Efecto de la hipoxia en el metabolismo de purinas en células de músculo esquelético de humanos

ABSTRACT
Mammals experience some degree of hypoxia during their lifetime. In response to hypoxic challenge, mammalian cells orchestrate specific responses at transcriptional and posttranslational level, which lead to changes in purine metabolites in order to cope with threatening conditions. The aim of this study was to evaluate the response of the enzymes involved in purine metabolism at human muscle cells under hypoxic conditions. Muscle cells in culture were exposed to hypoxia and the enzymatic activity of inosine monophosphate dehydrogenase (IMPDH), xanthine oxidase (XO), purine nucleoside phosphorylase (PNP) and hypoxanthine guanine phosphoribosyl transferase (HGPRT) as well as their transcript expression were quantified under normoxic and hypoxic conditions. Purine metabolite (hypoxanthine (HX), xanthine (X), uric acid (UA), inosine monophosphate (IMP), inosine, nicotinamide adenine dinucleotide (NAD^+), adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), guanosine diphosphate (GDP) and guanosine triphosphate (GTP)) concentrations were also quantified. Significant reduction of IMPDH activity as well as in HX and IMP concentrations (p < 0.05) were observed after hypoxia, suggesting a decrease in the purines de novo synthesis. After hypoxia a global reduction of transcripts was observed, suggesting a reduction of the metabolic machinery of purine metabolism to new steady states that balance ATP demand and ATP supply pathways.

Key words: hypoxia, cell culture, purines, salvage pathway.

RESUMEN
Los mamíferos experimentan cierto grado de hipoxia durante su vida. Como respuesta al reto de hipoxia, las células de mamíferos orquestan respuestas específicas a nivel transcripcional y postraducional que conducen a cambios en los metabolitos de purinas para hacer frente a las condiciones amenazantes. El objetivo de este estudio fue evaluar la respuesta de las enzimas involucradas en el metabolismo de las purinas de células musculares humanas a condiciones hipóxicas. Las células musculares en cultivo se expusieron a hipoxia y la actividad enzimática de la inosina monofosfato deshidrogenasa (IMPDH), la xantina oxidasa (XO), la purina nucleosido fosforilasa (PNP) y la hipoxantina guanina fosforribosil transferasa (HGPRT), así como su expresión de transcripción, se cuantificaron bajo condiciones de normoxia e hipoxia. Los metabolitos de purina (hipoxantina (HX), xantina (X), ácido úrico (UA), monofosfato de inosina (IMP), inosina, dinucleótido de nicotinamida y adena (NAD^+), adenosina, monofosfato de adenosina (AMP), difosfato de adenosina (ADP), trifosfato de adenosina (ATP), difosfato de guanosina (GDP) y trifosfato de guanosina (GTP)) también se cuantificaron. Se observó una reducción significativa de la actividad de IMPDH y de las concentraciones de HX e IMP (p < 0.05) después de la hipoxia, lo que sugiere una disminución de la síntesis de novo de purinas. Después de la hipoxia, se observó una reducción global de la expresión transcripcional, lo que sugiere una reducción de la maquinaria metabólica del metabolismo de las purinas a nuevos estados estacionarios que equilibran la demanda de ATP y las vías de suministro de ATP.

Palabras clave: hipoxia, cultivo celular, purinas, vía de rescate.

INTRODUCTION
Purine metabolism constitutes a key pathway for every organism. It is involved in DNA and RNA synthesis and degradation, and involves the production of ubiquitous metabolites, such as adenosine triphosphate (ATP) (Yin et al., 2018). There are two main routes for the synthesis of nucleotides, the de novo biosynthetic pathway and the complementary salvage pathway. The de novo biosynthetic pathway is responsible for the production of inosine monophosphate (IMP) in a twelve-step biosynthetic process with consumption of ATP (Pang et al., 2012; Yin et al., 2018). The complementary salvage pathway, however, converts extra-cellular nucleobases or degrades purine metabolites into the corresponding nucleosides and nucleotides (Nelson et al., 2008), and obviates energetically expensive de novo synthesis of these compounds.

When mammals are exposed to oxygen limiting situations, such as breath-hold diving induced hypoxia or ische-
mia, systemic and intracellular changes operate together to minimize injury and restore adequate oxygenation. During hypoxia, mammalian tissues cannot produce enough energy to maintain essential cellular processes (Giordano, 2005); thus, a reduction of ATP synthesis is produced, promoting the degradation of purine nucleotides (Nelson et al., 2008) which are accumulated in the cells (Raivio et al., 2001). The catabolic compounds derived from ATP breakdown, hypoxanthine (HX), inosine (I), xanthine (X), uric acid (UA) and adenine (A), have been widely used as markers of tissue hypoxia (Maiuolo et al., 2016).

Purine degradation involves the activation of purine nucleotide phosphorylase (PNP, E.C. 2.4.2.1) and xanthine oxidase (XO, E.C. 1.1.3.22) (Maiuolo et al., 2016). PNP catalyzes the reversible conversion of nucleosides (guanosine, xanthosine, inosine) to purine bases (guanine, xanthine, and hypoxanthine) (Dudzinska et al., 2006), while XO catalyzes the oxidation of hypoxanthine and xanthine to uric acid (Vorbach et al., 2003). Salvage of purines occurs through two main enzymes, adenine phosphoribosyltransferase (APRT), which mediates the transfer of phosphoribosyl-1-pyrophosphate (PrRPP), a high energy sugar phosphate, to adenine, to form adenosine monophosphate (AMP), and hypoxanthine-guanine phosphoribosyltransferase (HGPRt, E.C. 2.4.2.8), which forms IMP and guanosine monophosphate (GMP) (Nelson et al., 2008; Zhang et al., 2008). The enzyme inosine 5-monophosphate dehydrogenase (IMPDH, E.C. 1.1.1.205) uses IMP to produce xanthosine 5-monophosphate (XMP) and, ultimately, ATP (Cherin et al., 2006).

Oxygen limitation has a tissue-dependent effect (Wagner, 2008), which triggers transcriptional (Huang et al., 2004; Rocha, 2007) and posttranslational (Schumacker, 2011) modifications to the enzymes involved in the nucleotide synthesis and degradation. The de novo biosynthetic pathway is energy demanding, requiring five ATP molecules, two glutamine and formate molecules, and one glycine, aspartate and carbon dioxide molecules to generate one IMP molecule. During 24h hypoxia in human cells, a significant increase in hypoxanthine (HX) level is produced (Nagao et al., 2018), which is used by XO. Hypoxia, as well as other stimuli, including growth factors, cytokines and hormones, upregulates XO mRNA expression (Battelli et al., 2016). However, XO activity increases in response to hypoxia without changes in mRNA or protein levels (Berry and Hare, 2004), indicating that XO is modulated at a posttranslational level, mainly through phosphorylation (Kayyali et al., 2001).

Purine recycling in mammals increases in response to prolonged fasting and ischemia (López-Cruz et al., 2014). PNP activity increases significantly during ischemia (Vannoni et al., 2004) and its enzymatic deficiency in purine metabolism leads to several diseases, including Lesch-Nyhan syndrome, clinical gout, nephropathy and neurological disease (Nyhan, 2005). HPGRt activity in mammals plasma indicates an elevated purine recycling rate to avoid accumulation of non-recyclable purines (xanthine and uric acid) (López-Cruz et al., 2014), which rise the need for a comprehensive understanding of the regulation of enzymes involved in the purine metabolism.

The aim of this study was to analyze and compare the enzymatic activity and transcript expression of the proteins involved in purine degradation (XO, PNP) and salvage pathway (HPGRt), and the concentration of metabolites involved in purine metabolism after hypoxic conditions in human muscle cells.

MATERIAL AND METHODS

Cell culture

Human skeletal muscle cells (36 cell culture boxes) were cultured in DMEM/F12 50 (Cellgro) supplemented with 15% fetal bovine serum (FBS, Gibco), antibiotics (50 U mL⁻¹ penicillin, 50 µg mL⁻¹ streptomycin), 2 mM glutamax (Gibco), 25 mM HEPES (pH 7.4) and 100 µM sodium pyruvate (Gibco). Cells (18 culture boxes) were cultured in a humid atmosphere at 35°C and serially passaged upon 80% confluence. Hypoxic conditions were obtained by placing the cells (18 cell culture boxes) in a sealed hypoxic workstation for 1 h, medium degas time took around 5 minutes. Afterwards, control cells (normoxia) and hypoxic cells (hypoxia) with 80% confluence were detached from the box using trypsin 0.25% (GIBCO) by a 4 min incubation at 35°C, then trypsin was inactivated by the addition of fresh medium. Cell suspension was recovered in 15 mL tubes, centrifuged at 1200 rpm for 10 min. Supernatant was discarded and the pellet (cells) was re-suspended in 500 µL of medium containing 1% DMSO for metabolite and protein analysis, and in tubes containing TRIzol® Reagent (15596, Invitrogen, Carlsbad, CA, USA) for gene expression. This process was performed for nine cell culture boxes for each treatment. All samples were stored at -80°C until further analysis.

Protein extraction and quantification

Prior to analysis, cells (nine cell culture boxes per treatment) in medium containing DMSO were centrifuged at 1200 rpm for 15 min to remove the medium. Afterwards, 350 µL of deionized water were added and then vortexed for 30 s, followed by three cycles of freezing/thawing for cellular disruption. Finally, samples were centrifuged at 17900 x g for 15 min at 4°C. Supernatant, containing soluble proteins was recovered and stored at -80°C until further analysis. Total soluble protein was quantified using a commercial kit (Bio-Rad Laboratories, Inc. Hercules, CA, USA) based on the method described by Bradford (1976). Bovine serum albumin (BSA) was used as standard, and results expressed in mg mL⁻¹.

Purine metabolite concentration

Concentration of purines (HX, X, A, I, NAD⁺, UA, IMP, AMP, ADP, ATP, XMP, GDP and GTP) was quantified in human muscle cell culture samples from normoxia and hypoxia experiments by high performance liquid chromatography (HPLC) (Waters 2695, Milford, MA, USA) (Gianattassio et al., 2003). Samples were treated with 0.5 M cold perchloric acid to precipitate soluble protein, and the solution was
then neutralized with 0.5 M potassium hydroxide. The potassium perchlorate produced from the neutralization was eliminated by centrifugation at 17900 x g for 10 min at 4°C. The supernatant containing the purine metabolites was recovered and filtered through a Nylon membrane filter of 0.22 µm (Int. Millipore, Bedford, MA, USA) into a HPLC vial for analysis, the medium was also analyzed to evaluate if the metabolites were expelled from the cell during hypoxia. Purine metabolites were analyzed by HPLC in an analytical column Supelcosil C-18 (150 x 4.6 mm ID, 3 µm particle size; Waters). Metabolites were separated using a gradient from 0 to 19 min, beginning at 100% of buffer A (100 mM KH₂PO₄, 8 mM TBA pH 6.0) to 50% buffer B (100 mM KH₂PO₄, 8 mM TBA, 30% acetonitrile pH 6.0) at a flow rate of 1.3 mL min⁻¹. The eluate was monitored at 214 nm. The concentration of each purine metabolite was quantified using standard curves of a purine standards mixture (0.625 to 100 µM), expressing the metabolite concentrations as µM mg⁻¹ protein.

Enzymatic assays

Inosine 5’-monophosphate dehydrogenase (IMPDH, E.C. 1.1.1.205) activity

IMPDH activity was measured by quantifying the concentration of xanthosine 5’-monophosphate (XMP), resulting from the conversion of IMP, using HPLC (Waters 2695, Milford, MA, USA) (Glander et al., 2001). Enzymatic assays were performed in 40 mM sodium phosphate buffer pH 7.4, containing 0.5 mM IMP, 0.25 mM NAD⁺ and 50 mM KCl. Reactions were incubated for 3 h at 37°C, subsequently 4 M cold perchloric acid was added to stop the reaction; the reaction mixture was neutralized with 5 M potassium carbonate and potassium perchlorate produced from the neutralization was eliminated by centrifugation at 17900 x g for 10 min at 4°C. The supernatant containing the purine metabolites was recovered and filtered through a Nylon membrane filter of 0.22 µm (Int. Millipore, Bedford, MA, USA) into a HPLC vial for analysis, the medium was also analyzed to evaluate if the metabolites were expelled from the cell during hypoxia. Purine metabolites were analyzed by HPLC in an analytical column Supelcosil C-18 (150 x 4.6 mm ID, 3 µm particle size; Waters). Metabolites were separated using a gradient from 0 to 19 min, beginning at 100% of buffer A (100 mM KH₂PO₄, 8 mM TBA pH 6.0) to 50% buffer B (100 mM KH₂PO₄, 8 mM TBA, 30% acetonitrile pH 6.0) at a flow rate of 1.3 mL min⁻¹. The eluate was monitored at 214 nm. The concentration of each purine metabolite was quantified using standard curves of a purine standards mixture (0.625 to 100 µM), expressing the metabolite concentrations as µM mg⁻¹ protein.

Purine nucleotide phosphorylase (PNP, E.C. 2.4.2.1) activity

PNP activity was measured indirectly by the oxidation of 3, 5-dichloro-2-hydroxybenzenesulfonic acid/4-amino-phenazone to N-(4-antipyril)-3-chloro-5-sulfonate-b-benzoquinonammonio, by the hydrogen peroxide formed in the PNP-xanthine oxidase reaction (Chu et al., 1989). Briefly, the enzymatic reaction was performed in 22 mM potassium phosphate buffer pH 7.5, containing 25 U XO, 2000 U of horseradish peroxidase (HRP), 160 mM 4-aminoantipyrine, 120 µM potassium ferricyanide, 8 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, and 12 mM imosine. Production of N-(4-antipyril)-3-chloro-5-sulfonate-p-benzoquinone-monoimine was recorded at 520 nm in a spectrophotometer (Beckman Coulter DU 800, Fullerton, CA, USA) and the change of absorbance was recorded every 5 s for 3 min. The activity of PNP expressed as units (U) mg⁻¹ protein, defining a unit of PNP activity as the amount of enzyme necessary to deplete 1 µM of imosine per min at 25°C.

Hypoxanthine-guanine phosphoribosyltransferase (HGPRT, E.C. 2.4.2.8) activity

HGPRT activity was measured by quantifying the rate of IMP production, which is oxidized by IMPDH, using the PIERCE HPRT Assay Kit (NOVOCIB, Lyon, France) following the manufacturer’s instructions. Briefly, enzymatic assay was performed in reaction buffer (1X) containing IMPDH, DTT, NAD⁺ and PRPP. The reaction mixture was recorded every 5 min for 120 min at 340 nm using a microplate reader (Multiskan FC, Thermo Scientific, Finland), and HGPRT activity expressed as nmol mg⁻¹ protein h⁻¹.

Xanthine oxidase (XO, E.C. 1.17.3.2) activity

XO activity was measured using an Amplex® Red Xanthine/Xanthine oxidase assay kit (A22182; Invitrogen, Paisley, UK). The quantification is based on the superoxide anions (O₂⁻) produced by XO, which are degraded to hydrogen peroxide (H₂O₂), which reacts stoichiometrically with Amplex® Red reagent to generate the oxidation product, resorufin. Briefly, the enzymatic assay was performed in reaction buffer (1X) containing 100 µmol L⁻¹ Amplex Red reagent solution, 0.4 U mL⁻¹ HRP and 200 µmol L⁻¹ hypoxanthine, incubated for 30 min at 37°C. The presence of resorufin was recorded at 550 nm using a microplate reader (Multiskan FC, Thermo Scientific, Finland), and the XO activity expressed as mU mg⁻¹. One unit of XO activity is defined as the amount of enzyme required to produce 1 µM of UA from XH per min at 25°C.

Quantification of XO, PNP, IMPDH and HGPRT transcripts RNA extraction and cDNA synthesis

Total RNA was isolated from 1 x 10⁶ cells of skeletal muscle cells from human cell cultures (normoxia and hypoxia) using TRIzol® Reagent (15596, Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Contaminating genomic DNA was removed using DNase I (AMPD-1, Sigma-Aldrich Chemical), and total RNA concentration was spectrophotometrically determined at 260 nm. RNAs (1 µg) were reverse transcribed into cDNA using the QuantiTect® Reverse Transcription kit (205313, Qiagen) using random hexamer primers and according to the manufacturer instructions. cDNA samples were stored at −80°C until further analysis.

Real-time PCR analysis

Determination of relative mRNA expression of IMPDH, XO, HGPRT and PNP was performed by real-time PCR, using
the ABI 7000 Real Time PCR (Applied Biosystems). cDNA was 
diluted (1:5) and analyzed using the QuantiTect® SYBR® Green 
PCR Kit (204145, Qiagen). The reaction mixture contained 10 
µL of SYBR® Green, 1.0 µL of forward and reverse primers (10 
µM each), 5.0 µL of cDNA (50 ng), and 4.0 µL of sterile, distilled 
water. Controls without template were included to ensure 
absence of contaminating DNA. PCR reactions were carried 
out as follows, an initial cycle at 95°C for 15 min to activate 
the HotStarTaq® DNA polymerase, followed by the denatural-
ization of the cDNA at 94°C for 15 s, 40 cycles at 94°C for 15 s 
each, 60°C for 30 s, and 72°C for 30 s. The specificity of each 
primer set was validated by the construction of a melting 
curve, constructed after PCR by increasing the temperature 
from 75°C to 95°C at a rate of 0.3°C every 20 s. The expression 
of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 
mRNA was determined as housekeeping gene. The relative 
expression of target mRNA was normalized to the amount of 
GAPDH in the same cDNA using the standard curve method. 
Amplification efficiency for each analyzed gene was ~98%, 
using dilution from 0.25 x 10^{-2} ng µL^{-1} to 0.25 x 10^{-6} ng µL^{-1} of 
PCR fragments. Each RT-PCR data point is average of three 
independent biological replicates. Primer sequences and 
accession numbers for the housekeeping gene GAPDH and 
for the different enzyme genes are included in Table 1.

**Ethical aspects**

Muscle samples were obtained in accordance with 
institutional ethical standards and with the 1964 Helsinki 
declaration and its later amendments from healthy volun-
teers subjected to programmed cesarean section at the 
Hospital de Especialidades Médicas Fidepaz in La Paz, Baja 
California Sur, México. All participants in the study signed the 
informe consent form, which, together with the research 
project, were approved by the Baja California Sur Chapter of 
the National Mexican Academy of Bioethics and registered 
with the Comité de Ética en Investigación (CONBIOÉTI-
CA-09-CEI-009-20160601).

**Statistical analyses**

Data were tested for normality and homoscedasti-
city using Kolmogorov-Smirnov (p < 0.05) and Levene (p < 
0.05) tests, respectively. Significant differences in the non-
parametric data were determined with Kruskal-Wallis and 
Mann-Whitney U tests (Zar, 2009). Statistical significance was 
considered when p ≤ 0.05, determined using GraphPad Prism 
version 3.03.

**RESULTS**

**Enzyme activity**

Figure 1 show the activity of IMPDH, PNP, XO and 
HGPRT from human skeletal muscle cells after normoxia and 
hypoxia conditions. IMPDH activity was significantly higher 
in normoxic (46.59 ± 11.95 µM mg⁻¹ protein h⁻¹) than hypoxic 
cells (22.09 ± 2.74 µM mg⁻¹ protein h⁻¹) (p < 0.036). There were 
no significant differences in PNP, XO and HGPRT activities 
between treatments (p > 0.05).

**Purine metabolite concentration**

The concentration of purine bases (HX, X), nucleosi-
des (I, A), nucleotides (AMP, GDP, ADP, GTP, ATP), NAD⁺ and 
UA were measured by HPLC after HPLC of human skeletal muscle cells (Table 2). UA was 
not detected in neither normoxic nor hypoxic cells. There

![Figure 1](image-url)

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

| Target gene | Sense-primer (5´-3´) | Antisense-primer (5´-3´) | GenBank accession no. | Size (bp) |
|-------------|---------------------|--------------------------|-----------------------|-----------|
| IMPDH       | GGCTCCATCTGCATCAACCA| CCATGGCCCCGATCTTCTTG     | NM_000883             | 262       |
| HPRT        | GACTGAGAGCTAGTTAATG| CACACAGCAAGTGTCAACC      | NM_000194             | 198       |
| PNP         | CTGTGTTGATGACGGCAGG| TCAATGGGCCCTCTGAGAGG     | NM_000270             | 225       |
| XO          | ACAGAACACCATGAGACCC| CTGCCACAGTTATCAGCATG     | NM_000379             | 224       |
| GADPH       | GGAAACGTGGCCGATGAGG| CTCGCTTACACACCTTCCTTG    | AF261085              | 216       |
Hypoxia downregulates gene expression of enzymes that participate in purine metabolism. Transcripts encoding for IMPDH, PNP, XO and HPGRT were obtained from human skeletal muscle cells cDNA. The BLAST analysis of the sequences from the obtained fragments (~300 bp), confirmed the identity of the coding sequences which correspond to the enzymes of purine metabolism. Quantitative real-time PCR was used to analyze the levels of expression of mRNAs encoding IMPDH, PNP, XO and HPGRT enzymes involved in purine metabolism (Fig. 2). IMPDH and XO were the most abundant transcripts, showing the highest significant reduction (p < 0.01) after hypoxia in contrast to normoxic cells (~99% each). HPGRT mRNA showed a 58% reduction (p < 0.01) under hypoxic conditions, while PNP presented the lowest expression from all the transcripts analyzed, and it did not show significant differences in contrast to hypoxic cells.

**DISCUSSION**

Mammals have developed effective strategies to cope with oxygen limiting situations. To achieve tolerance to acute or chronic hypoxia, the organism reduces metabolism, to prevent cellular injury and maintain functional integrity (Ramirez et al., 2007). Failure of these strategies results in different physiological and pathophysiological conditions (e.g. ischemia, inflammation and tumors) (Lahat et al., 2011).

The primary response to hypoxia within the cell ultimately leads to alternative routes of ATP generation (Wheaton and Chandel, 2011). IMPDH is a key enzyme in the purine nucleotide pathway, since it controls the gateway to guanine nucleotides (Hedstrom, 2009). IMPDH activity has been widely studied in several tissues, including plasma, erythrocytes, lymphocytes, spleen and thymus (Jonsson and Carlsten, 2001; Fukuda et al., 2016). Differential activity differences does not affect their kinetic properties (Hedstrom, 2009). In the present study, a partial sequence of IMPDH from human skeletal muscle cells, amplified and sequenced contained a conserved functional region, shared by the two

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**Table 2.** Purine metabolite concentrations in human skeletal muscle cells exposed to hypoxia.

| Metabolite     | Normoxia     | Hypoxia     |
|----------------|--------------|-------------|
| Xanthine       | 2.04 (0.99, 3.35) | 1.52 (0.98, 2.06) |
| HX             | 1.54 (0.81, 2.17)  | 0.76 (0.62, 0.99)* |
| UA             | ND           | ND          |
| IMP            | 1.04 (0.55, 2.12)  | 0.42 (0.29, 0.62)* |
| Inosine        | 4.71 (2.5, 7.22)  | 3.33 (1.86, 4.38) |
| NAD+           | 0.25 (0.08, 0.50)  | 0.21 (0.09, 0.78) |
| Adenosine      | 0.18 (0.02, 8.34)  | 1.11 (1.00, 1.15) |
| AMP            | 0.18 (0.02, 8.34)  | 1.11 (1.10, 1.15) |
| GDP            | 0.07 (0.03, 0.11)  | 0.05 (0.02, 0.08) |
| ADP            | 0.37 (0.06, 7.17)  | 0.09 (0.03, 2.68) |
| GTP            | 4.56 (2.24, 6.88)  | 0.08 (0.01, 2.20) |
| ATP            | 0.31 (0.17, 0.87)  | 0.16 (0.09, 0.60) |

**Figure 2.** Effect of hypoxia on the expression of the enzymes involved in purine metabolism. A) Inosine 5’-monophosphate dehydrogenase mRNA (IMPDH), B) purine nucleotide phosphorylase mRNA (PNP), C) xanthine oxidase mRNA (XO), and D) hypoxanthine-guanine phosphoribosyltransferase mRNA (HPGRT). Skeletal muscle cells were incubated under normoxic or hypoxic conditions for 1 h, afterwards cells were collected and transcripts were quantified by real-time PCR. Data were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene. Data are shown as mean ± S.E. of three independent biological experiments by triplicate. *=statistical differences between treatments (p < 0.05).
IMPDH genes. Normoxic cells showed high expression of IMPDH mRNA and IMPDH activity. After hypoxia treatment, IMPDH mRNA and IMPDH activity, as well as the endogenous levels of IMP, were downregulated, suggesting that oxygen limitation transcriptionally affects the de novo production of purines, as has been shown in normal lymphocyte cells (Jain et al., 2004) and hypoxia-treated human leukemic cells (Han et al., 2006).

PNP and XO are involved in purine degradation. PNP catalyzes the inosine breakdown to hypoxanthine (Dudzinska et al., 2006), the substrate for xanthine oxidase (Moriwaki et al., 1999). PNP activity has been reported in erythrocytes, liver and plasma of mammals (Schomaker et al., 2016). In this study, muscle cell PNP activity was 12.37 ± 2.71 U mg⁻¹ under normoxic conditions, with lower PNP activity observed (6.79 ± 1.20 U mg⁻¹), although this decrease was not significant. The same pattern was observed when the PNP mRNA was analyzed. In erythrocytes from rats, changes in PNP activity were evident after seven days of hypoxia (Hosek et al., 1986), while from the same species required an hour to stimulate its activity (Rao et al., 1989). In human adipocytes, PNP mRNA expression increased after 24h hypoxia (Nagao et al., 2018). The differential activity and expression pattern of PNP among tissues, might reflect different metabolic demands of each tissue and responses to stress conditions. XO activity in muscle tissue has been reported as less than 0.5 µU g⁻¹ tissue (Morion et al., 1993); in this study, higher levels of XO activity were obtained (17.55 ± 3.40 µU mg⁻¹). The hypoxia treatment resulted in a mild increase of XO activity (21.44 ± 4.13 µU mg⁻¹); however, this increase was not statistically significant. Previous reports indicate that moderate hypoxia significantly enhances XO activity in endothelial arterial cells (Kelley et al., 2006) and skeletal muscle and human plasma (Terada et al., 1992). The lack of increased activity in this study might be related to the significant reduction of HX levels observed after hypoxia (1.54 µM mg⁻¹ under normoxia and 0.76 µM mg⁻¹ under hypoxic conditions), limiting the catalytic capability of the enzyme. This level is consistent with the absence of HX and low levels of X. Interestingly, XO mRNA expression is modulated by hypoxia in a dose- and time-dependent manner, either by enhancing their expression (moderate hypoxia) or not affecting the gene expression (Poss et al., 1996; Kelley et al., 2006). In this study, XO mRNA in muscle cells was downregulated in hypoxic conditions; these differences might relate to the time of exposure to hypoxia and to the level of regulation that XO presents, which is at transcriptional and posttranslational level (Kayyali et al., 2001; Poss et al., 1996).

The alternate pathway for purine nucleotide formation is the purine salvage pathway, in which instead of generating new purines, preexisting purine bases are taken up for the generation of nucleotides. HGPRT activity in human muscle cells has been previously reported to be 4.8 ± 0.015 nmoles mg⁻¹ h⁻¹ (Jacobs et al., 1988), which is in agreement with the levels obtained in the muscle cell culture under basal conditions (9.90 ± 3.6 nmoles mg⁻¹ h⁻¹) in this study. An increase of HGPRT activity has been observed after prolonged fasting (Soñanez-Organis et al., 2012) and as a compensatory response to hypoxia in mice heart tissue (Wu et al., 2015). Interestingly, cerebral hypoxia-ischemia experiments in rabbits did not show changes in HGPRT activity (Cherin et al., 2006). In this study, HGPRT activity decreased after hypoxia exposure; however, this reduction was not significant at the protein level, but it was significant at the transcriptional level.

The analysis of the purine metabolites showed a significant increase in HX content, which correlates with the low activity of XO observed. These results correlated with previous findings in adipocyte tissues exposed to hypoxia, which showed an increase in HX and acid uric secretion during prolonged hypoxia (Nagao et al., 2018). The significantly reduction of IMP concentration in this study, might be related to an active uptake of this compound by IMPDH, as has been shown in other mammalian tissues, including erythrocytes, placenta, liver and heart (Traut, 1994; Pérez-Milicua et al., 2015).

CONCLUSIONS

The observed reduction in enzyme activities for purine metabolism as well as their transcripts, along with the significant reduction of HX and IMP levels suggest that skeletal muscle cells respond differently to oxygen limitation than other tissues previously studied. This reduction might relate to a decrease of the metabolic machinery to avoid the excessive production of metabolites, which may lead to apoptotic signaling. This could suggest that skeletal muscle cells are able to withstand longer periods of hypoxia, before developing alternative mechanisms to cope with hypoxia effects via the salvage pathway, as has been observed in erythrocytes and plasma. However, further research is necessary to understand the mechanism underlying purine metabolism under hypoxia conditions.

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