UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

DEPARTAMENTO DE CIÊNCIAS FISIOLÓGICAS

PROGRAMA INTERINSTITUCIONAL DE PÓS-GRADUAÇÃO EM CIÊNCIAS

FISIOLÓGICAS UFSCar/UNESP

HIPERAMONEMIA ATIVA HIF-1α PELA VIA NF-kB: UM POSSÍVEL

MECANISMO DE SARCOPENIA EM CIRROSE

Rafaella Nascimento e Silva

São Carlos – SP

2016

HIPERAMONEMIA ATIVA HIF-1α PELA VIA NF-kB: UM POSSÍVEL MECANISMO DE SARCOPENIA EM CIRROSE

Rafaella Nascimento e Silva

Tese de doutorado apresentada ao Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas associação ampla UFSCar/UNESP do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos como parte dos requisitos para a obtenção do título de Doutora em Ciências Fisiológicas.

Orientadora: Profª Drª Ângela Merice de Oliveira Leal

Co-orientador: Dr. Srinivasan Dasarathy (Cleveland Clinic - Cleveland - OH, USA)

São Carlos –SP

2016

Folha de Aprovação

Assinaturas dos membros da comissão examinadora que avaliou e aprovou a Defesa de Tese de Doutorado da candidata Rafaella Nascimento e Silva, realizada em 15/09/2016:

Profa. Dra. Ângela Merice de Oliveira Leal
UFSCar

Profa. Dra. Silvana Gama Florêncio Chachá
UFSCar

Prof. Dr. Fernando Gomes Romeiro
UNESP

Profa. Dra. Tania de Fatima Salvini
UFSCar

Profa. Dra. Rita de Cassia Martins Alves da Silva
FAMERP

"Dedico esse trabalho primeiramente à Deus por ter me dado força e discernimento para a realização deste trabalho e aos meus queridos pais, Haroldo e Eva, e meu irmão Raphael, por acreditarem em mim investindo neste sonho e pelos primeiros ensinamentos na vida."

AGRADECIMENTOS

A meu co-orientador, Dr. Srinivasan Dasarathy, primeiramente por me acolher em seu laboratório na Cleveland Clinic, pela colaboração com seus ensinamentos e por todo o suporte dado para a realização de todos os experimentos deste trabalho.

Aos meus pais, Haroldo Gracio e Eva Creuza, e ao meu irmão, Raphael Augusto, que mesmo distantes estão sempre presentes me apoiando e me incentivando. Amo vocês!

Ao meu namorado e parceiro, João Paulo Rebouças, esse doce de pessoa que Deus colocou no meu caminho num momento em que eu só tinha foco para trabalho, veio com seu carinho, equilíbrio e serenidade e me deu muito mais força e incentivo para finalizar o doutorado.

À Maria Fernanda Cury e Graziéle Derrigi Pisani, pela grande amizade e por estarem sempre disponíveis me ajudando sempre, em todos os sentidos.

À Larissa Oliveira Guimarães e Vanessa Nicolete minhas parceiras que foram fundamentais na minha jornada nos Estados Unidos (Cleveland-OH), pois foram pessoas que estavam sempre comigo em todos os momentos alegres ou tristes.

À família Blanton, que me acolheu como uma filha e irmã em Cleveland-OH e que considero minha segunda família. Nunca os esquecerei!!!

Aos meus amigos e familiares da Paraíba, pela amizade verdadeira, que mesmo na distância se mantém forte, e sinto como se vocês estivessem sempre comigo.

À todo o pessoal do Laboratório de Bioquímica e Biologia Molecular-UFSCar, pela amizade e paciência em tirar minhas dúvidas sempre que solicitadas.

HIPERAMONEMIA ATIVA HIF-1α PELA VIA NF-kB: UM POSSÍVEL MECANISMO DE SARCOPENIA EM CIRROSE

RESUMO

Resumo: Um dos grandes mediadores de sarcopenia em cirrose é a hiperamonemia. Esta anormalidade metabólica é frequente em doenças hepáticas promovendo conversão danificada de amônia em uréia prejudicando a síntese protéica e induzindo autofagia do músculo esquelético pela up-regulação de miostatina via fator nuclear kappa B (NF-kB). O metabolismo de amônia no músculo esquelético ocorre via síntese de glutamato e glutamina através do intermediário metabólico α-cetoglutarato do ciclo de Krebs, que regula o Fator Induzido por Hipóxia (HIF-1α). Além disso, existe um interação entre os dois fatores de transcrição HIF-1α and NF-kB. **Objetivo:** Este estudo avalia os efeitos da hiperamonemia no *cross-talking* entre NF-kB and HIF-1α. **Métodos:** Para examinar os efeitos do aumento da concentração de amônia na expressão de HIF-1α através da via NF-kB foi realizado um knockdown estável de NF-kB, e as subunidades IKKα e IKKβ do complexo I kappa B quinase (IKK) em células C_2C_{12} avaliando as atividades de HIF-1α e miostatina. **Resultados:** A expressão protéica de HIF-1α foi significativamente alta em células C_2C_{12} sob tratamento com acetato de amônia comparado com controle. Uma vez que ocorre o silenciamento de NF-kB, IKKα and IKKβ em células C_2C_{12}, não é observada a expressão protéica de HIF-1α, sugerindo um *cross-talking* entre eles. A expressão protéica de miostatina foi significativamente alta em células C_2C_{12} silenciadas para IKKα sob tratamento com acetato de amônia comparado com células C_2C_{12} random (controle), sugerindo que miostatina não é dependente da quinase IKKα. **Conclusão:** Foi concluído que hiperamonemia é um ativador normóxico de HIF-1α através da via de sinalização NF-kB resultando em sarcopenia via up-regulação de miostatina.

Palavras-chaves: Hiperamonemia. Fator induzido por hipóxia 1α. Factor nuclear kappa B. Miostatina. Músculo esquelético.

FEDERAL UNIVERSITY OS SÃO CARLOS
BIOLOGICAL SCIENCES AND HEALTH CENTER
DEPARTMENT OF PHYSIOLOGICAL SCIENCES
INTERINSTITUTIONAL PROGRAM OF POST GRADUATE IN PHYSIOLOGICAL SCIENCES

Rafaella Nascimento e Silva

Supervisor: Professor. Ph.D. Ângela Merice de Oliveira Leal

Co-Supervisor: Professor. Ph.D. Srinivasan Dasarathy

HYPERAMMONEMIA ACTIVATES HIF-1α VIA A NF-kB PATHWAY: A POSSIBLE MECHANISM FOR SARCOPENIA IN CIRRHOSIS

Abstract: Hyperammonemia impairs skeletal muscle protein synthesis and induces autophagy by upregulating myostatin via nuclear factor-kappaB (NF-kB). Skeletal muscle ammonia metabolism occurs via synthesis of glutamate and glutamine via critical TCA intermediate α-KG, that regulates increase expression of hypoxia inducible factor 1α (HIF-1α). Furthermore, there is a interaction between HIF-1α and NF-kB. **Objective:** This study evaluated the effects of hyperammonemia in NF-kB and HIF-1α *cross-talking*. **Methods:** To examine the effects of ammonium acetate intervention under HIF-1α signaling through NF-kB pathway it has generated a stable knockdown cell line for NF-kB and the subunits α and β of the I kappa B kinase (IKK) complex (IKKα and IKKβ) evaluating the HIF-1α and myostatin activities. **Results:** The protein expression of HIF-1α was significantly higher in C_2C_{12} murine myotubes under ammonium acetate intervention compared to control. Once the deletion of NF-kB, IKKα and IKKβ occurs, the HIF-1α is not expressed, suggesting a *cross-talking* between them. The protein expression of myostatin was significantly higher in C_2C_{12} IKKα deletion under ammonium acetate intervention compared to C_2C_{12} random suggesting that myostatin is not IKKα dependent. **Conclusion:** We conclude that hyperammonemia is a normoxemic activator of HIF-1α through NF-kB pathway that results in sarcopenia via up-regulation of myostatin expression.

Keywords: Hyperammonemia. Hypoxia inducible factor 1α. Nuclear factor-kappaB. Myostatin. Skeletal muscle.

FIGURES LIST

TABLES LIST

ABBREVIATIONS LIST

α-KG: α-ketoglutarate

Arnt: Aryl Hydrocarbon Receptor Nuclear Translocator

DMEM: Dulbecco's modified Eagle medium

FBS: Fetal Bovine Serum

FH: Fumarate Hydratase

GAPDH: Glyceraldehyde-3-phosphate Dehydrogenase

HCC: Hepatocellular Carcinoma

HE: Hepatic Encephalopathy

HEK-293T: Human Embryonic Kidney 293 cells

HIF-1α: Hypoxia Inducible Factor 1α

HRE: Hypoxia Response Elements

IKKs: IkappaB kinases

MMPs: Matrix Metalloproteinases

mTOR: Mammalian Target of Rapamycin

NF-kB: Nuclear factor-kappaB

ODD: Oxygen Degradation Domain

PHDs: Prolyl-Hydroxylases

pVHL: Tumour Suppressor von Hippel–Lindau protein

SDH: Succinate Dehydrogenase

TBT: TATA-binding protein

TCA: Tricarboxylic Acid Cycle

TGF-β: Transforming Growth Factor beta Family

TIMPs: Tissue Inhibitors of MMPs

SUMMARY

1 INTRODUCTION

1.1 Liver Cirrhosis

The liver is the major parenchymal organ in the body that performs essential functions for survival and has large storage capacity, booking and regeneration (ROBSON, 2011). It is perfused by approximately 1050 ml of blood per minute through the portal vein and also 300 mL of blood per minute through the hepatic artery, the equivalent of 27% of the cardiac output. Anatomically, located in the cranial portion of the abdomen, the liver comprises four lobes: left, right, quadrate and caudate lobes (CHAMBERLAIN, 2013).

Cirrhosis is considered the final stage of chronic liver diseases where normal architecture is replaced by regenerative nodules separated by bands of fibrous tissue (SCHUPPAN e AFDHAL, 2008; DASARATHY, 2012), which causes the loss of both synthesis and excretion functions of the liver. It is a dynamic process associated with infections, vascular remodeling, portal hypertension development, fibrogenesis and, eventually, liver failure. The most common causes of cirrhosis are alcoholic and non-alcoholic fatty liver disease and viral hepatitis (B or C) (figure 1) (SCHUPPAN e AFDHAL, 2008; MORMONE et al., 2011; WIEGAND e BERG, 2013). The exact prevalence of cirrhosis worldwide is unknown; however, recent epidemiological data show that prevalence of cirrhosis in the United States was approximately 0.27%, corresponding to 633,323 adults. It is considered a serious public health problem, and interestingly sixty-nine percent were unaware of having the disease (SCAGLIONE et al., 2015).

Figure 1 - Causes of liver cirrhosis.
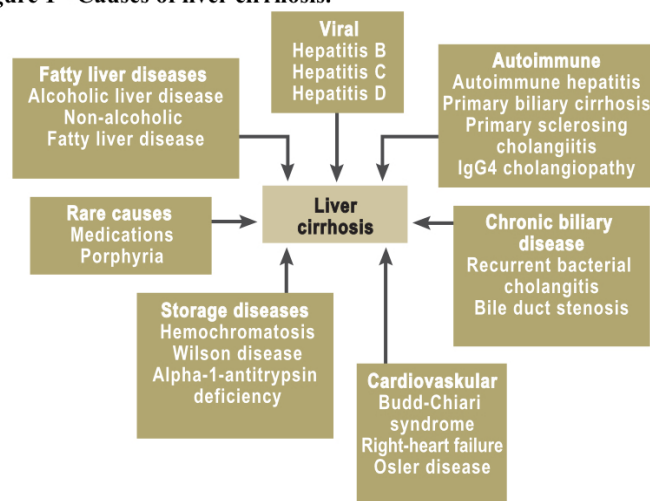
Reference: adapted from Wiegand e Berg, 2013.

The major clinical consequences of cirrhosis are impaired hepatocyte function, an increased intra-hepatic resistance (portal hypertension), ascites, hepatic encephalopathy, spontaneous bacterial peritonitis and the development of hepatocellular carcinoma (HCC). The general circulatory abnormalities in cirrhosis such as increased cardiac output and water and salt retention are associated to the hepatic vascular alterations, and the resulting portal hypertension (Figure 2). These complications caused by cirrhosis are responsible for high mortality rates (SCHUPPAN e AFDHAL, 2008).

Figure 2 - Vascular architecture in cirrhosis.
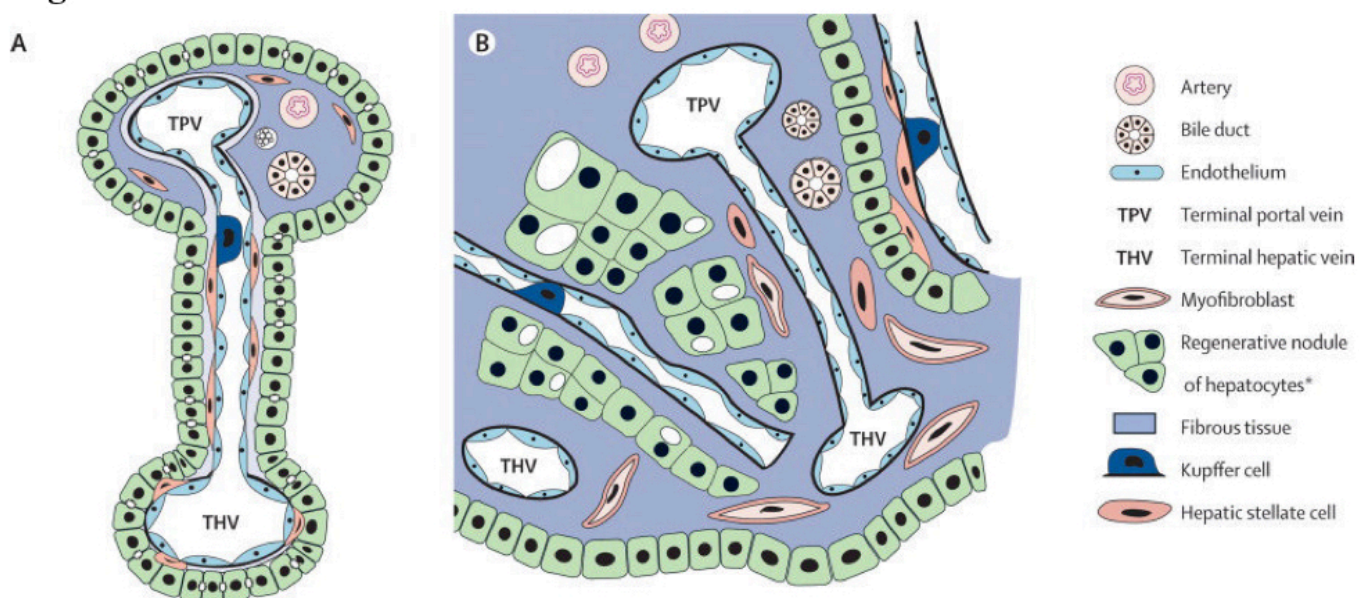
Reference: adapted from Schuppan e Afdhal, 2008.

Fibrosis is an evolutionary response to limit tissue damage and develops in consequence of the injury, but the pattern of hepatic fibrosis is similar regardless of the etiology (FALLOWFIELD e IREDALE, 2004). The formation of fibrosis occurs by an abnormal extracellular matrix remodeling in response to chronic liver injury (SCHUPPAN et al., 2001). First of all, fibrogenesis is regulated by removal of excess extracellular matrix by proteolytic enzymes, such as matrix metalloproteinases (MMPs) (BENYON e ARTHUR, 2001). Chronic liver damage promotes fibrogenesis, with upregulation of tissue inhibitors of MMPs (TIMPs) (BENYON e ARTHUR, 2001).

The liver has several cell types that are able to synthesize and deposit extracellular matrix components; however, stellate cells play an important role in the fibrotic pro-

cess, since it is directly related to the excessive synthesis of extracellular matrix and fibrosis (KNITTEL et al., 1999; SCHUPPAN et al., 2001). The fibrogenic stimuli include reactive oxygen species (EROS), hypoxia, inflammatory and immune responses, apoptosis and steatosis (GHIASSI-NEJAD e FRIEDMAN, 2008). Evidence suggests that cirrhosis retardation, stoppage or even reversal is possible. The reversal of liver fibrosis requires the loss of fibrogenic cells as well as the degradation of extracellular matrix, and the remodeling of hepatic architecture (WANLESS et al., 2000).

The clinical evaluation of patients with cirrhosis includes questions about risk factors and a thorough physical examination for detection of chronic liver disease stigmas. The diagnosis includes laboratory tests, imaging methods, endoscopy and liver biopsy. The ultrasonography provides important information on hepatic architecture, is cheap and widely available. However, the biopsy is considered the gold standard for diagnosis of cirrhosis (REGEV et al., 2002), but it is dispensable when evident signs of cirrhosis, such as ascites, coagulopathy, and shrunken nodular-appearing liver are present.

1.2 Sarcopenia in Cirrhosis

Sarcopenia is defined as a reduction in skeletal muscle mass and function as severe as two standard deviation stages below the healthy young adult, and it is associated with aging, malignancy and chronic disease (MARCELL, 2003). Skeletal muscle mass will not reduce if the protein synthesis rate is balanced with amino acid degradation rates, combined with dietary absorption. Imbalances between synthesis and degradation over many years are necessary to eventually result in the significant loss of muscle mass (MITCH et al., 1999; MOSONI et al., 1999). Muscle wasting is a common characteristic of chronic liver disease, found in approximately 40% of patients with cirrhosis (KALAFATELI et al., 2015).

Evidence shows that the prevalence of sarcopenia is increased in patients with cirrhosis (DURAND et al., 2014; HANAI et al., 2015). Furthermore, as previously demonstrated, patients with cirrhosis and sarcopenia have elevated mortality, and one of the reasons is increased infection in elderly patients with sarcopenia than in those without (COSQUERIC et al., 2006; DASARATHY, 2012). Due to the fact that loss of

skeletal muscle compromises immunity and may be associated with sepsis, sarcopenia is considered one of the leading causes of death in cirrhotic patients (HOLECEK et al., 1995; COSQUERIC et al., 2006; PERIYALWAR e DASARATHY, 2012).

Several factors contribute to increase sarcopenia in cirrhotic individuals, including inadequate dietary intake, metabolic disturbances, and malabsorption (O'BRIEN e WILLIAMS, 2008). The appetite decrease, caused by the increase in pro-inflammatory cytokine concentration, as well as sodium and water restrictions to prevent fluid accumulation, or even decrease in protein intake, noticed in patients with hepatic encephalopathy, are related to deficient food intake (O'BRIEN e WILLIAMS, 2008; DASARATHY, 2012). Another mechanism involved in sarcopenia development is an increase in plasma and skeletal muscle tissue myostatin. This protein is member of transforming growth factor beta family (TGF-β), which inhibits protein synthesis through impaired mammalian target of rapamycin (mTOR) signaling (LEE e MCPHERRON, 2001; MC-CROSKERY et al., 2003). In this context, hyperammonemia one of the main findings of cirrhotic patients, is a condition that is closely related to and involved with the regulation of myostatin, as well as increasing the autophagy in skeletal muscle (QIU et al., 2012).

Some therapies to attenuate sarcopenia in cirrhotic patients, including myostatin antagonists are waiting to be evaluated in randomized controlled trials. In a preliminary investigation, myostatin levels were significantly higher in cirrhotics compared with healthy controls (GARCÍA et al., 2010). In addition, animal model studies have shown that impairment of liver function increased significantly the expression of myostatin (DASARATHY et al., 2011).

1.3 Hyperammonemia and Ammonia Metabolism

Ammonia (NH_3) is a toxic weak basic compound that is produced during cells metabolism and needs to be detoxified and eliminated from the human body. Ammonia derives from the metabolism of amino acids (ADEVA et al., 2012; HOLECEK, 2013; MATOORI e LEROUX, 2015).

Many organs including liver, kidney, skeletal muscle, brain, lung and others are involved in ammonium homeostasis in the body (ADEVA et al., 2012). The liver plays a

central role in whole body nitrogen metabolism, which arises in several biochemical pathways, including deamination of many amino acid, protein synthesis, the breakdown of purine and pyrimidine derivatives, polyamines and by gut bacterial metabolism. The latter generates ammonia, an important nitrogen source that, at elevated levels, is toxic mainly to the central nervous system, and is the key disturbance involved in hepatic encephalopathy (HE) (COOPER e PLUM, 1987; OLDE DAMINK et al., 2002; MATOORI e LEROUX, 2015; TAPPER et al., 2015). Ammonia, a neurotoxic agent, is continually produced mainly by skeletal muscle and intestinal mucosa in mammals, and consumed during cell metabolism in human body tissues. It is normally excreted as urea by periportal liver and in most extrahepatic tissues is detoxified by glutamine formation (HOLECEK et al., 1995; ADEVA et al., 2012; TAPPER et al., 2015). Once the capacity for detoxification of ammonia is insufficient, a vicious cycle occurs, increasing systemic ammonia that leads to loss of appetite and vomiting. Then, protein synthesis is reduced and catabolism increases, leading to weight loss in patients with liver function impairment (HÄBERLE, 2011).

Severe liver diseases, including acute liver failure and cirrhosis, lead to hepatocellular dysfunction. The liver dysfunction results in disturbed body nitrogen homeostasis, which impairs hepatic urea synthesis capacity and modifies intra- and/or extra-hepatic shunting of portal blood into the systemic circulation, leading to increased blood and tissue concentrations of ammonia or hyperammonemia (RUDMAN et al., 1973, ZIEVE, 1987; MATOORI e LEROUX, 2015).

Hyperammonemia is a metabolic disorder that can affect patients at any age. Elevated levels of ammonia in plasma indicate overproduction and/or an inefficient detoxification process (COOPER e PLUM, 1987; ALFADHEL et al., 2016). The concentration of ammonia in human plasma varies in venous, arterial or capillary blood (ADEVA et al., 2012). It has been reported in healthy individual the arterial ammonia concentration to be about 45 μM and when in hepatic disorders this concentration can reach about 600-700 μM, but it can vary based on the patient's age and gender (CLEMMESEN et al., 2000; HÄBERLE, 2011; ALFADHEL et al., 2016). The patients with increased concentration of ammonia present many different clinical features that depend on the age of the individual, and on the type and severity of the cause (HÄ-

BERLE, 2011). Most of the clinical signs and symptoms under elevated plasma ammonia levels are neurological and occur in many diseases, including urea cycle defects and liver disease (BRAISSANT et al., 2013; ALFADHEL et al., 2016).

This metabolic disorder is one of the potential mediators of skeletal muscle mass loss in cirrhosis that impairs skeletal muscle protein synthesis and induces autophagy. Qiu et al (2012) have previously reported that ammonia is a mediator of the *liver-muscle* axis, but the molecular mechanisms of sarcopenia associated to hyperammonemia are not well-defined. In other study, Qiu et al (2013) have reported that hyperammonemia transcriptionally upregulates myostatin via a nuclear factor-kappaB (NF-kB) mediated mechanism. The mechanism by which ammonia mediated chemical-signaling transduction occurs are not known and an integrated metabolic-molecular approach is required for these studies.

The hepatic urea cycle is the main pathway to detoxify ammonia but, in patients with cirrhosis, the skeletal muscle becomes an alternate organ for ammonia disposal. During hyperammonemia, muscle takes up ammonia and plays a major role in (temporarily) detoxifying ammonia to glutamine. Glutamine synthesis is the most important alternative pathway for ammonia detoxification (OLDE DAMINK et al., 2002; HOLECEK, 2014). Since ureagenesis occurs only in the hepatocytes, skeletal muscle ammonia metabolism occurs via the synthesis of glutamate and then glutamine from critical tricarboxylic acid cycle (TCA) intermediate, α-ketoglutarate (α-KG) (FAFF-MICHALAK e ALBRECHT, 1993; BUTTERWORTH, 1998; HOLECEK, 2014). The first step of ammonia disposal in the muscle is the mitochondrial glutamate synthesis catalyzed by glutamate dehydrogenase. Ammonia entry into mitochondria has been reported to occur via aquaporin 8 in the liver, but its transport into skeletal muscle mitochondria is not known (SORIA et al., 2014). Conversion of α-KG to glutamate results in increased consumption of α-KG with perturbations in TCA cycle and mitochondrial function (ZAGANAS et al., 2013; WANG et al., 2014). Regulation of signaling pathways by α-KG, a metabolic intermediate, is mediated by increased expression of the transcription factor, hypoxia inducible factor 1α (HIF-1α) (SUTENDRA et al., 2013; DROMPARIS et al., 2014).

1.4 Hypoxia-inducible factor (HIF) family of transcription factors

An adequate oxygen supply to cells and tissues is crucial for the function and survival of aerobic organisms. Hypoxia is a state where oxygen availability/delivery is below the necessary threshold to maintain physiological oxygen tension for a particular tissue. It is well-established that an extensive transcriptional response regulating angiogenesis, glucose metabolism, cell growth, metastasis and others processes is induced by increased expression of HIFs to regulate the oxygen homeostasis (LOBODA et al., 2012; KIETZMANN et al., 2016; WIGERUP et al., 2016). Canonical regulation of HIFs are mediated by cellular oxygen content or hypoxia, but also in response to other stimuli, such as reactive oxygen species, metal ions, growth and coagulation factors, hormones, or cytokines under normoxic conditions (WAGATSUMA et al., 2011; LOBODA et al., 2012; KIETZMANN et al., 2016).

The HIFs are proteins that act as transcription factors in response to oxygen deficiency, promoting a transcriptional regulation mediated by heterodimerization of a α-subunit, whose expression is crucially stabilized by hypoxia, with a β-subunit that is constitutively expressed even in normal oxygen conditions (HUANG et al., 1996; LOBODA et al., 2012; WIGERUP et al., 2016). It has been reported three different α-subunit isoforms HIF-1α, HIF-2α and HIF-3α among which HIF-1α and HIF-2α have been most comprehensively studied, and three isoforms of β-subunit, also known as the aryl hydrocarbon receptor nuclear translocator (Arnt1, Arnt2 and Arnt3) (WANG et al., 1995; HUANG et al., 1996). The HIF-1α and HIF-2α play a special role in the cellular response to lack of oxygen, but both isoforms may have some unique characteristics mainly in structure and functions, regulating the expression of many common genes whereas HIF-1α preferentially induces glycolytic pathway and HIF-2α regulates important genes for tumor growth, cell cycle progression and maintaining stem cell pluripotency (HU et al., 2003; WANG et al., 2005). The HIF α-subunit proteins contribute to the development of hypoxia-related diseases including anemia, myocardial infarction, thrombosis, atherosclerosis, diabetes mellitus, or cancer and to physiological processes, which include high altitude living, intense exercise and embryo development that also demonstrate changes in oxygen (KAELIN, 2011; MASSON e RATCLIFFE, 2014).

Oxygen sensitivity is achieved by a class of dioxygenases enzymes, called prolyl-hydroxylases 1, 2 and 3 (PHDs) that regulate directly HIF-1α stability. In physiological states under normoxia and in presence of α-KG, as well as cofactors: iron and ascorbate, the PHDs catalyze the hydroxylation of specific proline residues Pro[402] and Pro[564] within the oxygen degradation domain (ODD) of HIF-1α, creating a high-affinity binding site for the tumour suppressor von Hippel–Lindau protein (pVHL) which recruits other proteins of E3 ubiquitin ligase complex (BRUICK e MCKNIGHT, 2001; ZAGÓRSKA e DULAK, 2004; WAGATSUMA et al., 2011; HOU et al., 2014). The HIF-1α is subsequently ubiquitinated and degraded by the 26S proteasome. However, under hypoxic conditions the PHDs are inactivated, which prevents binding of pVHL. Therefore, HIF-1α escapes ubiquitination and proteasomal degradation, and can be transported to the nucleus where, after dimerization with HIF-1β, forms heterodimeric transcription factors binding to hypoxia response elements (HRE) that stimulate target genes transcription (Figure 3) (ZAGÓRSKA e DULAK, 2004; KIETZMANN et al., 2016).

Figure 3 - HIF-1α signaling under normoxia and hypoxia condition.
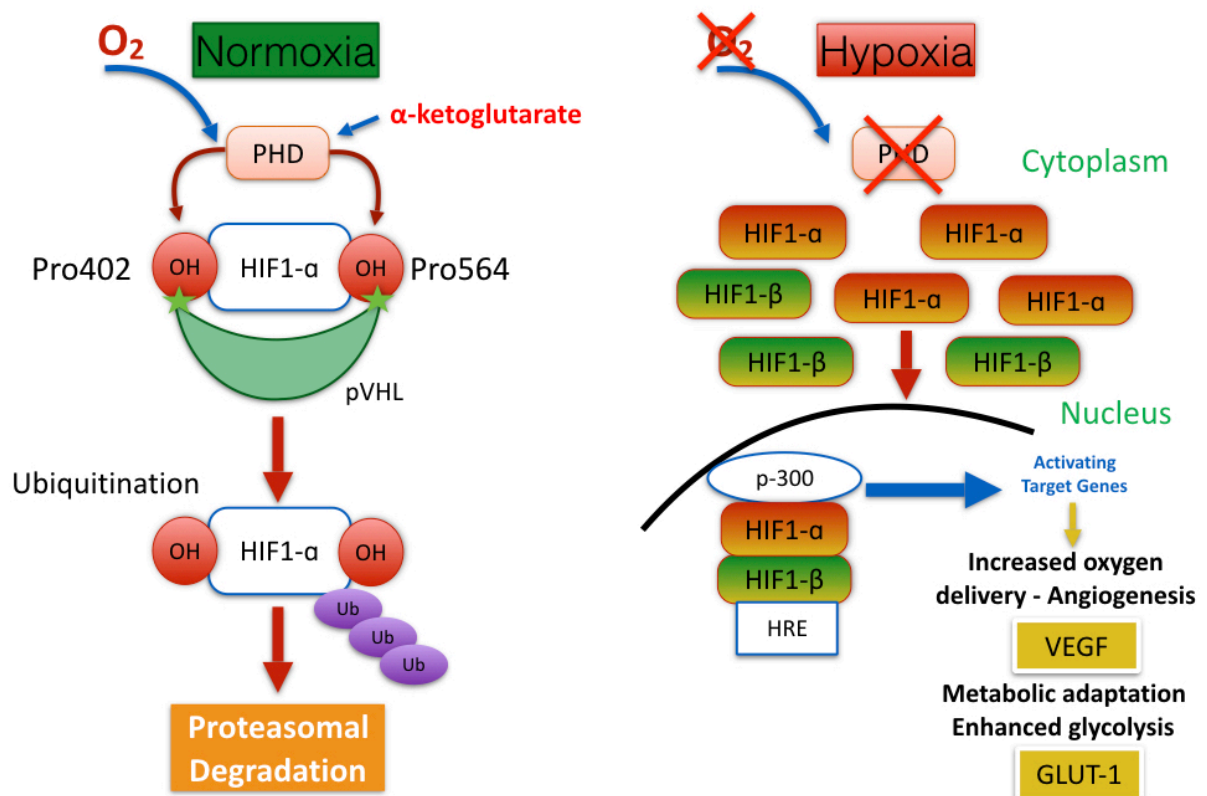
Since α-KG is a critical metabolic requirement for proline hydroxylation by PHDs, a deficiency of α-KG is also likely to result in impaired proline hydroxylation and consequent stabilization of HIF-1α (STROKA et al., 2001; LOBODA et al., 2012; HOU et al., 2014). In cancer diseases, mutation of the enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH) causes succinate or fumarate accumulation that competes with α-KG for PHD binding, thereby inhibiting PHD and stabilizing HIF-1α (POLLARD et al., 2005; SELAK et al., 2005; XIAO et al., 2012; KIM et al, 2013). In severe liver diseases, elevation of ammonia concentration can be noticed. Bessman & Bessman (1955) proposed that this characteristic results in the TCA cycle being deprived of α-KG. Therefore, regulation of signaling pathways by α-KG, a metabolic intermediate, could be mediated by increased expression of HIF-1α (SUTENDRA et al., 2013).

However, it is not only HIF that responds to hypoxia, but many other transcription factors have been participated of cellular response to low oxygen, and one of these is the family of NF-κB (KENNETH e ROCHA, 2008; BIDDLESTONE et al., 2015; D'IGNAZIO et al., 2016). The NF-κB is considered the main transcription factor activated by inflammatory pathways that is involved in several pathological conditions (RIUS et al., 2008). It has been reported that exists an extensive crosstalk between HIF and NF-κB mainly in inflammatory diseases and immune responses (D'IGNAZIO et al., 2016).

2 OBJECTIVE

The aim of this study was evaluate the effects of hyperammonemia in HIF-1α activity in murine C_2C_{12} cells line.

1. to investigate the HIF-1α expression under hyperammonemic condition.
2. to explore the HIF-1α and target genes mRNA levels under hyperammonemic condition.
3. to examine the *cross-talking* between HIF-1α and NF-kB under hyperammonemic condition.

3 MATERIALS AND METHODS

3.1 *In vitro* study

The C_2C_{12} is a mouse myoblast cell line that is capable of differentiation. The murine C_2C_{12} cells line were obtained from American Type Culture Collection (ATCC®, Manassas, VA, USA) and grown to confluence in proliferation medium consisting of Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) FBS (Fetal Bovine Serum). After 80-90% of confluence, the medium was changed to differentiation medium [DMEM with 2% (vol/vol) horse serum]. Following 48h of differentiation, cells were exposed to freshly prepared 10 mM ammonium acetate in different time points (0, 3h, 6h and 24h). For positive control, the cells were exposed to freshly prepared 200 µM cobalt chloride. The DMEM and cellular pH in response to ammonium acetate was performed as described (QIU et al., 2012).

3.2 Stable Knockdown of p65 NF-kB in C_2C_{12} Cells

Stable cell lines expressing shRNA to the p65 subunit of NF-kB or scrambled shRNA were generated by lentivirus transduction using protocols approved by the Institutional Biosafety Committee at the Cleveland Clinic. In brief, lentivirus was produced in Human Embryonic Kidney 293 (HEK-293T) cells transfected with a MISSION p65 shRNA construct (NM_009045.4-2313s21c1; Sigma, St. Louis, MO, USA), and the lentivirus packaging constructs RSV-Rev, MDGLG-RRE, and HCMV-G by using Polyfect (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Medium containing viral particles was then collected 48h post-transfection, passed through 0.4-µm filter, and used to infect C_2C_{12} cells in the presence of polybrene (20 µg/mL). Stable cell lines were selected with puromycin (2 µg/mL) for 10 days.

3.3 IkappaB kinases α and β (IKKs) and scrambled shRNA

Murine C_2C_{12} cells line were plated in 2 ml of DMEM medium supplemented with 10% FBS without antibiotics and incubated overnight at 37 ºC with 5% CO_2. After 50% of confluence, the DMEM medium was changed to 1 ml of freshly 10% DMEM medium. In different tubes, 4 µl of TransfeX™ (ATCC®, Manassas, VA, USA) were mi-

xed with 100 µl OPTI-MEM serum-free medium and 1 µg of shRNA (IKK-α or IKK-β or scrambled). This mixture was incubated for 20 min at room temperature and then added to the cells. The medium was changed 24h after transfection.

3.4 RNA isolation and RT-PCR analysis

Total RNA was extracted by using the TRIzol® reagent, following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The cDNA was generated by using the BD Clontech kit (Clontech Inc., Mountain View, CA, USA) containing avian leukemia reverse transcriptase and quantified by spectrophotometry following described methods (DASARATHY et al., 2004). Real-time quantitative PCR was performed on a Stratagene Mx 3000P (Stratagene Inc., La Jolla, CA, USA) by using the Brilliant III SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) protocol, and relative changes in mRNA expression was quantified as described (DASARATHY et al., 2004). The primer sequences (forward; reverse) for the mouse genes used in the study are as follows in Table 1.

Table 1 - Primer sequences.

Gene	Primer Sequence	Product Size (bp)	Accession Number
HIF-1α	Forward, 5'-tcaagtcagcaacgtggaag-3' Reverse, 5'-tatcgaggctgtgtcgactg-3'	198	NM_010431.2
VEGF	Forward, 5'-caggctgctgtaacgatgaa-3' Reverse, 5'-aatgctttctccgctctgaa'-3'	207	M95200.1
PDK1	Forward, 5'-ggcggctttgtgatttgtat-3' Reverse, 5'-acctgaatcgggggataaac-3'	199	NM_172665.5

3.5 Total cell lysis, cytoplasmic and nuclear fractionation

Cells were placed on ice, rinsed with phosphate-buffered saline, and harvested. Pellets were lysed in RIPA® buffer [25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS] (Thermo Fisher Scientific, Rockford, IL, USA) containing protease inhibitor cocktail and phosphatase inhibitors (Sigma-Aldrich, St. Louis, USA) and stored at -80°C. Cytoplasmic and nuclear protein was extracted by using the low salt buffer [1% Nonidet P-40, 150 mM NaCl2, 50 mM tris-HCL (pH 7.5),

1.5 mM MgCl2] containing protease inhibitor cocktail and phosphatase inhibitors (Sigma-Aldrich, St. Louis, USA). The cells were placed on ice for 30 minutes with low salt buffer. Nuclei fraction were pelleted by centrifugation at 600 x g for 5 minutes at 4°C. The supernatant containing cytoplasmic protein was collected and stored at -80°C and nuclei pellet was resuspended in a low-salt buffer and centrifuge at 600 x g for 5 minutes at 4°C 3 times to remove the cytoplasmic extract completely. The nuclei pellet was sonicated by a metallic Soundproof Box (BIORUPTOR® PLUS - Sonication System, Diagenode Inc. USA NORTH AMERICA, Denville, NJ, USA) at medium speed for 5 minutes at 4°C. The homogenate containing nuclear protein was collected and stored at -80°C.

3.6 Western blotting

The proteins were quantified, resolved by SDS/PAGE, and immunoblotted as described (DASARATHY et al., 2004). The membranes were probed in the following primary antibodies: polyclonal rabbit HIF-1α (D2U3T), polyclonal rabbit TATA-binding protein (TBT) antibody, monoclonal rabbit Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (14C10), were obtained from Cell Signaling Technology® (Danvers, MA, USA), polyclonal goat Actin (I-19) (Santa Cruz Biotechnology, Dallas, TX, USA) and polyclonal rabbit GDF-8-myostatin (Abcam, Cambridge, MA, USA) were diluted to 1:2000, except for monoclonal rabbit Anti-IKKα [Y463] (Abcam, Cambridge, MA, USA), monoclonal mouse Anti-IKKβ (10AG2) (EMD Millipore®, Billerica, MA, USA) and polyclonal goat NF-kB p65 (C-20)X (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies (1:5000 dilution). Secondary antibodies anti-rabbit, anti-mouse and anti-goat conjugated with horseradish peroxidase were used at a dilution of 1:10000 and obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The antigen-antibody complexes were detected by enhanced chemiluminescence (ECL®, EMD Millipore Headquarters, Billerica, MA, USA).

3.7 Statistics Analysis

All experiments were performed in triplicate. Statistical analyses were performed by paired and unpaired Student's t tests. Data are reported as means ± SEM. $P < 0.05$, $P < 0.01$ and $P < 0.001$ in order to be considered statistically significant.

4 RESULTS

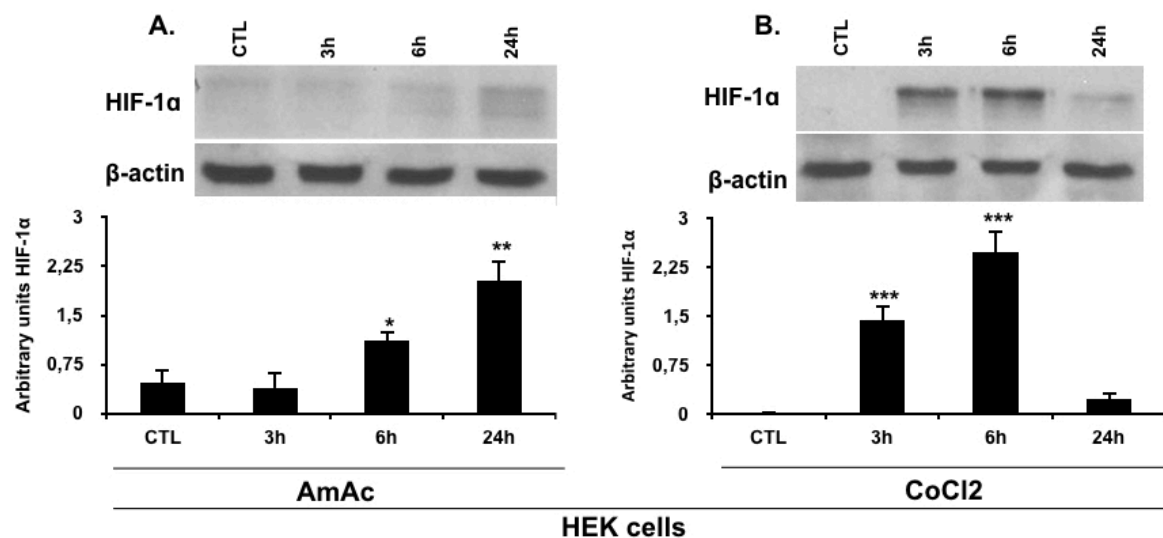### 4.1 Increased HIF-1α activity under hyperammonemic condition

To observe the activity HIF-1α under hyperammonemia *in vitro*, we used C_2C_{12} murine myotubes under ammonium acetate intervention, demonstrating HIF-1α activation in total lysate and cytoplasmic and nuclear protein fractions. Our results show that hyperammonemia led to increased protein expression of HIF-1α in C_2C_{12} cells under 6h and 24h of intervention compared to control (Figure 4A). As a positive control, we used cobalt chloride, a known inhibitor of PHD degradation that increases HIF-1α (Figure 4B). To demonstrate that ammonia mediated perturbations in metabolic regulation of HIF-1α, we also showed that, in Human Embryonic Kidney (HEK) 293 cells, hyperammonemia increased HIF-1α expression (Figure 5A). In HEK 293 cells we can suggest that HIF-1α expression have the same regulation in human cells under elevated levels of ammonia. We then show that hyperammonemia increased nuclear translocation
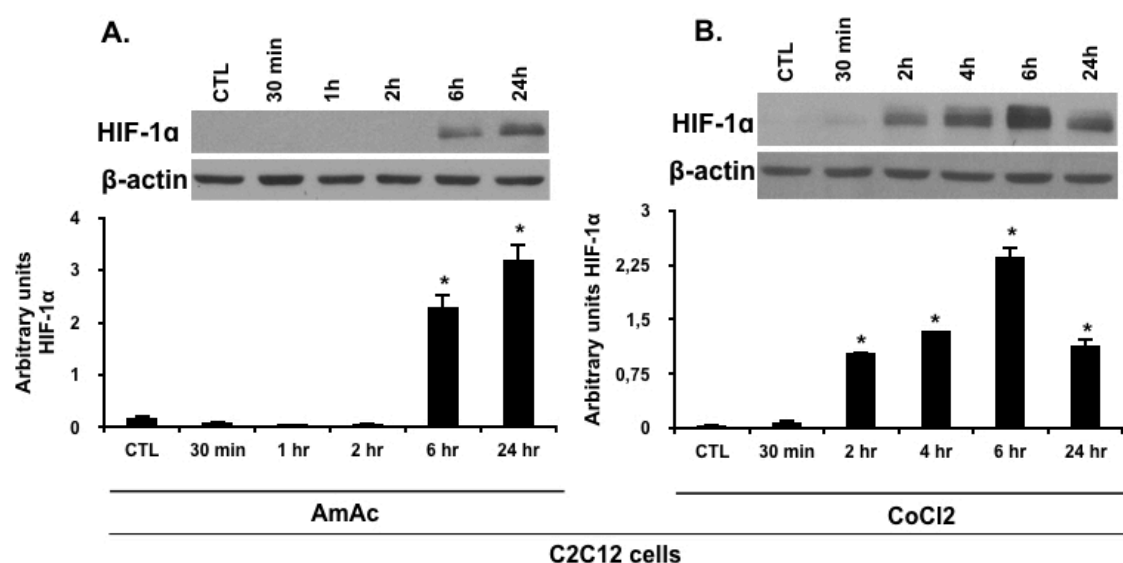
Figure 4. HIF-1α total protein expression in C_2C_{12} myotubes. A. C_2C_{12} myotubes were treated with 10 mM Ammonium Acetate. Western blot analysis for the indicated proteins was then performed. B. C_2C_{12} myotubes were treated with 200 µM Cobalt Chloride. Western blot analysis for the indicated proteins was then performed. A Student's t test was performed and *$P < 0.001$ when compared with the control.

of HIF-1α under normoxic conditions (Figure 6A). To confirm our data we showed the same alteration in the positive control (Figure 6B).

Figure 5. HIF-1α total protein expression in HEK293 cells. A. HEK293 cells were treated with 10 mM Ammonium Acetate. Western blot analysis for the indicated proteins was then performed on these extracts. B. HEK293 cells were treated with 200 μM Cobalt Chloride. Western blot analysis for the indicated proteins was then performed on these extracts. A Student's t test was performed and *P < 0.05; **P < 0.01 and ***P < 0.001 when compared with the control.

Figure 6. HIF-1α cytoplasmic and nuclear protein expression. A. C_2C_{12} myotubes were treated with 10 mM ammonium acetate. Western blot analysis for the indicated proteins was then performed on these extracts. B.C_2C_{12} myotubes were treated with 200 μM cobalt chloride. Western blot analysis for the indicated proteins was then performed on these extracts. A Student's t test was performed. a, P < 0.05; b, P < 0.01 and c, P < 0.001 when compared with the control.

4.2 Effects of hyperammonemia in HIF-1α and target genes mRNA levels

HIF-1α subunit mRNA was significantly higher in C_2C_{12} murine myotubes under ammonium acetate intervention in 6h and 24h than in non-intervention cells, but no difference was observed between CTL and 6h $CoCl_2$. The target genes mRNA levels were significantly higher in 24h AmAc and 6h $CoCl_2$ compared to non-intervention cells (Figure 7).
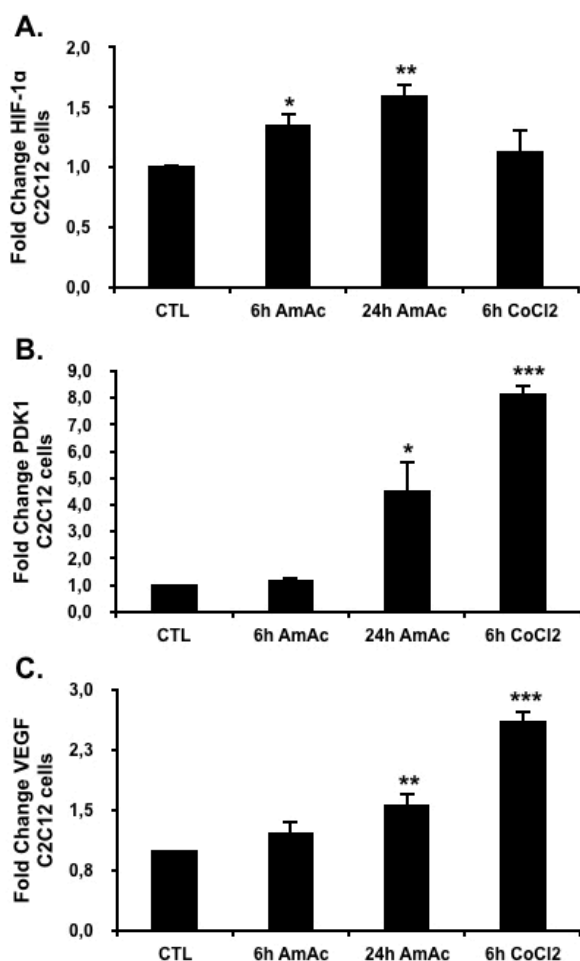
Figure 7. HIF-1α and target genes mRNA levels. C_2C_{12} myotubes were treated with 10 mM ammonium acetate. RT-PCR analysis for indicated gene expression was then performed on these extracts. A. HIF-1α gene expression. B. PDK1 gene expression. C. VEGF gene expression. Student's t test was performed and $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$ when compared with the control.

4.3 HIF-1α activation through NF-kB pathway in hyperammonemia

To demonstrate the interaction between HIF-1α and NF-kB, we used knockdown C_2C_{12} cells in NF-kB and IKKs alpha and beta under ammonium acetate intervention. The result has demonstrated that when the deletion of NF-kB and IKKs occurs the HIF-1α is not expressed, compared to scrambled shRNA cells in different time points of intervention (Figure 8 and 9).
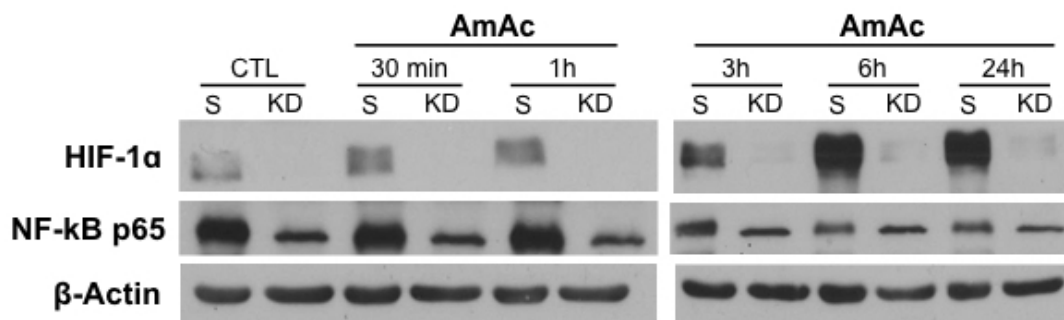
Figure 8. HIF-1α protein expression in scramble and NF-kB p65 knockdown cells. C_2C_{12} myotubes NF-kB p65 knockdown and scrambled cells as control under ammonium acetate intervention in different time points. Western blot analysis for the indicated proteins was then performed on these extracts. S = cells transfected with scrambled ShRNA for NF-kB p65 / KD = knockdown cells.
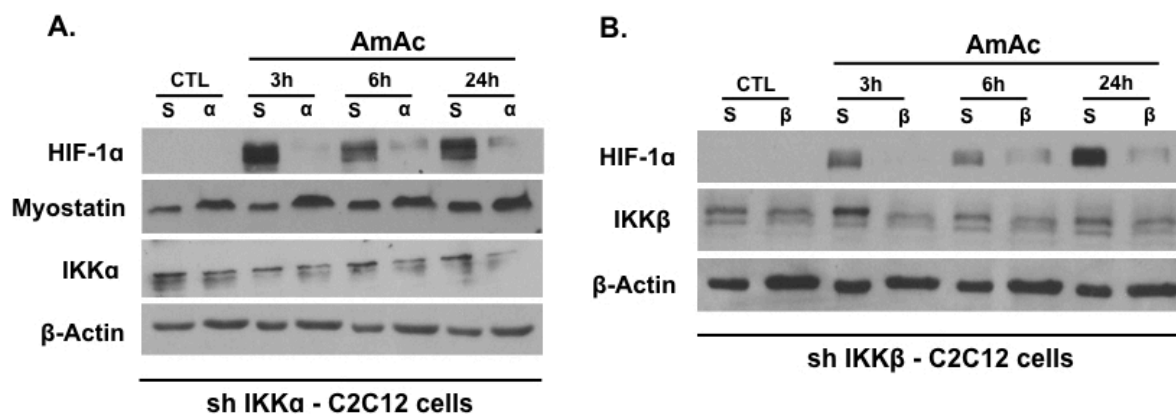
Figure 9. HIF-1α protein expression in scramble, IKKα and IKKβ knockdown cells. C_2C_{12} myotubes IKKα and IKKβ knockdown and scrambled cells as control under ammonium acetate intervention in different time points. Western blot analysis for the indicated proteins was then performed on these extracts. S = cells transfected with scrambled ShRNA for IKKα or IKKβ / α= IKKα knockdown cells / β = IKKβ knockdown cells.

5 DISCUSSION

The present data shows that hyperammonemia is a normoxemic activator of HIF-1α signaling through NF-kB pathway. Hyperammonemia is an abnormality in cirrhosis due to reduced urea synthesis because of hepatocellular dysfunction and portosystemic shunting (LOCKWOOD et al, 1979; SHANGRAW E JAHOOR, 1999). Given that the liver is not able to metabolize ammonia, the skeletal muscle, as an alternate organ, removes the ammonia from circulation and, instead, the substance is metabolized via conversion of glutamine to α-KG on TCA. (FAFF-MICHALAK E ALBRECHT, 1993; CHATAURET et al, 2006; HOLECEK, 2014). According, Holecek (2014) ammonia detoxification to glutamine is activated in skeletal muscle, brain, and lungs. It is well established that in hypoxia condition HIF-1α regulates energy homeostasis and cellular adaptation inducing specific genes expression as GLUT1, VEGF and PDK1 (CUMMINS E TAYLOR, 2005; HOU et al, 2014). Recently, it has been reported high expression of HIF-1α in cortical neurons of hyperammonemic animals (pre-hepatic portal hypertension) (TALLIS et al, 2014). Our results show that elevated concentration of ammonia in skeletal muscle cells (murine C_2C_{12} cells line) activates accumulation of HIF-1α and induces target genes as VEGF and PDK1 as previously demonstrated. We postulated that the lack of α-KG regulates HIF-1α accumulation under hyperammonemic condition (SUTENDRA et al, 2013; DROMPARIS et al, 2014). However, mechanistically, the effect of hyperammonemia on HIF-1α signaling is incompletely understood.

Hyperammonemia stimulates NF-kB a transcriptional factor that is typically activated by cytokines, others pro-inflammatory factors and hypoxia (CHEN et al, 2003; CUMMINS E TAYLOR, 2005; CUMMINS et al, 2006; RIUS et al, 2008; QIU et al, 2013). NF-kB activation is controlled by IkappaB kinases alpha or beta (IKKs) needed for phosphorylation-induced degradation of IkappaB inhibitors in response to infection and inflammation. It has been observed that, in response to hypoxia due to PHD activity inhibition, a negatively regulator of IKK-β, an increase of IKK-β activity can be noticed. Therefore, IKK-β is essential for HIF-1α accumulation, linking the hypoxia response to innate immunity and inflammation (CUMMINS et al, 2006, RIUS et al, 2008).

Previous findings demonstrated a connection between HIF-1α and innate immunity and inflammation (CRAMER et al, 2003). Cramer et al (2003) have examined the inflammatory response in mice with conditional knockouts of HIF-1α indicating that HIF-1α is essential for regulation of glycolytic capacity in myeloid cells and for their infiltration and activation. These data demonstrate that HIF-1α regulates directly the function and survival in the inflammatory microenvironment. Our data have shown that in NF-kB, IKK-α and IKK-β deficiency there is a defective activation of HIF-1α suggesting that occur a *cross-talking* between the IKKs alpha and beta/NF-kB/HIF-1α under hyperammonemic *in vitro* condition.

Recents studies have shown that myostatin is a critical regulator of protein synthesis and muscle loss in cirrhosis (DASARATHY et al, 2004; DASARATHY et al, 2011; QIU et al, 2012). It has been established that HIF-1α regulates skeletal myogenesis (MAJMUNDAR et al, 2015). Moreover, HIF regulares muscle glycolytic metabolism through NF-kB activation (REMELS et al, 2015). According, Qiu et al (2013) hyperammonemia causes transcriptional up-regulation of myostatin in a p65 NF-κB–dependent manner. We speculate that hyperammonemia activates NF-κB signaling stimulating sarcopenia through activation of the hypoxic regulator HIF-1α. Despite this, our data has shown in IKK-α knockdown C_2C_{12} cells an effective activation of myostatin indicating that not occurs a link between myostatin and the kinase IKK-α.

In conclusion, the observations of this study suggest that hyperammonemia is a normoxemic activator of HIF-1α that may regulates myostatin expression through NF-kB pathway resulting in sarcopenia.

6 REFERENCES

ADEVA, M.M. et al. Ammonium metabolism in humans. **Metabolism,** v. 61, n. 11, p. 1495-1511, 2012.

ALFADHEL, M. et al. Guidelines for acute management of hyperammonemia in the Middle East region. **Ther Clin Risk Manag,** v. 31, n. 12, p. 479-487, 2016.

BENYON, R.C.; ARTHUR, M.J. Extracellular matrix degradation and the role of hepatic stellate cells. **Semin Liver Dis,** v. 21, n. 3, p. 373-384, 2001.

BESSMAN, S.P.; BESSMAN, A.N. The cerebral and peripheral uptake of ammonia in liver disease with an hypothesis for the mechanism of hepatic coma. **J Clin Invest,** v. 34, n. 4, p. 622-628, 1955.

BIDDLESTONE, J.; BANDARRA, D.; ROCHA, S. The role of hypoxia in inflammatory disease (review). **Int J Mol Med,** v. 35, n. 4, p. 859-869, 2015.

BRAISSANT, O.; MCLIN, V.A.; CUDALBU, C. Ammonia toxicity to the brain. **J Inherit Metab Dis,** v. 36, n. 4, p. 595-612, 2013.

BRUICK, R.K.; MCKNIGHT, S.L. A conserved family of prolyl-4-hydroxylases that modify HIF. **Science,** v. 294, n. 5545, p. 1337-1340, 2001.

BUTTERWORTH, R.F. Effects of hyperammonaemia on brain function. **J Inherit Metab Dis,** v. 21, n. Suppl 1, p. 6-20, 1998.

CHAMBERLAIN, R. S. Essential Functional Hepatic and Biliary Anatomy for the Surgeon. ABDELDAYEM, H. (Ed.). In: **Hepatic Surgery,** Chapter 2, 2013. DOI: 10.5772/53849. Disponível em: < http://www.intechopen.com/books/hepatic-surgery/

essential-functional-hepatic-and-biliary-anatomy-for-the-surgeon>. Acesso em: 30.jun. 2016.

CHATAURET, N. et al. Direct molecular and spectroscopic evidence for increased ammonia removal capacity of skeletal muscle in acute liver failure. **J Hepatol,** v. 44, n. 6, p. 1083-1088, 2006.

CHEN, L.W. et al. The two faces of IKK and NF-kappaB inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. **Nat Med,** v. 9, n. 5, p. 575-581, 2003.

CLEMMESEN, J.O.; KONDRUP, J.; OTT, P. Splanchnic and leg exchange of amino acids and ammonia in acute liver failure. **Gastroenterology,** v. 118, n. 6, p. 1131-1139, 2000.

COLOMBO, J.P. et al. Plasma ammonia concentrations in newborns and children. **Clin Chim Acta,** v. 138, n. 3, p. 283-291, 1984.

COOPER, A.J.; PLUM, F. Biochemistry and physiology of brain ammonia. **Physiol Rev,** v. 67, n. 2, p. 440-519, 1987.

COSQUÉRIC, G. et al. Sarcopenia is predictive of nosocomial infection in care of the elderly. **Br J Nutr,** v. 96, n. 5, p. 895-901, 2006.

CRAMER, T. et al. HIF-1alpha is essential for myeloid cell-mediated inflammation. **Cell,** v. 112, n. 5, p. 645-657, 2003.

CUMMINS, E.P.; TAYLOR, C.T. Hypoxia-responsive transcription factors. **Pflugers Arch,** v. 450, n. 6, p. 363-371, 2005.

CUMMINS, E.P. et al. Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. **Proc Natl Acad Sci U S A,** v. 103, n. 48, p. 18154-18159, 2006.

DASARATHY, S. et al. Skeletal muscle atrophy is associated with an increased expression of myostatin and impaired satellite cell function in the portacaval anastamosis rat. **Am J Physiol Gastrointest Liver Physiol,** v. 287, n. 6, p. G1124-G1130, 2004.

DASARATHY, S. et al. Sarcopenia associated with portosystemic shunting is reversed by follistatin. **J Hepatol,** v. 54, n. 5, p. 915-921, 2011.

DASARATHY, S. Consilience in sarcopenia of cirrhosis. **J Cachexia Sarcopenia Muscle,** v. 3, n. 4, p. 225-237, 2012.

D'IGNAZIO, L.; BANDARRA, D.; ROCHA, S. NF-κB and HIF crosstalk in immune responses. **FEBS J,** v. 283, n. 3, 413-424, 2016.

DROMPARIS, P. et al. Pioglitazone inhibits HIF-1α-dependent angiogenesis in rats by paracrine and direct effects on endothelial cells. **J Mol Med (Berl),** v. 92, n. 5, p. 497-507, 2014.

DURAND, F. et al. Prognostic value of muscle atrophy in cirrhosis using psoas muscle thickness on computed tomography. **J Hepatol,** v. 60, n. 6, p. 1151-1157, 2014.

FAFF-MICHALAK, L.; ALBRECHT, J. Hyperammonemia and hepatic encephalopathy stimulate rat cerebral synaptic mitochondrial glutamate dehydrogenase activity specifically in the direction of glutamate oxidation. **Brain Res,** v. 618, n. 2, p. 299-302, 1993.

FALLOWFIELD, J.A.; IREDALE, J.P. Reversal of liver fibrosis and cirrhosis--an emerging reality. **Scott Med J,** v. 49, n. 1, p. 3-6, 2004.

GARCÍA, P.S. et al. Brief-reports: elevated myostatin levels in patients with liver disease: a potential contributor to skeletal muscle wasting. **Anesth Analg,** v. 111, n. 3, p. 707-709, 2010.

GHIASSI-NEJAD, Z.; FRIEDMAN, S.L. Advances in antifibrotic therapy. **Expert Rev Gastroenterol Hepatol,** v. 2, n. 6, p. 803-816, 2008.

HÄBERLE, J. Clinical practice: the management of hyperammonemia. **Eur J Pediatr,** v. 170, n. 1, p. 21-34, 2011.

HANAI, T. et al. Sarcopenia impairs prognosis of patients with liver cirrhosis. **Nutrition,** v. 31, n. 1, p.193-199, 2015.

HOLECEK, M.; SKOPEC, F.; SPRONGL, L. Protein metabolism in cirrhotic rats: effect of dietary restriction. **Ann Nutr Metab,** v. 39, n. 6, p. 346-354, 1995.

HOLECEK, M. Branched-chain amino acids and ammonia metabolism in liver disease: therapeutic implications. **Nutrition,** v. 29, n. 10, p. 1186-1191, 2013.

HOLECEK, M. Evidence of a vicious cycle in glutamine synthesis and breakdown in pathogenesis of hepatic encephalopathy-therapeutic perspectives. **Metab Brain Dis,** v. 29, n. 1, p. 9-17, 2014.

HOU, P. et al. Intermediary metabolite precursor dimethyl-2-ketoglutarate stabilizes hypoxia-inducible factor-1α by inhibiting prolyl-4-hydroxylase PHD2. **PLoS One,** v. 9, n. 11, p. e113865, 2014.

HU, C.J. et al. Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. **Mol Cell Biol,** v. 23, n. 24, p. 9361-9374, 2003.

HUANG, L.E. et al. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. **J Biol Chem,** v. 271, n. 50, p. 32253-32259, 1996.

KAELIN, W.G. JR. Cancer and altered metabolism: potential importance of hypoxia-inducible factor and 2-oxoglutarate-dependent dioxygenases. **Cold Spring Harb Symp Quant Biol,** v. 76, p. 335-345, 2011.

KALAFATELI, M. et al. Impact of muscle wasting on survival in patients with liver cirrhosis. **World J Gastroenterol,** v. 21, n. 24, p. 7357-7361, 2015.

KENNETH, N.S.; ROCHA, S. Regulation of gene expression by hypoxia. **Biochem J,** v. 414, n.1, p. 19-29, 2008.

KIETZMANN, T.; MENNERICH, D.; DIMOVA, E.Y. Hypoxia-Inducible Factors (HIFs) and Phosphorylation: Impact on Stability, Localization, and Transactivity. **Front Cell Dev Biol,** v. 4, n. 11, 2016.

KIM, S. et al. Succinate dehydrogenase expression in breast cancer. **Springerplus,** v. 2, n. 1, p. 299, 2013.

KIM, H.Y.; JANG, J.W. Sarcopenia in the prognosis of cirrhosis: Going beyond the MELD score. **World J Gastroenterol,** v. 21, n. 25, p. 7637-7647, 2015.

KNITTEL, T. et al. Rat liver myofibroblasts and hepatic stellate cells: different cell populations of the fibroblast lineage with fibrogenic potential. **Gastroenterology,** v. 117, n. 5, p. 1205-1221, 1999.

LEE, S. J.; MCPHERRON, A.C. Regulation of myostatin activity and muscle growth. **Proc Natl Acad Sci U S A,** v. 98, n. 16, p. 9306-9311, 2001.

LOBODA, A.; JOZKOWICZ, A.; DULAK, J. HIF-1 versus HIF-2--is one more important than the other? **Vascul Pharmacol,** v. 56, n. 5-6, p. 245-251, 2012.

LOCKWOOD, A. H. et al. The dynamics of ammonia metabolism in man. Effects of liver disease and hyperammonemia. **J Clin Invest,** v. 63, n. 3, p. 449-460, 1979.

MAJMUNDAR, A. J. et al. HIF modulation of Wnt signaling regulates skeletal myogenesis in vivo. **Development,** v. 142, n. 14, p. 2405-2412, 2015.

MARCELL ,T. J. Sarcopenia: causes, consequences, and preventions. **J Gerontol A Biol Sci Med Sci,** v. 58, n. 10, p. M911-916, 2003.

MASSON, N.; RATCLIFFE, P. J. Hypoxia signaling pathways in cancer metabolism: the importance of co-selecting interconnected physiological pathways. **Cancer Metab,** v. 2, n. 1, p. 3, 2014.

MATOORI, S.; LEROUX, J. C. Recent advances in the treatment of hyperammonemia. **Adv Drug Deliv Rev,** v. 1, n. 90, p. 55-68, 2015.

MCCROSKERY, S. et al. Myostatin negatively regulates satellite cell activation and self-renewal. **J Cell Biol,** v. 162, n. 6, p. 1135-1147, 2003.

MITCH, W. E. et al. Evaluation of signals activating ubiquitin-proteasome proteolysis in a model of muscle wasting. **Am J Physiol,** v. 276, p. C1132-C1138, 1999.

MORMONE, E.; GEORGE, J.; NIETO, N. Molecular pathogenesis of hepatic fibrosis and current therapeutic approaches. **Chem Biol Interact,** v. 193, n. 3, p. 225-231, 2011.

MOSONI, L. et al. Lower recovery of muscle protein lost during starvation in old rats despite a stimulation of protein synthesis. **Am J Physiol,** v. 277, n. 4 Pt 1, p. E608-E616, 1999.

O'BRIEN, A.; WILLIAMS, R. Nutrition in end-stage liver disease: principles and practice. **Gastroenterology,** v. 134, n. 6, p. 1729-1740, 2008.

OLDE DAMINK, S.W. et al. Interorgan ammonia metabolism in liver failure. **Neurochem Int,** v. 41, n. 2-3, p. 177-188, 2002.

PERIYALWAR, P.; DASARATHY, S. Malnutrition in cirrhosis: contribution and consequences of sarcopenia on metabolic and clinical responses. **Clin Liver Dis,** v. 16, n. 1, p. 95-131, 2012.

POLLARD, P. J. et al. Accumulation of Krebs cycle intermediates and over-expression of HIF1alpha in tumours which result from germline FH and SDH mutations. **Hum Mol Genet,** v. 14, n. 15, p. 2231-2239, 2005.

QIU, J. et al. Hyperammonemia-mediated autophagy in skeletal muscle contributes to sarcopenia of cirrhosis. **Am J Physiol Endocrinol Metab,** v. 303, n. 8, p. E983-E993, 2012.

QIU, J. et al. Hyperammonemia in cirrhosis induces transcriptional regulation of myostatin by an NF-κB-mediated mechanism. **Proc Natl Acad Sci U S A,** v. 110, n. 45, p. 18162-18167, 2013.

REGEV, A. et al. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. **Am J Gastroenterol,** v. 97, n. 10, p. 2614-2618, 2002.

REMELS, A.H. et al. TNF-α-induced NF-κB activation stimulates skeletal muscle glycolytic metabolism through activation of HIF-1α. **Endocrinology,** v. 156, n. 5, p. 1770-1781, 2015.

RIUS, J. et al. NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. **Nature,** v. 453, n. 7196, p. 807-811, 2008.

ROBSON, S. C. Liver Endothelial Cells: Hemostasis, Thrombosis and Hepatic Vascular Diseases. DELEVE, L. D. e GARCIA-TSAO, G. (Ed). In: **Vacular Liver Disease: Mechanisms and Management,** New York: Springer, p. 3-23, 2011.

RUDMAN, D. et al. Maximal rates of excretion and synthesis of urea in normal and cirrhotic subjects. **J Clin Invest,** v. 52, n. 9, p. 2241-2249, 1973.

SCAGLIONE, S. et al. The Epidemiology of Cirrhosis in the United States: A Population-based Study. **J Clin Gastroenterol,** v. 49, n. 8, p. 690-696, 2015.

SCHUPPAN, D. et al. Matrix as a modulator of hepatic fibrogenesis. **Semin Liver Dis,** v. 21, n. 3, p. 351-372, 2001.

SCHUPPAN, D.; AFDHAL, N.H. Liver cirrhosis. **Lancet,** v. 371, n. 9615, p. 838-851, 2008.

SELAK, M. A. et al. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. **Cancer Cell,** v. 7, n. 1, p. 77-85, 2005.

SHANGRAW, R. E.; JAHOOR, F. Effect of liver disease and transplantation on urea synthesis in humans: relationship to acid-base status. **Am J Physiol,** v. 276, n. 5 Pt 1, p. G1145-G1152, 1999.

SORIA, L. R. et al. Lipopolysaccharide impairs hepatocyte ureagenesis from ammonia: involvement of mitochondrial aquaporin-8. **FEBS Lett,** v. 588, n. 9, p. 1686-1691, 2014.

STROKA, D.M. et al. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. **FASEB J,** v. 15, n. 13, p. 2445-2453, 2001.

SUTENDRA, G. et al. Mitochondrial activation by inhibition of PDKII suppresses HIF1a signaling and angiogenesis in cancer. **Oncogene,** v. 32, n. 13, p. 1638-1650, 2013.

TALLIS, S. et al. Changes in CNS cells in hyperammonemic portal hypertensive rats. **J Neurochem,** v. 128, n. 3, p. 431-444, 2014.

TAPPER, E. B.; JIANG, Z. G.; PATWARDHAN, V. R. Refining the ammonia hypothesis: a physiology-driven approach to the treatment of hepatic encephalopathy. **Mayo Clin Proc,** v. 90, n. 5, p. 646-658, 2015.

WANG, G. L. et al. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. **Proc Natl Acad Sci U S A,** v. 92, n. 12, p. 5510-5514, 1995.

WANG, V. et al. Differential gene up-regulation by hypoxia-inducible factor-1alpha and hypoxia-inducible factor-2alpha in HEK293T cells. **Cancer Res,** v. 65, n. 8, p. 3299-3306, 2005.

WANG, Q. et al. Ammonia-induced energy disorders interfere with bilirubin metabolism in hepatocytes. **Arch Biochem Biophys,** v. 555-556, p. 16-22, 2014.

WAGATSUMA, A.; KOTAKE, N.; YAMADA, S. Spatial and temporal expression of hypoxia-inducible factor-1α during myogenesis in vivo and in vitro. **Mol Cell Biochem,** v. 347, n. 1-2, p. 145-155, 2011.

WANLESS, I. R.; NAKASHIMA, E.; SHERMAN, M. Regression of human cirrhosis. Morphologic features and the genesis of incomplete septal cirrhosis. **Arch Pathol Lab Med,** v. 124, n. 11, p. 1599-1607, 2000.

WIEGAND, J.; BERG, T. The etiology, diagnosis and prevention of liver cirrhosis: part 1 of a series on liver cirrhosis. **Dtsch Arztebl Int,** v. 110, n. 6, p. 85-91, 2013.

WIGERUP, C.; PÅHLMAN, S.; BEXELL, D. Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. **Pharmacol Ther,** v. S0163-7258, n. 16, p. 30055-30059, 2016.

XIAO, M. et al. Inhibition of α-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. **Genes Dev,** v. 26, n. 12, p. 1326-1338, 2012.

ZAGANAS, I. et al. The effect of pH and ADP on ammonia affinity for human glutamate dehydrogenases. **Metab Brain Dis,** v. 28, n. 2, p. 127-131, 2013.

ZAGÓRSKA, A.; DULAK, J. HIF-1: the knowns and unknowns of hypoxia sensing. **Acta Biochim Pol,** v. 51, n. 3, p. 563-585, 2004.

ZIEVE, L. Pathogenesis of hepatic encephalopathy. **Metab Brain Dis,** v. 2, n. 3, p. 147-165, 1987.

7 ANNEX - MANUSCRIPT

Hyperammonemia activates HIF-1α via a NF-kB pathway: a possible mechanism for sarcopenia in cirrhosis

Rafaella Nascimento e Silva[1,2], Gangarao Davuluri[1], Angela Merice Leal[3], Srinivasan Dasarathy[1#]

[1] Department of Pathobiology, Cleveland Clinic, Cleveland, Ohio, USA.

[2] Current address: Department of Physiology, University of Sao Carlos, Sao Carlos, SP, Brazil.

[3] Department of Medicine, University of Sao Carlos, Sao Carlos, SP, Brazil.

Running title: Hyperammonemia activated HIF1a in cirrhosis

Characters excl. spaces: 8939   **Abstract:** 163words

Introduction/Results/Discussion: 1449 words

Materials and methods: 651 words

KEYWORDS

Hyperammonemia / Hypoxia inducible factor 1α / Nuclear factor-kappaB / Myostatin / Skeletal muscle

[#]Address correspondence to:

Srinivasan Dasarathy

Department of Pathobiology, Cleveland Clinic Foundation, Lerner Research Institute, 9500 Euclid Avenue / A30, Cleveland, 44195 Ohio, United States

Tel: +1 216 444-2980

Fax: +1 216 445-3889

E-mail: dasaras@ccf.org

Abstract

Hyperammonemia impairs skeletal muscle protein synthesis and induces autophagy by upregulating myostatin via an nuclear factor-kappaB (NF-kB). Skeletal muscle ammonia metabolism occurs via the synthesis of glutamate and then glutamine from critical TCA intermediate α-KG a metabolic intermediate that regulates increase expression of hypoxia inducible factor 1α (HIF-1α). Furthermore, there is a interaction between HIF-1α and NF-kB. To examine the effects of hyperammonemia under HIF-1α signaling through NF-kB pathway we have generated a stable knockdown cells line in NF-kB, IKKα and IKKβ to see the myostatin and HIF-1α activities. The protein expression of HIF-1α was significantly higher in C_2C_{12} murine myotubes under ammonium acetate intervention. Once the deletion of NF-kB occurs the HIF-1α is not expressed suggesting a *cross-talking* between them. We hypothesize that hyperammonemia is a normoxemic activator of nuclear translocation of HIF-1α through NF-kB pathway that results in sarcopenia via up-regulation of myostatin expression.

INTRODUCTION

Sarcopenia or loss of skeletal muscle mass is the most frequent complication in cirrhosis and adversely affects survival, quality of life and post-transplant outcomes (13, 10, 20, 30). One of potential mediators of sarcopenia in cirrhosis is hyperammonemia, a consistent abnormality in liver disease, because of hepatocellular dysfunction and portosystemic shunting that results in impaired conversion of ammonia to urea (17, 25). Qiu et al, 2012 (21) has previously reported that ammonia is a mediator of the *liver-muscle* axis. Hyperammonemia impairs skeletal muscle protein synthesis and induces autophagy, but the molecular mechanisms of sarcopenia are not known. Qiu et al, 2013 (22) has also reported that hyperammonemia transcriptionally upregulates myostatin via a nuclear factor-kappaB (NF-kB) mediated mechanism. The mechanism by which ammonia mediated chemical-signaling transduction occurs are not known and an integrated metabolic-molecular approach is required for these studies.

Ammonia is a toxic chemical that is generated primarily during amino acid catabolism and by gut bacterial metabolism. Purine metabolism and gastrointestinal bleeding also generate ammonia (13, 29). In physiological states, ammonia is converted to urea in the liver. Severe liver diseases including acute liver failure and cirrhosis result in impaired conversion of ammonia to urea with increased circulating and tissue concentrations of ammonia (19). In patients with cirrhosis, the skeletal muscle becomes an alternate organ for ammonia disposal. Since ureagenesis occurs only in the hepatocytes, skeletal muscle ammonia metabolism occurs via the synthesis of glutamate and then glutamine from critical tricarboxylic acid cycle (TCA) intermediate, α-ketoglutarate (α-KG) (2, 12, 14). The first step of ammonia disposal in the muscle is the mitochondrial glutamate synthesis catalyzed by glutamate dehydrogenase. Ammonia entry into mitochondria has been reported to occur via aquaporin 8 in the liver but its transport into skeletal muscle mitochondria is not known (26). Conversion of α-KG to glutamate results in increased consumption of α-KG with perturbations in TCA cycle and mitochondrial function (32, 33). Regulation of signaling pathways

by α-KG, a metabolic intermediate, is mediated by increased expression of transcription factor, hypoxia inducible factor 1α (HIF-1α) (28, 11).

Transcriptional regulation by HIF is mediated by heterodimerization of HIF-1α with a nuclear factor, HIF-1β. HIF-1α subunit is located in cells cytoplasm and its stability is controlled by oxygen availability. Canonical regulation of HIF-1α is mediated by cellular oxygen content. In physiological states under normoxia and in presence of iron and α-KG, HIF-1α is hydroxylated in proline residues Pro^{402} and Pro^{564} in the oxygen degradation domain (ODD) of HIF1α by a group of dioxygenases, prolyl hydroxylases 1, 2 and 3 (1, 15, 28, 31). Hydroxylation of the ODD requires α-KG that is a recognized metabolic-signaling interface. Even though hypoxia is recognized to be the major regulator of HIF-1α expression and transcriptional effects, non-canonical activation of HIF-1α have been reported and include UV radiation, cytokines, and reactive oxygen species (16, 27). Once the proline residues on HIF-1α are hydroxylated, the von Hippel-Lindau tumor supressor (pVHL) E3 ligase complex recognizes HIF-1α leading to ubiquitylation and degradation by the proteasome. However, under hypoxic condition, HIF-1α is accumulated into the cytoplasmic because the reduced activity of PHD, inhibiting HIF-1α hydroxylation and degradation that is translocated to the nucleus increasing the production of target genes (15, 27). Since α-KG is a critical metabolic requirement for proline hydroxylation by PHDs, a deficiency of α-KG is also likely to result in impaired proline hydroxylation and consequent stabilization of HIF-1α.

Our findings indicate that hyperammonemia is a normoxemic activator of nuclear translocation of HIF-1α through NF-kB pathway that results in sarcopenia via up-regulation of myostatin expression.

RESULTS

HIF-1α activity under hyperammonemic condition

To observe the activity HIF-1α under hyperammonemia *in vitro*, we used C_2C_{12} murine myotubes under ammonium acetate intervention demonstrating HIF-1α activation in total lysate and cytoplasmic and nuclear protein fractions. Our results have showed that hyperammonemia resulted in increased protein expression of HIF-1α (Figure 1A). As a positive control, we used cobalt chloride, a known inhibitor of PHD degradation that increases HIF-1α (Figure 1B). To demonstrate that ammonia mediated perturbations in metabolic regulation of HIF-1α, we also showed that in Human Embryonic Kidney 293 cells (HEK 293 cells) hyperammonemia increased expression of HIF-1α (Figure 2A). We then showed that hyperammonemia increased nuclear translocation of HIF-1α under normoxic conditions (Figure 3A). To confirm our data we showed the same alteration in the positive control (Figure 3B).

Effects of hyperammonemia in HIF-1α and target genes mRNA levels

HIF-1α subunit mRNA was significantly higher inC_2C_{12} murine myotubes under ammonium acetate intervention in 6 and 24 hours than in non-intervention cells, but no difference was observed between CTL and 6 h $CoCl_2$. The target genes mRNA levels were significantly higher in 24 h AmAc and 6 h $CoCl_2$ compared to non-intervention cells (Figure 4).

HIF-1α activation through NF-kB pathway in hyperammonemia

To demonstrated the interaction between HIF-1α and NF-kB, used knockdown C_2C_{12} cells in NF-kB and IKKs alpha and beta under ammonium acetate intervention. We could observe that when the deletion of NF-kB and IKKs occurs the HIF-1α is not expressed compared to scrambled shRNA cells in different time points of intervention (Figure 5 and 6).

DISCUSSION

The present data show that hyperammonemia is a normoxemic activator of HIF-1α signaling through NF-kB pathway. Hyperammonemia is an abnormality in cirrhosis due to reduced urea synthesis because of hepatocellular dysfunction and portosystemic shunting (17, 25). However, the skeletal muscle as an alternate organ removes the ammonia from circulation and it has been metabolized via conversion of glutamine to α-KG on TCA (3, 12, 14). It is well established that in hypoxia condition HIF-1α regulates energy homeostasis and cellular adaptation inducing specific gene expression as GLUT1, VEGF and PDK1 (6, 15). Our results show that hyperammonemia *in vitro* activates accumulation of HIF-1α and induces target genes as VEGF and PDK1 as previously demonstrated. We postulate that α-KG regulates HIF-1α accumulation under hyperammonemic condition (11, 28). However, mechanistically, the effect of hyperammonemia on HIF-1α signaling is incompletely understood.

Hyperammonemia stimulates NF-kB a transcriptional factor that is typically activated by cytokines, others pro-inflammatory factors and hypoxia (4, 6, 7, 22, 24). NF-kB activation is controlled by IkappaB kinases (IKK) needed for phosphorylation-induced degradation of IkappaB inhibitors in response to infection and inflammation. It has been observed that in response to hypoxia through inhibiting PHD activity that is a negatively regulator of IKK-β, increases the activity of KK-β. Therefore, IKK-β is essential for HIF-1α accumulation linking the hypoxia response to innate immunity and inflammation (7, 24). Previous findings demonstrated a connection between HIF-1α and innate immunity and inflammation (5). Our data demonstrate that in NF-kB, IKK-α and IKK-β deficiency there is a defective activation of HIF-1α suggesting that there is a *cross-talking* between the IKKs alpha and beta/NF-kB/HIF-1α under hyperammonemic *in vitro* condition.

We also have been studied the link between HIF-1a and myostatin. Recents studies have shown that myostatin is a critical regulator of protein synthesis and muscle loss in cirrhosis (8, 9, 21). It has been established that HIF-1α regulates skeletal myogenesis (18). Moreover, the hypoxic transcriptional factor regulares muscle glycolytic metabolism through NF-kB activation (23). Qiu et al, 2013 (22)

demonstrated that hyperammonemia causes transcriptional up-regulation of myostatin in a p65 NF-κB–dependent manner. We speculate that hyperammonemia activates NF-κB signaling stimulating sarcopenia through activation of the hypoxic regulator HIF-1α. Despite this, our data showed that in IKK-α knockdown C2C12 cells occurs a effective activation of myostatin indicating that there isn't a link between myostatin and the kinase IKK-α.

In conclusion, the observations of this study suggest that hyperammonemia is a normoxemic activator of HIF-1α that may regulates myostatin expression through NF-kB pathway that results in sarcopenia.

MATERIALS AND METHODS

Cell Culture

Murine C_2C_{12} cell lines were obtained from ATCC and grown to confluence in proliferation medium consisting of Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) FBS. After 80-90% of confluence, the medium was changed to differentiation medium [DMEM with 2% (vol/vol) horse serum]. Following 48 h of differentiation, cells were exposed to freshly prepared 10 mM ammonium acetate in different time points (0, 30 min, 1 h, 3 h, 6 h and 24 h). For positive control the cells were exposed to freshly prepared 200 μM cobalt chloride for 3 h, 6 h and 24 h. Medium and cellular pH in response to ammonium acetate was performed as described (21).

Stable Knockdown of p65 NF-kBin C_2C_{12} Cells.

Stable cell lines expressing shRNA to the p65 subunit of NF-kB or scrambled shRNA were generated by lentivirus transduction using protocols approved by the Institutional Biosafety Committee at the Cleveland Clinic. In brief, lentivirus was produced in HEK-293T cells transfected with a MISSION p65 shRNA construct (NM_009045.4-2313s21c1; Sigma), and the lentivirus packaging cons-

tructs RSV-Rev, MDGLG-RRE, and HCMV-G by using Polyfect (Qiagen) according to manufacturer's instructions. Medium containing viral particles was then collected 48 h post-transfection, passed through 0.4-µm filter, and used to infect C_2C_{12} cells in the presence of polybrene (20 µg/mL). Stable cell lines were selected with puromycin (2 µg/mL) for 10 d. All studies were performed in trans-fectedC_2C_{12} cells that were grown to confluence and differentiated for 48 h to myotubes before being treated with ammonium acetate or cobalt chloride or vehicle alone.

IKK-α, IKK-β and scrambled shRNA.

Murine C_2C_{12} cell lines were plated in 2 ml of DMEM medium supplemented with 10% FBS without antibiotics and incubated overnight at 37 °C with 5% CO_2. After 50% of confluence the DMEM medium was changed to 1 ml of freshly 10% DMEM medium. For each construct 4 µl of TransfeX™ (ATCC®) were mixed with 100 µl OPTI-MEM serum-free medium and 1 µg of shRNA IKK-α or IKK-β or scrambled in different tubes. This mixture was incubated for 20 min at room temperature and then added to the cells. The medium was changed 24h after transfection.

RNA isolation and RT-PCR analysis

Total RNA was extracted by using the TRIzol reagent following the manufacturer's protocol (Molecular Research Center). cDNA from the RNA was generated by using the BD Clontech kit (Clontech Inc.) containing avian leukemia reverse transcriptase and quantified by spectrophotometry using described methods (8). Real-time quantitative PCR was performed on a Stratagene Mx 3000P (Stratagene Inc.) by using the Sybr Green protocol, and relative changes in mRNA expression was quantified as described (8). The primer sequences (forward; reverse) for the mouse genes used in the study are as follows in Table 1.

Western Blots

Protein was extracted by using RIPA buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors. The proteins were quantified, resolved by SDS/PAGE, and immunoblotted as described (8). Cytoplasmic and Nuclear protein was extracted by using the low salt buffer [1% Nonidet P-40, 150 mM NaCl2, 50 mM tris-HCL (pH 7.5), 1.5 mM MgCl2] containing protease and phosphatase inhibitors. Primary antibodies HIF-1α (D2U3T), TBT antibody, GAPDH (14C10) obtained from Cell Signaling Technology, Actin (I-19) (Santa Cruz Biotechnology) and GDF-8-myostatin (Abcam), were diluted to 1:2000 except Anti-IKKα (Abcam), Anti-IKKβ (10AG2) (EMD Millipore) and NF-kB p65 (C-20)X (Santa Cruz Biotechnology)antibodies (1:5000 dilution). Secondary antibodies were used at a dilution of 1:10000 and obtained from Santa Cruz Biotechnology.

Statistics Analysis

All experiments were performed in triplicate. Statistical analyses were performed by paired and unpaired Student's t tests. Data are reported as means ± SEM. P < 0.05 was considered statistically significant.

REFERENCES

1. **Bruick RK, McKnight SL.**2001. A conserved family of prolyl-4-hydroxylases that modify HIF. Science **294(5545):**1337-1340.
2. **Butterworth RF.** 1998. Effects of hyperammonaemia on brain function. J Inherit Metab Dis **21 Suppl 1**:6-20.
3. **Chatauret N, Desjardins P, Zwingmann C, Rose C, Rao KV, Butterworth RF.** 2006. Direct molecular and spectroscopic evidence for increased ammonia removal capacity of skeletal muscle in acute liver failure. J Hepatol **44(6):**1083-1088.

4. **Chen LW, Egan L, Li ZW, Greten FR, Kagnoff MF, Karin M.** 2003. The two faces of IKK and NF-kappaB inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. Nat Med **9(5):**575-581.

5. **Cramer T, Yamanishi Y, Clausen BE, Förster I, Pawlinski R, Mackman N, Haase VH, Jaenisch R, Corr M, Nizet V, Firestein GS, Gerber HP, Ferrara N, Johnson RS.** 2003. HIF-1alpha is essential for myeloid cell-mediated inflammation. Cell **112(5):**645-657.

6. **Cummins EP, Taylor CT.** 2005. Hypoxia-responsive transcription factors. Pflugers Arch **450(6):**363-371.

7. **Cummins EP, Berra E, Comerford KM, Ginouves A, Fitzgerald KT, Seeballuck F, Godson C, Nielsen JE, Moynagh P, Pouyssegur J, Taylor CT.** 2006. Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. Proc Natl Acad Sci U S A **103(48):**18154-18159.

8. **Dasarathy S, Dodig M, Muc SM, Kalhan SC, McCullough AJ.** 2004. Skeletal muscle atrophy is associated with an increased expression of myostatin and impaired satellite cell function in the portacaval anastamosis rat. Am J Physiol Gastrointest Liver Physiol **287(6):**G1124-G1130.

9. **Dasarathy S, McCullough AJ, Muc S, Schneyer A, Bennett CD, Dodig M, Kalhan SC.** 2011. Sarcopenia associated with portosystemic shunting is reversed by follistatin. J Hepatol **54(5):**915-921.

10. **Dasarathy S.** 2012. Consilience in sarcopenia of cirrhosis. J Cachexia Sarcopenia Muscle **3(4):**225-237.

11. **Dromparis P, Sutendra G, Paulin R, Proctor S, Michelakis ED, McMurtry MS.** 2014. Pioglitazone inhibits HIF-1α-dependent angiogenesis in rats by paracrine and direct effects on endothelial cells. J Mol Med (Berl) **92(5):**497-507.

12. **Faff-Michalak L, Albrecht J.** 1993. Hyperammonemia and hepatic encephalopathy stimulate rat cerebral synaptic mitochondrial glutamate dehydrogenase activity specifically in the direction of glutamate oxidation. Brain Res **618(2):**299-302.

13. **Holecek M, Skopec F, Sprongl L.** 1995. Protein metabolism in cirrhotic rats: effect of dietary restriction. Ann Nutr Metab **39(6):**346-354.

14. **Holecek M.** 2014. Evidence of a vicious cycle in glutamine synthesis and breakdown in pathogenesis of hepatic encephalopathy-therapeutic perspectives. Metab Brain Dis **29(1):**9-17.

15. **Hou P, Kuo CY, Cheng CT, Liou JP, Ann DK, Chen Q.** 2014. Intermediary metabolite precursor dimethyl-2-ketoglutarate stabilizes hypoxia-inducible factor-1α by inhibiting prolyl-4-hydroxylase PHD2. PLoS One **9(11):**e113865.

16. **Jang Y, Jeong SH, Park YH, Bae HC, Lee H, Ryu WI, Park GH, Son SW.** 2013. UVB induces HIF-1α-dependent TSLP expression via the JNK and ERK pathways. J Invest Dermatol **133(11):**2601-2608.

17. **Lockwood AH, McDonald JM, Reiman RE, Gelbard AS, Laughlin JS, Duffy TE, Plum F.** 1979. The dynamics of ammonia metabolism in man. Effects of liver disease and hyperammonemia. J Clin Invest **63(3):**449-460.

18. **Majmundar AJ, Lee DS, Skuli N, Mesquita RC, Kim MN, Yodh AG, Nguyen-McCarty M, Li B, Simon MC.** 2015. HIF modulation of Wnt signaling regulates skeletal myogenesis in vivo. Development **142(14):**2405-2412.

19. **Matoori S, Leroux JC.** 2015. Recent advances in the treatment of hyperammonemia. Adv Drug Deliv Rev **90:**55-68.

20. **Periyalwar P, Dasarathy S.** 2012. Malnutrition in cirrhosis: contribution and consequences of sarcopenia on metabolic and clinical responses. Clin Liver Dis **16(1):**95-131.

21. **Qiu J, Tsien C, Thapalaya S, Narayanan A, Weihl CC, Ching JK, Eghtesad B, Singh K, Fu X, Dubyak G, McDonald C, Almasan A, Hazen SL, Naga Prasad SV, Dasarathy S.** 2012. Hyperammonemia-mediated autophagy in skeletal muscle contributes to sarcopenia of cirrhosis. Am J Physiol Endocrinol Metab **303(8):**E983-E993.

22. **Qiu J, Thapaliya S, Runkana A, Yang Y, Tsien C, Mohan ML, Narayanan A, Eghtesad B, Mozdziak PE, McDonald C, Stark GR, Welle S, Naga Prasad SV, Dasarathy S.** 2013. Hyperammonemia in cirrhosis induces transcriptional regulation of myostatin by an NF-κB-mediated mechanism. Proc Natl Acad Sci U S A **110(45):**18162-18167.

23. **Remels AH, Gosker HR, Verhees KJ, Langen RC, Schols AM.** 2015. TNF-α-induced NF-κB activation stimulates skeletal muscle glycolytic metabolism through activation of HIF-1α. Endocrinology **156(5):**1770-1781.

24. **Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, Johnson RS, Haddad GG, Karin M.** 2008. NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. Nature **453(7196):**807-811.

25. **Shangraw RE, Jahoor F.** 1999. Effect of liver disease and transplantation on urea synthesis in humans: relationship to acid-base status. Am J Physiol **276(5 Pt 1):**G1145-G1152.

26. **Soria LR, Marrone J, Molinas SM, Lehmann GL, Calamita G, Marinelli RA.** 2014. Lipopolysaccharide impairs hepatocyte ureagenesis from ammonia: involvement of mitochondrial aquaporin-8. FEBS Lett **588(9):** 1686-1691.

27. **Stroka DM, Burkhardt T, Desbaillets I, Wenger RH, Neil DA, Bauer C, Gassmann M, Candinas D.** 2001. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. FASEB J **15(13):**2445-2453.

28. **Sutendra G, Dromparis P, Kinnaird A, Stenson TH, Haromy A, Parker JM, McMurtry MS, Michelakis ED.** 2013. Mitochondrial activation by inhibition of PDKII suppresses HIF1a signaling and angiogenesis in cancer. Oncogene **32(13):**1638-1650.

29. **Tapper EB, Jiang ZG, Patwardhan VR.** 2015. Refining the ammonia hypothesis: a physiology-driven approach to the treatment of hepatic encephalopathy. Mayo Clin Proc **90(5):**646-658.

30. **Tessari P.** 2003. Protein metabolism in liver cirrhosis: from albumin to muscle myofibrils. Curr Opin Clin Nutr Metab Care **6(1):**79-85.

31. **Wagatsuma A, Kotake N, Yamada S.** 2011. Spatial and temporal expression of hypoxia-inducible factor-1α during myogenesis in vivo and in vitro. Mol Cell Biochem **347(1-2):**145-155.

32. **Wang Q, Wang Y, Yu Z, Li D, Jia B, Li J, Guan K, Zhou Y, Chen Y, Kan Q.** 2014. Ammonia-induced energy disorders interfere with bilirubin metabolism in hepatocytes. Arch Biochem Biophys **555-556:**16-22.

33. **Zaganas I, Pajęcka K, Wendel Nielsen C, Schousboe A, Waagepetersen HS, Plaitakis A.** 2013. The effect of pH and ADP on ammonia affinity for human glutamate dehydrogenases. Metab Brain Dis **28(2):**127-131.

Figure legends

Table 1. Primer sequences

Figure 1. HIF-1α total protein expression. A. C_2C_{12} myotubes were treated with 10 mM Ammonium Acetate. Western blot analysis for the indicated proteins was then performed on these extracts. B.C_2C_{12} myotubes were treated with 200 μM Cobalt Chloride. Western blot analysis for the indicated proteins was then performed on these extracts. A Student's t test was performed and ***$P < 0.0010$ when compared with the control.

Figure 2. HIF-1α total protein expression in HEK293 cells. A. HEK293 cells were treated with 10 mM Ammonium Acetate. Western blot analysis for the indicated proteins was then performed on these extracts. B. HEK293 cells were treated with 200 μM Cobalt Chloride. Western blot analysis for the indicated proteins was then performed on these extracts. A Student's t test was performed and *$P < 0.050$; **$P < 0.010$ and ***$P < 0.0010$ when compared with the control.

Figure 3. HIF-1α cytoplasmic and nuclear protein expression. A. C_2C_{12} myotubes were treated with 10 mM ammonium acetate. Western blot analysis for the indicated proteins was then performed on these extracts. B.C_2C_{12} myotubes were treated with 200 μM cobalt chloride. Western blot analysis for the indicated proteins was then performed on these extracts. A Student's t test was

performed. a, P < 0.050; b, P < 0.010 and c, P < 0.0010 when compared with the control.

Figure 4.HIF-1α and target genes mRNA levels.C_2C_{12} myotubes were treated with 10 mM ammonium acetate. RT-PCR analysis for the indicated gene expression was then performed on these extracts. A Student's t test was performed and *P < 0.050; **P < 0.010 and ***P < 0.0010 when compared with the control.

**Figure 5. HIF-1α protein expression in scramble and NF-kB p65 knockdown cells.** It was   generated C_2C_{12} myotubes NF-kB p65 knockdown and scrambled cells as control under ammonium acetate intervention in different time points. Western blot analysis for the indicated proteins was then performed on these extracts. S = scrambled cells / KD = knockdown cells.

Figure 6. HIF-1α protein expression in scramble and NF-kB p65 knockdown cells. It was generated C_2C_{12} myotubes IKKα and IKKβ knockdown and scrambled cells as control under ammonium acetate intervention in different time points. Western blot analysis for the indicated proteins was then performed on these extracts. S = scrambled cells / α= IKKα knockdown cells / β = IKKβ knockdown cells.

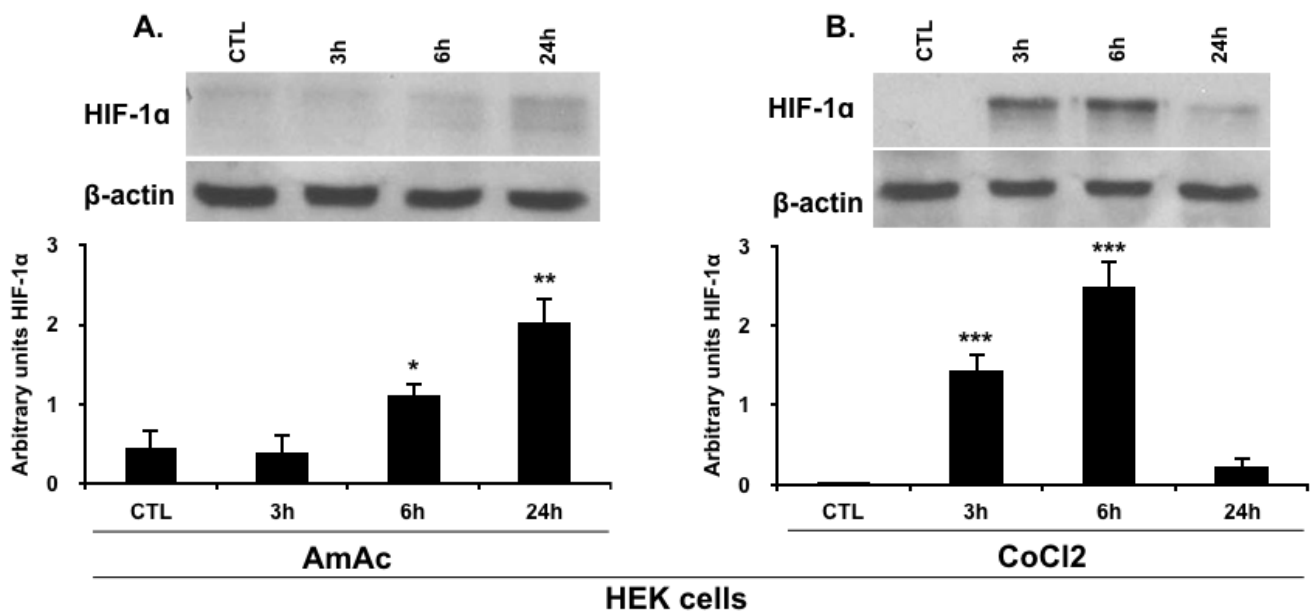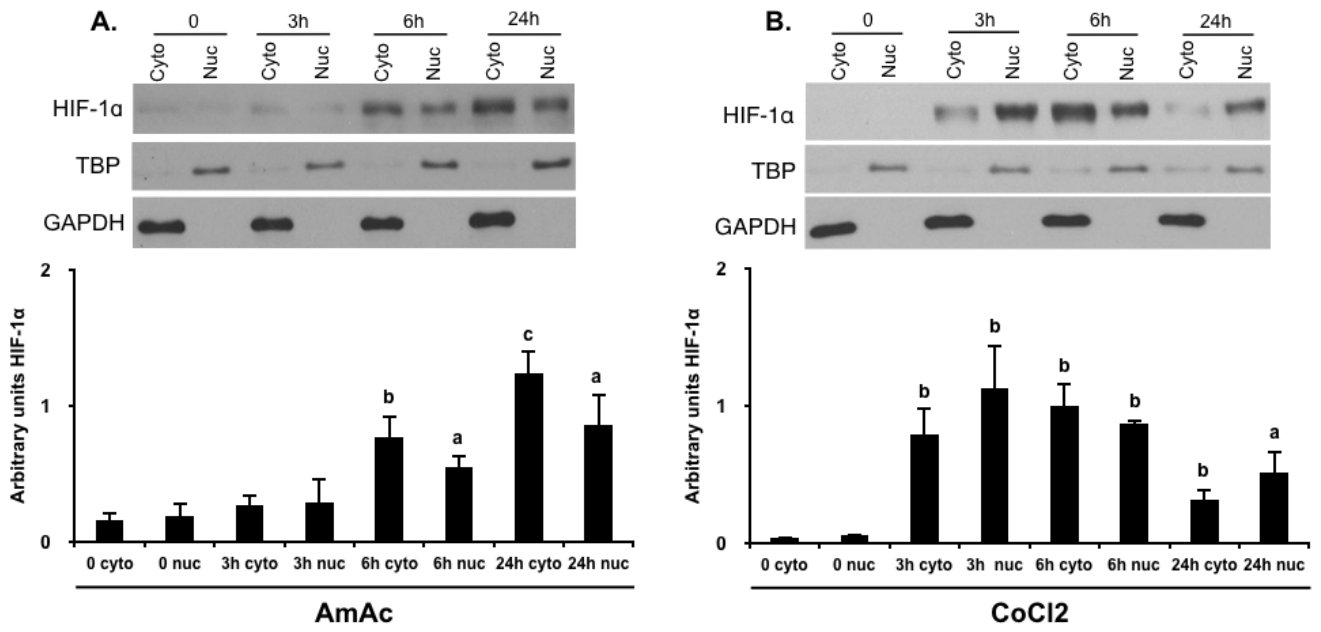| Gene	Primer Sequence	Product Size (bp)	Accession Number
HIF-1α	Forward, 5'-tcaagtcagcaacgtggaag-3' Reverse, 5'-tatcgaggctgtgtcgactg-3'	198	NM_010431.2
VEGF	Forward, 5'-caggctgctgtaacgatgaa-3' Reverse, 5'-aatgctttctccgctctgaa'-3'	207	M95200.1
PDK1	Forward, 5'-ggcggctttgtgatttgtat-3' Reverse, 5'-acctgaatcgggggataaac-3'	199	NM_172665.5

Silva et al. Figure 1
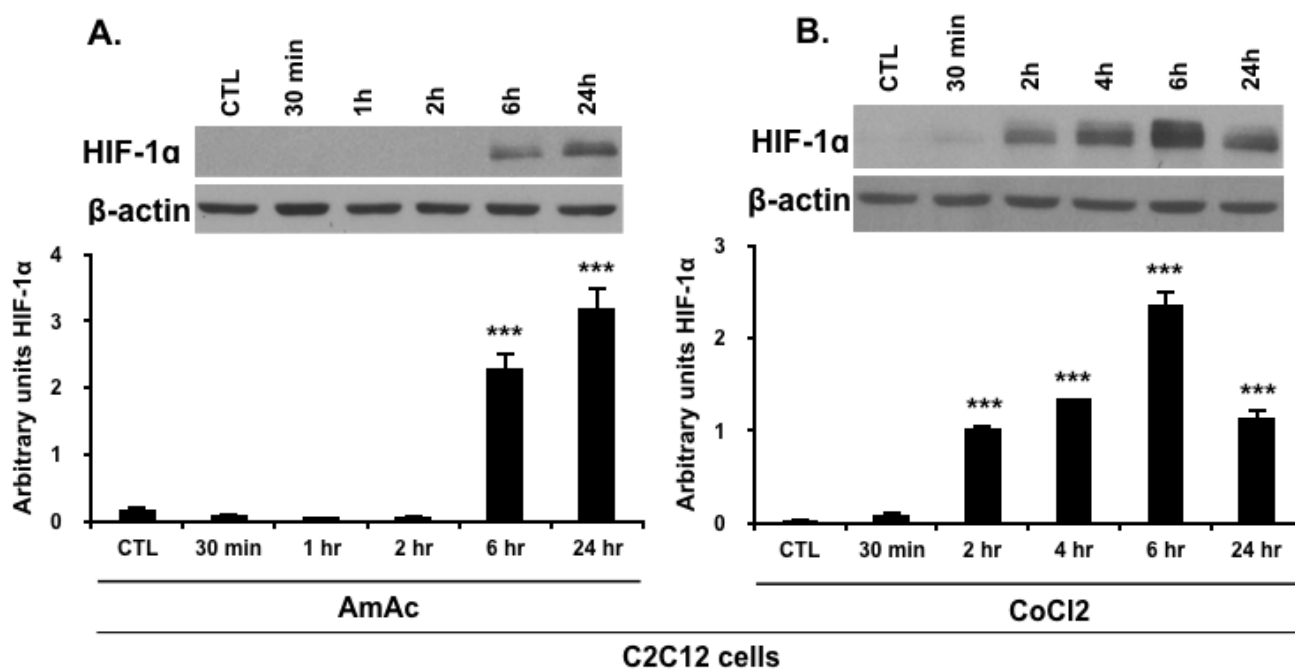
Silva et al. Figure 2

Silva et al. Figure 4

Silva et al. Figure 5

Silva et al. Figure 6