candidates in drug development.

Herein, to study the molecular mechanisms of malaria-induced injury, 10 gene-signaling pathways were simultaneously monitored in mouse skeletal muscles infected with *Plasmodium berghei* and *Plasmodium chabaudi*. PCR Arrays are reliable tools for analyzing focused panels of genes in signal transduction, biological processes or disease research pathways. Furthermore, We also applied our new targeted lipidomic approach using liquid chromatography with tandem mass spectrometry (LC-MS/MS) to profile total 158 LMs, and further quantify 16 key LMs directly associated with inflammation and tissue healing in skeletal muscles. Our results showed a high level of concordance between the RT-PCR gene arrays and Lipidomics. Malaria infected muscles present a conclusive signature of enhanced inflammation signaling and reduced signaling to resolve inflammation. More specifically, a crosstalk between arachidonic acid (AA) related lipid mediators and Acsl4 gene was revealed as a potential mechanism modulating inflammation in malarial infected muscles.

**Methods**

**Chemicals and reagents**

Sixteen isotope-labelled lipid mediator (LM) internal standards (IS) including arachidonic acid-d₈ (AA-d₈), 6-keto prostaglandin F₁₆-d₄ (6-keto-PGF₁₆-d₄), prostaglandin F₂α-d₄ (PGF₂α-d₄), prostaglandin E₂-d₄ (PGE₂-d₄), prostaglandin D₂-d₄ (PGD₂-d₄), thromboxane B₂-d₄ (TXB₂-d₄), leukotriene B₄-d₄ (LTB₄-d₄), leukotriene C₄-d₅ (LTC₄-d₅), 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d₈ acid (5-HETE-d₈), 15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic-5,6,8,9,11,12,14,15-d₈ acid (15-HETE-d₈), and 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d₈ acid (12-HETE-d₈), platelet-activating factor C-16-d₄ (PAF C-16-d₄), tetrnanor-prostaglandin E metabolite-d₆ (tetrnanor-PGEM-d₆), oleoyl ethanolamide-d₄ (OEA-d₄), docosahexaenoic acid-d₅ (DHA-d₅), and eicosapentaenoic
Acid- $d_5$ (EPA-$d_5$), and eighteen LM standard compounds including 6-keto-PGF$_{1\alpha}$, PGF$_{2\alpha}$, PGE$_2$, PGD$_2$, PGA$_2$, TXB$_2$, LTB$_4$, LTC$_4$, 12(S)-hydroxyeptadecatrienoic acid (12-HHT), 9-hydroxy-10,12-octadecadienoic acid (9-HODE), 13(S)-hydroxyoctadecadienoic acid (13-HODE), 17,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (17,18-DiHETE), 8-hydroxy-4Z,6E,10Z,13Z,16Z,19Z-docosahexaenoic acid (8-HDoHE), N-arachidonylethanolamine (AEA), EPA, DHA, OEA and AA were purchased from Cayman Chemical Co. (Ann Arbor, MI). Formic acid (reagent grade, $\geq$ 95%) was obtained from Sigma–Aldrich (St. Louis, MO, USA). HPLC-MS grade acetonitrile, water, methanol, and ethanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Tri-reagent from Molecular Research Center, Inc. (Cincinnati, OH, USA). High-capacity cDNA reverse-transcription kit from Applied Biosystems (Foster City, CA, USA). Mouse Signal Transduction Pathway Finder PCR Array. RT2 First Strand Kit. RT2 Real-Time SYBR green/Rox PCR master mix from SABiosciences (Valencia, CA, USA). RNeasy Mini Kit from Qiagen (Valencia, CA, USA).

**Animals and infection with malaria**

Male mice from 4-5 months of age (Swiss Webster), weighing between 25-30g were infected with malaria parasites *Plasmodium berghei* (strain ANKA 2.34) and *Plasmodium chabaudi* (strain CR), uninfected mice used as controls of the experiments. *Plasmodium berghei* ANKA isolated from rats Grammomys surdates 2.34, has been maintained by Swiss Webster mice passage in the lab, and *Plasmodium chabaudi* in Balb/c mice, as described by Hoffmann et al. [25]. For the infections, the mice were intravenously inoculated with identical rates (200 $\mu$l) frozen red cell with 25% parasitaemia of malaria parasites diluted in PBS. The parasitaemias were monitored daily by microscopic examination of blood smears stained with GIEMSA. Mice with parasitemia of approximately 35% were selected for the analyses, based on our previous findings [7], that these levels of parasitemia lead to muscle dysfunction, but with no signs of brain dysfunction (i.e. deviation of head, seizures and coma followed by death). Therefore, in this study, only the mice without symptoms of cerebral malaria were used. These levels of parasitaemias were achieved 3-6 days after injection of parasites. Mice were euthanized by cervical dislocation and then intact muscles (gastrocnemius) were isolated, kept
stabilized in RNA later and shipped to University of Texas at Arlington.

**RNA isolation and RT-PCR gene arrays**

The Mouse Signal Transduction Pathway-Finder PCR Array from SABiosciences was used to simultaneously detect gene expression changes of 10 signaling pathways (see Table 1, and details in Results). Total RNA was extracted from the cells using the Tri reagent according to manufacturer’s protocol, quantified in a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) by determining absorbance at 260 nm in triplicate. RNA purity was indicated by the A260/280 nm absorbance ratio of 1.9 to 2.1 and A260/230 nm absorbance ratio ≥1.8. Using 0.5 μg of RNA, each sample was reverse transcribed in a 20-μL reaction volume. cDNA was synthesized using the RT2 First Strand Kit and the PCR Array was run according to the manufacturer’s protocol, including a threshold of 0.25 and validation of each gene tested by the identification of single peaks in melting curves. As above, data were analyzed using RT2 Profiler PCR Array Data Analysis Software (SABiosciences); CT values were normalized to built-in six reference housekeeping genes, genomic DNA control, reverse transcription control, and positive PCR control [26]. We used this analytical software to set the statistical significance of upregulation or downregulation of all tested genes at fivefold difference. A major advantage of this technology is the pre-validation of these pathways at the protein level by the manufacturer and the robust utilization of internal and reference controls.

**Lipidomics**

All components of LC-MS/MS system are from Shimadzu Scientific Instruments, Inc. (Columbia, MD, USA). LC system was equipped with four pumps (Pump A/B: LC-30AD, Pump C/D: LC-20AD XR), a SIL-30AC autosampler (AS), and a CTO-30A column oven containing a 2-channel six-port switching valve. The LC separation was conducted on a C8 column (Ultra C8, 150 × 2.1 mm, 3 μm, RESTEK, Manchaca, TX, USA) along with a Halo guard column (Optimize Technologies, Oregon City, OR, USA). The MS/MS analysis was performed on Shimadzu LCMS-8050 triple quadrupole mass spectrometer. The instrument was operated and optimized under both positive and negative electrospray and multiple reaction monitoring modes (+/− ESI MRM). The settings of flow rate and gradient program for the LC system as well as MS/MS conditions are recommended by a software method package for 158 lipid
mediators (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) and further optimized following our previously published quantification method [27]. All analyses and data processing were completed on Shimadzu LabSolutions V5.91 software (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA).

**Tissue preparation for lipidomic analyses**

Striated muscles were isolated after mice sacrifice, then snap frozen in liquid nitrogen immediately and stored at -80°C. Before the experiment, the aliquoted frozen muscle tissue (50-100 mg) were defrosted on ice and in the dark, weighed carefully, and minced into small pieces on ice. The minced muscle was placed into a 2.0 mL round-bottom low retention microcentrifuge tube (Fisher Scientific, Waltham, MA) and 1.0 mL of ice-cold 80% methanol in water (v/v) will be added. The mixture was homogenized using a Tissue Lyser II homogenizer (Qiagen, Germantown, MD) at the frequency of 30 s⁻¹, in 8×30-s bursts, waiting 20 s in between to avoid high temperature. The obtained homogenate was mixed with 5 µL of isotope-labelled LM internal standards (IS) mixture stock solution (5 µg/mL for AA-d₈, 2 µg/mL for DHA-d₅ and EPA-d₅, and 0.5 µg/mL for the rest IS), and then agitated on ice and in the dark for 1-2 h, followed by centrifugation at 6000×g at 4°C for 10 min to remove any tissue residue and precipitated proteins.

All muscle samples need to be cleaned and concentrated by Solid Phase Extraction (SPE) before being injected into LCMS. Ice-cold 0.1% formic acid (4 mL) was added in the obtained supernatant to fully protonate the LM species before sample was loaded to the preconditioned SPE cartridges (Strata-X 33 µm polymeric reversed phase, Phenomenex, Torrance, CA, USA). Once the sample had been totally loaded, cartridges were washed with 0.1% formic acid followed by 15% (v/v) ethanol in water to remove excess salts. Then the LMs from the SPE sorbent bed were eluted by methanol. Solvents were removed using an Eppendorf® 5301 concentrator centrifugal evaporator (Eppendorf, Hauppauge, NY, USA). The dried extracts were stored at -80°C immediately for future LC-MS/MS analysis.

**Statistics analysis**

For lipidomic analyses data is presented as mean ± SD of all samples in multiple experiments. One-
way ANOVA with post hoc Tukey's test ($\alpha = 0.05$) was performed for data analysis. Differences were considered statistically significant at $p < 0.05$.

| Signaling Pathways | Genes |
|--------------------|-------|
| TGFß Pathway       | Atf4, Cdkn1b (p27Kip1), Emp1, Gadd45b, Herpud1, Ifrd1, Myc, Tnfsf10 |
| WNT Pathway        | Axin2, Ccnd1, Ccnd2, Dab2, Fosl1 (Fra-1), Mmp7 (Matrilysin), Myc, Ppard, Wisp1 |
| NFκB Pathway       | Bcl2a1a (Bfl-1/A1), Bcl2l1 (Bcl-x), Birc3 (c-IAP1), Ccnd1 (Rantesc), Csf1 (Mcfsf), Icam1,Ifng, Stat1, Tnf |
| JAK/STAT Pathway   | Ifi1 |
|                   | Bcl2l1 (Bcl-x), Ccnd1, Cebpd, Lrg1, Mcl1, Socs3 |
|                   | Bcl2l1 (Bcl-x), Ccnd1, Socs3 |
|                   | Fcer2a, Gata3 |
| p53 Pathway        | Bax, Bbc3, Btg2, Cdkn1a (p21Cip1/Waf1), Egrf, Fas (Tnfrsf6), Gadd45a, Pcna, Rbl1 |
| Notch Pathway      | Hes1, Hes5, Hey1, Hey2, Heyl, Id1, Jag1, Ifng, Notch1 |
| Hedgehog Pathway   | Bcl2, Bmp2, Bmp4, Ptc1, Wnt1, Wnt2b, Wnt3a, Wnt5a, Wnt6 |
| PPAR Pathway       | Acs13, Acs4, Acs5, Cpt2, Fabp1, Olr1, Slic27a4, Sorbs1 |
| Oxidative Stress   | Fth1, Gclc, Gclm, Gsr, Hmox1, Nqo1, Sqstm1, Txn1, Txnr1 |
| Hypoxia            | Adm, Arnt, Car9, Epo, Hmox1, Ldha, Serpine1 (PAI-1), Slic2a1, Vegfa |

**Table 1**

Table 1 shows the 10 signaling pathways and the specific genes monitored in each pathway through the Mouse Signal Transduction Pathway-Finder PCR Array from SABiosciences. We implemented this methodology to simultaneously detect gene expression changes in striated muscles of mice infected with *P. chabaudi* or *P. berghei* parasites.

The analysis showed three genes (*Gadd45b, Ppard, Slic27a4*) were significantly up-regulated compared to controls in skeletal muscles from mice infected with *P. berghei*, while twenty other genes (*Ifrd1, Axin2, Mmp7, Bcl2l1, Ccnd1, Mcl1, Socs3, Gata3, Bax, Bbc3, Btg2, Gadd45a, Rb1, Jag1, Wnt1, Wnt3a, Olr1, Hmox1, Txn1, Serpine1*) were up-regulated only in muscles from mice infected with *P. chabaudi* (Fig. 1).

Seven common genes (*Acs14, Bmp2, Cebpd, Cdkn1a, Sqstm1, Stat1 and Txnrd1*) had their expression significantly altered in skeletal muscles from mice infected with either *P. chabaudi* or *P. berghei* parasites, comparing to uninfected mouse controls, with higher expressions on *P. chabaudi* infected mice, as illustrated in Fig.2.
Compared to negative control, the expression of Acsl4 gene was downregulated by -2.37-fold \((p=0.005674)\) and -3.22-fold \((p=0.018156)\), respectively, in muscles infected with \(P. \) berghei and \(P. \) chabaudi parasites, respectively (Fig. 2). The other six genes were upregulated comparing to the uninfected mouse muscles, with Bmp2 gene altered by 3.99-fold \((p=0.012177)\) and 9.04-fold \((p=0.006948)\), respectively in \(P. \) berghei and \(P. \) chabaudi infected muscles. Cebpd was significantly altered by 3.72-fold \((p=0.025971)\) and 51.02-fold \((p=0.006948)\) in \(P. \) berghei and \(P. \) chabaudi infected muscles, respectively.

The expression of Cdkn1a gene was significantly up-regulated by 12.48-fold \((p=0.047676)\) and 40.70-fold \((p=0.009800)\), respectively in \(P. \) berghei and \(P. \) chabaudi infected muscles. Stat1 gene was significantly altered by 11.20-fold \((p=0.021470)\) in \(P. \) berghei infected muscles, and 9.16-fold \((p=0.005999)\), in \(P. \) chabaudi infected muscles. Regarding the gene Sqstm1, it was upregulated related to the controls, by 4.33-fold \((p=0.008631)\) and 11-fold \((p=0.022897)\), respectively, in \(P. \) berghei and \(P. \) chabaudi infected muscles. Finally, the expression of Txnrd1 gene upregulated by 3.14-fold \((p=0.030464)\) in \(P. \) berghei infected muscles and 6.77-fold \((p=0.009119)\) in \(P. \) chabaudi infected muscles.

**Lipid Signaling Mediators (LMs)**

Due to the essential roles of lipid signaling in health and disease, we compared the profiles of total 158 lipid signaling mediators between the muscles from mice infected with two types of malaria and uninfected mice (control) through a LC-ESI-MS/MS based targeted lipidomic analytical method, and 58 were detectable (Table S1, supplementary material), mostly arachidonoids, eicosanoids and docosanoids. Then we further determined the concentrations of 18 key LMs, and in which 16 LMs, from six metabolic pathways, were finally detected and quantified from mouse skeletal muscles in this study. We recently applied this quantification method and showed substantial differences during aging [27]. The analysis of the concentrations of LMs indicated significant differences in the content of LMs in skeletal muscles of mice infected with \(P. \) berghei and \(P. \) chabaudi compared to that of the muscles from the control mice (Table 2, Fig. 3).

In these 16 detectable LMs, the muscle levels of four arachidonic acid-derived (AA, 20:4, \(\omega-6\)) LMs,
including 6-keto-PGF$_{1\alpha}$, PGF$_{2\alpha}$, PGD$_{2}$, 12-HHT, were observed to be remarkably higher in mice infected with *P. berghei* than uninfected mice. As for the two LMs showing significantly lowered concentrations, EPA belongs to ω-3 PUFA eicosapentaenoic acid (EPA, 20:5, ω-3) metabolic pathway, and AEA belongs to fatty acid ethanolamide (EA)-derived metabolic pathway (Table 2, Fig. 3).

Analysis of LM quantification from muscles of *P. chabaudi* infected mice showed similar results. The concentration of 4 LMs from AA metabolic pathway, including PGF$_{2\alpha}$, PGD$_{2}$, PGA$_{2}$ and 12-HHT, were significantly higher than the controls, while two of ω-3 PUFA metabolites (EPA and docosahexaenoic acid (DHA, 22:6, ω-3)) and one EA metabolite (AEA) were found in lower concentrations comparing to the muscles from uninfected mice (Table 2, Fig. 3).

All LMs related to ω-6 PUFA metabolic pathways, including both AA (20:4, ω-6) and linoleic acid (LA, 18:2, ω-6) determined in this study, exhibited elevated levels in striated muscles from mice with two types of malaria, even though no statistically significant differences (*p*<0.05) were observed (Table 2). Two hydroxyoctadecadienoic acids 13-HODE and 9-HODE, recently being considered as lipid peroxidation biomarkers [28,29], showed much higher concentrations in malaria infected muscles, about 6-fold (*P. chabaudi*) and 2-fold (*P. berghei*) higher than the control, respectively, for both 13-HODE and 9-HODE (Table 2).

The AA/EPA ratio, an indicator of inflammation and skeletal muscle depletion [30,31], was drastically higher (by 5-6 fold) in muscles from malaria infected mice, either with *P. berghei* (16.7±7.7, *p*=0.10133) or *P. chabaudi* (19.9±11.9, *p*=0.03887) when compared to the control group (3.6±0.8) (Table 2).
Table 2
Quantification of lipid mediator (pg mg\(^{-1}\) muscle) in striated muscles from mice with or without different malaria infections and the associated metabolic pathways.

| Metabolic pathways       | Lipid mediator | Control       | P. chabaudi  | P. berghei   |
|--------------------------|----------------|---------------|--------------|--------------|
| Arachidonic acid (AA)    | 6-keto-PGF\(_{1\alpha}\) | 13.4 ± 7.9    | 43.9 ± 15.6  | 105.1 ± 77.6* |
|                          | TXB\(_{2}\)     | 3.4 ± 1.7     | 12.1 ± 12.8  | 26.9 ± 23.3  |
|                          | PGF\(_{2\alpha}\) | 7.9 ± 2.1     | 38.7 ± 19.5* | 38.7 ± 11.7* |
|                          | PGD\(_{2}\)     | 7.0 ± 0.6     | 53.2 ± 23.5* | 34.7 ± 6.6*  |
|                          | PGA\(_{2}\)     | 2.4 ± 0.4     | 10.2 ± 6.4*  | 5.8 ± 1.6    |
|                          | 12-HHT          | 43.7 ± 5.9    | 145.1 ± 23.1* | 182.7 ± 70** | # |
| Linoleic acid (LA)       | 13-HODE         | 911.6 ± 183.9 | 5724.6 ± 5486.6 | 1934.3 ± 989.8 |
|                          | 9-HODE          | 1159.2 ± 314.2 | 7427.6 ± 6820.3 | 2521.9 ± 1288.6 |
| Eicosapentaenoic acid (EPA)| 17,18-DiHETE   | 11.5 ± 1.4    | 8.6 ± 5.9    | 9.2 ± 4.1    |
|                          | EPA             | 4702.9 ± 954.4 | 1160 ± 292.7*** | 1483.4 ± 391.1*** |
| Docosahexaenoic acid (DHA)| 8-HDoHE        | 136.8 ± 52.9  | 208.3 ± 85.6 | 221.9 ± 49.7 |
|                          | DHA             | 73814.6 ± 16486.1 | 50135.2 ± 9021.8* | 57149.6 ± 8011.9 |
| α-Linolenic acid (ALA)   | 9-HOTrE         | 16.9 ± 1.2    | 121.4 ± 110.5 | 42.1 ± 13.8  |
| Ethanolamide (EA)        | AEA             | 21.1 ± 2.2    | 6.4 ± 2.9*** | 9.9 ± 4.4*** |
|                          | OEA             | 36.5 ± 10.5   | 62.9 ± 22.2  | 72.1 ± 25    |
| Ratio of AA/EPA          | 3.6 ± 0.8       | 19.9 ± 11.9*  | 16.7 ± 7.7   |              |

Mean ± SD, n = 5. One-way ANOVA with Tukey post-hoc test (α = 0.05) was applied to compare means between multiple groups. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 represent statistically significant difference between uninfected mice (Control) and P. berghei or P. chabaudi infected mice; # p ≤ 0.05 represents statistically significant difference between P. berghei and P. chabaudi infected mice.

Discussion
Malaria is one of the most prevalent infectious disease in the world, causing splenomegaly, a recognized malaria symptom, and damage in other organs such as liver and kidney [8]. Currently at least 3.2 billion humans are at risk of exposure to malaria. Remarkably, malaria infection can also cause muscle damage due to the combination of ischemia, inflammation, and oxidative stress [8].

Considering the vital importance of the skeletal muscles as the largest organ-system in the body, comprising nearly 50% of its mass and largely responsible for the regulation and modulation of overall metabolism, we first investigated the potential molecular mechanisms of plasmodia infection affecting skeletal muscles of mice. We employed the Mouse Signal Transduction Pathway Finder PCR Array to monitor gene expression changes of 10 essential signaling pathways in skeletal muscles from mice infected with *Plasmodium berghei* and *P. chabaudi* (Table 1).

Among all the genes analyzed, the expression of six genes, *AcsL4*, *Bmp2*, *Cebpd*, *Cdkn1a*, *Sqstm1*, *Stat1* and *Txnrd1*, were significantly up-regulated (Table 1, and Fig. 3) in both *P. berghei* and *P. chabaudi* infected mice muscles, compared to the negative controls. Only one gene, *AcsL4*, was downregulated in the infected mice muscles with both plasmodia parasites, compared to the negative
controls.

We discovered significant expression changes in genes related to the inflammatory and immune response of the host. For instance, *Cebpd* gene encodes an important protein related to the regulation of genes involved in inflammatory and immune responses [32]. *Bmp2* gene belongs to TGFβ signaling pathway, which has pleiotropic functions, including regulating immune response [33]. The gene *Txnrd1* encodes a protein associated to oxidative stress pathways and is involved in the regulation of lipids metabolism via the PPAR signaling pathway [34].

*Stat1* gene it is a signal transducer and transcription activator that mediates cellular responses to interferons (IFNs), cytokines and other growth factors, being directly associated with the NF-kB pathway [35]. This result indicated that malaria infection may function through the NF-kB pathway to regulate myoblast differentiation, since this pathway was affected by both *P. berghei* and *P. chabaudi* infections. NF-kB is one of the most important signaling pathways linked to the loss of skeletal muscle mass in normal physiological and pathophysiological conditions [36]. *Sqstm1* gene is associated to oxidative stress and to the NF-kB signaling pathway.

Another gene, *Cdkn1*, was upregulated in infected muscles with both plasmodia species. This gene is related to the p53 signaling pathway, encoding a potent cyclin-dependent kinase inhibitor, and it might be an important intermediate by which p53/TP53 mediates its role as an inhibitor of cellular proliferation in response to DNA damage. The activation of NF-kB and p53 can activate protein kinase (MAPK) pathways leading to skeletal muscle atrophy [37]. The nuclear factor NF-κB pathway has long been considered a prototypical proinflammatory signaling pathway, largely based on the role of NF-κB in the expression of proinflammatory genes including cytokines, chemokines, and adhesion molecules, playing a key role in regulating the immune response to infection [38]. These results corroborated with our previous studies, which had shown a detrimental effects of malaria on skeletal muscles associated with significant decrease in content of key contractile proteins (reduced from 15 to 45%) in the skinned fibers from malaria mice [7], and also are in agreement with findings from other studies of the presence of muscle specific proteins in the circulation [13,14,16].

The results of mouse signal transduction pathway finder PCR array have shown a higher number of
over regulated genes in skeletal muscles of *P. chabaudi* infected mice when compared to *P. berghei* infected mice. It is important to mention that the plasmodia strains were selected according to their characteristics and effect on the experimental mice models. Infections with *P. berghei* ANKA 2.34 strain can affect the brain and cause complications in laboratory mice, comparable to human cerebral malaria caused by *Plasmodium falciparum* [39], whereas malaria caused by *Plasmodium chabaudi*, strain CR, is not lethal, and defined as an experimental malaria self-control. This self-control characteristic of *P. chabaudi* parasite may explain the higher number of over expressed genes found in striated muscles infected with *Plasmodium chabaudi* compared to the low number of over expressed genes when the mice were infected with *Plasmodium berghei* ANKA strain.

The detrimental effects of malaria parasites on skeletal and cardiac muscles are related to their pathogenic mechanism. *Plasmodium berghei* is a mice model, compared to the human *Plasmodum falciparum*, that causes a severe malaria, provoked by microvascular sequestration of parasitized red blood cells causing microcirculatory obstruction [3], leading to decreasing oxygen delivery, obstruction of blood flow and tissue hypoxia [15]. Due to this aspect, skeletal muscle necrosis and rhabdomyolysis, which are directly or indirectly caused by muscle injury or death, have been reported in patients with severe falciparum malaria [3,5,6]. Considering that necropsies occur in *P. falciparum* patients, but not in humans infected by the non-lethal *P. vivax* malaria, it was expected that some genes were only significantly altered in muscles of mice infected with the *P. chabaudi* parasites, a parasite comparable to *P. vivax*. For instance, the function related to *Mcl1* gene could explained the fact that in mice infected with this parasite do not go through an apoptosis process, since this gene encodes an anti-apoptotic protein.

*Acs14* gene encodes an isozyme of the long-chain fatty-acid-coenzyme synthase involved in the peroxisome proliferator-activated receptor (PPAR) signaling pathway (*Table 1*) and fatty acid pathway. PPARs play essential roles in the regulation of cellular differentiation, development, and metabolism of bio molecules such as carbohydrates, lipids and proteins of vertebrates [40-42]. This isozyme has marked preference for arachidonate acid as its substrate, which is a polyunsaturated ω-6 fatty acid, related to a metabolic pathway directly related to inflammatory effects (*Table 2*).
Polyunsaturated fatty acids (PUFAs) are important constituents of the phospholipids of all cell membranes and generally considered to have beneficial effects. However, it is believed that ω-3 and ω-6 PUFAs have opposing effects on metabolic functions in the body. Typically, ω-6 PUFAs are associated with inflammatory responses, constriction of blood vessels, and platelet aggregation [43]. In contrast, ω-3 PUFAs help resolve inflammation and alter the function of vascular and carcinogen biomarkers, thus reducing the risk of cancer [44]. Therefore, our results corroborated with those assessments, since all LPs found on higher concentrations compared to the controls are from ω-6 pathway, while the lipids found on lower concentrations comparing to the controls are related to ω-3 pathway.

The profiling analysis of lipids revealed 58 detectable lipids, most of them related to AA and DHA metabolic pathways. These studies led to us to conduct quantitative analysis of LMs in skeletal muscle models of malaria to elucidate the fundamental mechanisms of the different pathways of lipid metabolism in the skeletal muscle of mice infected with two types of malaria, a lethal mouse malaria caused by *P. berghei* and another form of mice malaria, non-lethal, caused by *P. chabaudi*. This may lead to better understanding of the cause and potential of treatments for harmful effects of malaria on skeletal muscle. Our analysis of the concentrations of lipid mediators indicate significant differences in the levels of the determined LMs in the striated muscles of mice infected with both parasites, *P. berghei* and *P. chabaudi*, compared to the mice control muscles ([Table 2], [Fig. 3]). Few studies have shown evidence that lipid mediators may regulate skeletal muscle mass and function and potentially protect against muscle wasting in response to various infected diseases, but so far, no information of on LM levels in malaria infected skeletal muscles has been reported. In the *P. berghei* infected mice group, the levels of 5 AA-metabolized LMs, including 6-keto-PGF$_{1α}$, PGF$_{2α}$, PGD$_2$, PGA$_2$, and 12-HHT, were remarkably higher in muscles as compared to uninfected mice ([Table 2], [Fig. 3]). On the other hand, EPA, DHA and AEA, derived from either ω-3 PUFA (EPA and DHA) or fatty acid ethanolamide, respectively, had statistically significant lower concentrations comparing to uninfected mice muscles. EPA concentration was statistically lower ($p≤0.001$) on muscles of mice infected with either malaria parasites. The same was found regarding to AEA concentration on muscles infected
with either plasmodia species ($p \leq 0.001$). Regarding to DHA, both infected malaria muscles were found on lower concentrations, but statistically lower ($p \leq 0.05$) only on muscles from *P. chabaudi* infected mice (*Table 2, Fig. 3f, 3g, 3h*).

Eicosanoids is a family of signaling mediators metabolized from arachidonic acid (AA, 20:4, $\omega$-6) or other PUFAs that contain 20 carbon units in length, such as dihomo-γ-linolenic acid (DGLA, 20:3, $\omega$-6) and eicosapentaenoic acid (EPA, 20:5, $\omega$-3). Some well-known eicosanoids such as prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs) and related oxygenated derivatives have a wide range of biological actions including potent effects on inflammation [45]. Generally, AA-derived eicosanoids promote inflammation and exhibit pro-inflammatory activities, while those derived from EPA and DGLA are less inflammatory, or even show anti-inflammatory and pro-resolving activities to resolve inflammation effectively [46,47]. Our results indicated a significantly elevated AA/EPA ratio linked with malaria. Malaria infection can cause muscle damage due to the combination of ischemia, inflammation, and oxidative stress [8]. Malaria is known as an inflammatory disease, with recognized malaria symptoms, such as splenomegaly, and damage in other organs such as liver and kidney [8]. Additionally, this significantly increased AA/EPA ratios may also suggest an association between malaria infection and skeletal muscle depletion. It has been reported that an elevated serum AA/EPA ratio is correlated with decreased psoas muscle area volume and the possibility of sarcopenia and muscle-depleting cachexia in patients with advanced cancer [30].

Our results of lipid profiles also indicate the potentially increased peroxidation in skeletal muscles infected with malaria parasites. Recently, hydroxyoctadecadienoic acids (HODEs) such as 13-HODE and 9-HODE have been reported as lipid peroxidation biomarkers [29]. HODEs are stable oxidation products of linoleic acid (LA, 18:2, $\omega$-6), a most abundant PUFA *in vivo*, by 15-lipoxygenase-1 (15-LOX-1) followed by glutathione peroxidases and phospholipases [28,48]. HODEs accumulate in atherosclerotic lesions and exert protective effects in atherogenesis by modulating macrophage lipid accumulation and inflammatory mediator generation through peroxisome proliferator-activated receptor-γ (PPAR-γ) activation [49,50]. Elevated levels of HODEs is always linked to oxidative stress-related diseases such as diabetes [49]. In our study, both 13-HODE and 9-HODE showed increased
levels in skeletal muscles from malaria infected mouse, confirming previous data showing that malaria infection induces a combination of inflammation and oxidative damage in skeletal muscles [14,15]. These data are also corroborated by our gene array results which showed an upregulated expression of Sqstm1 gene, associated to oxidative stress pathway.

Therefore, profiles of targeted lipid mediators obtained in this study provided crucial information related to the aspects of skeletal muscle infected with the malaria parasite P. berghei and P. chabaudi, with insights on the quantification of LMs involved with essential physiological functions ranging from tissue repair and inflammation due to malaria infection. Our studies further point to a crosstalk between AA-related lipid mediators and Acsl4 gene as a potential cellular mechanism of sustained inflammation in malarial muscles.

Conclusion
The gene profiling analyses showed expression of key genes altered by malaria infection associate with inflammation, oxidative stress, and atrophy. In support to these results, the lipidomic analyses revealed higher concentrations of LMs directly related to inflammatory responses, while on the levels of LMs crucial in resolving inflammation and tissue repair reduced significantly. Figure 4 shows a proposed model for the potential mechanism between AA derived lipid mediators and the Acsl4 gene; a gene directly related to inflammation process of malaria-infected muscles. The high concentration of the AA related lipid mediators found in malaria infected mice may act downregulating the Acsl4 [51], promoting inflammatory response. Therefore, our study helped to increase the knowledge of mechanisms underlying molecular mechanisms of malaria-induced muscle damage, targeting key molecules, such as genes and lipids, associated and leading to muscle damage. Together, this information could be useful in future works to provide better monitoring of disease progression and development of specific interventions for the mitigation of long-term chronic effects on skeletal muscle function.

Declarations
Authors’ contributions
MTM and MB conceived and designed the study. MTM oversaw the mice infections. ZW performed the
lipidomic experiments. JH, MTM performed the gene array experiments. JH, MTM and ZW analyzed the data. MTM wrote the first draft of the article. JH, ZW and MB made the scientific review of the article.

All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Consent for publication**

Not applicable.

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**Ethics approval and consent to participate**

The experimental protocols were conducted according to the Ethics in Animal Experimentation (COBEA/SBCAL - Brazilian College of Animal Experimentation) and approved by the Animal Research
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Figures
Figure 1

Venn diagram showing the common genes with altered expression in skeletal muscles from mice infected with either P. chabaudi or P. berghei parasites.
Figure 2

Key genes altered regulating inflammatory signaling pathways are present in skeletal muscles from mice infected with P. chabaudi and P. berghei parasites.
Figure 3

Comparison of the lipid concentration in muscles of control uninfected and infected mice with P. chabaudi and P. berghei parasites: (a) 6-keto-PGF1α, (b) PGF2α, (c) PGD2, (d) PGA2, (e) 12-HHT, (f) EPA, (g) DHA, (h) AEA.
Schematic representation of arachnoid acid (AA) related lipids downregulating Acs14 gene and modulating inflammatory process in malaria infected skeletal muscles. Blue arrow-ended line: stimulation/activation; red circle-ended line: inhibition; dash line: PPAR signaling pathway.
Supplementary Files

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