Effect of stress on the function of Na\(^{+}/K^{+}\)-ATPase and regulation of energy metabolism in skeletal muscle

DOCTORAL DISSERTATION

Vpliv stresnih razmer na delovanje Na\(^{+}/K^{+}\)-ATPaze in uravnavanje energijske presnove v skeletni mišici

DOKTORSKA DISERTACIJA

Vid JAN

Ljubljana, 2021
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Mentor was named at the UL senate session: 12.9.2017

Co-mentor was named at the UL senate session: 12.9.2017

Committee for evaluation and defence was named at the UL MF senate session: 13.3.2017

Date of defence: 17.6.2021

Mentor: assist. prof. Sergej Pirkmajer, MD, PhD

Co-mentor: prof. Matej Podbregar, MD, PhD

President of the committee: prof. Peter Veranič, PhD

Member: assoc. prof. Paola Lorenzon, PhD

Member: assoc. prof. Maja Šoštarič, MD, PhD
DECLARATION

I hereby declare that I am the author of the doctoral thesis titled **Effect of stress on the function of Na\(^+\)/K\(^+\)-ATPase and regulation of energy metabolism in skeletal muscle.** I conducted the work at the Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana in Ljubljana, Slovenia. Scientific papers that are part of this thesis were not used for the same purpose by any other co-author. I declare that the printed version of the doctoral thesis is identical to the electronic version.

Vid Jan

Ljubljana, 2021
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACL</td>
<td>anterior cruciate ligament</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-Aminoimidazole-4-carboxamide ribonucleoside</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B or PKB</td>
</tr>
<tr>
<td>aMEM</td>
<td>Advanced Minimum Essential Medium</td>
</tr>
<tr>
<td>AMPK</td>
<td>5' AMP-activated protein kinase / z AMP aktivirana protein-kinaza</td>
</tr>
<tr>
<td>AS160</td>
<td>Akt substrate of 160 kDa (also known as TBC1D4)</td>
</tr>
<tr>
<td>ATIC</td>
<td>5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchonic acid</td>
</tr>
<tr>
<td>BFR</td>
<td>training with blood flow restriction</td>
</tr>
<tr>
<td>CaMKKβ</td>
<td>Ca(^{2+})/calmodulin-dependent kinase kinase β</td>
</tr>
<tr>
<td>CST</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DHPR(\alpha_\text{1S})</td>
<td>(\alpha_{1S}) subunit of the dihydropyridine receptor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DPY</td>
<td>dipyridamole</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle’s Balanced Salt Solution</td>
</tr>
<tr>
<td>ENT1</td>
<td>equilibrative nucleoside transporters 1</td>
</tr>
<tr>
<td>ENT2</td>
<td>equilibrative nucleoside transporters 2</td>
</tr>
<tr>
<td>EPS</td>
<td>electrical-pulse stimulation</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinase 1/2 (also known as p42/44 mitogen-activated protein kinase (MAPK))</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FXYD1</td>
<td>phospholemman</td>
</tr>
<tr>
<td>FXYD5</td>
<td>dysadherin</td>
</tr>
<tr>
<td>GART</td>
<td>glycinamide ribonucleotide formyltransferase</td>
</tr>
<tr>
<td>GD</td>
<td>glucose deprivation</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter type 4</td>
</tr>
<tr>
<td>GSK-3</td>
<td>glycogen synthase kinase-3</td>
</tr>
<tr>
<td>HGD</td>
<td>hypoxia and glucose deprivation (artificial ischaemia)</td>
</tr>
<tr>
<td>HI-BFR</td>
<td>high intensity blood flow restriction training</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia-inducible factor-1α</td>
</tr>
<tr>
<td>HSMC</td>
<td>human skeletal muscle cells</td>
</tr>
<tr>
<td>HYP</td>
<td>hypoxia</td>
</tr>
<tr>
<td>I&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximal transport activity</td>
</tr>
<tr>
<td>IMP</td>
<td>inosine monophosphate</td>
</tr>
<tr>
<td>LKB1</td>
<td>liver kinase B1</td>
</tr>
<tr>
<td>LL-BFR</td>
<td>low load blood flow restriction training</td>
</tr>
<tr>
<td>MACS</td>
<td>magnetic-activated cell sorting</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney cells</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>MTX</td>
<td>methotrexate</td>
</tr>
<tr>
<td>NCAM</td>
<td>neural cell adhesion molecule (CD56)</td>
</tr>
<tr>
<td>NKA</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;/K&lt;sup&gt;+&lt;/sup&gt;-ATPase</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>NOR</td>
<td>normoxia</td>
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</table>
PAK  p21-activated kinase
PBS  phosphate-buffered saline
PGK1 phosphoglycerate kinase 1
PI3K phosphatidylinositol 3-kinase
PKCζ protein kinase Cζ
PP2A protein phosphatase 2A
PRPP 5-phosphoribosyl pyrophosphate
PVDF polyvinylidene fluoride
RT-qPCR quantitative reverse transcription polymerase chain reaction
SCB Santa Cruz Biotechnology
SDS sodium dodecyl sulfate
SDS-PAGE sodium dodecyl (lauryl) sulfate-polyacrylamide gel
SERCA sarcoplasmic reticulum Ca\(^{2+}\)-ATPase
Sp1 specificity protein 1
Sp3 specificity protein 3
SSZ sulfasalazine
TBST tris-buffered saline with Tween
VEGFA vascular endothelial factor A
\(V_{\text{max}}\) maximal ATPase activity
ZMP AICAR-monophosphate
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>ACC</td>
<td>acetil-CoA karboksilaza</td>
</tr>
<tr>
<td>ACL</td>
<td>sprednja križna vez</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazol-4-karboksamid ribonukleozid</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinaza Akt ali protein kinaza B (PKB)</td>
</tr>
<tr>
<td>aMEM</td>
<td>izboljšani minimalni esencialni medij</td>
</tr>
<tr>
<td>AMPK</td>
<td>z AMP aktivirana protein-kinaza</td>
</tr>
<tr>
<td>AS160</td>
<td>substrat kinaze Akt 160 kDa (znan tudi pod imenom TBC1D4)</td>
</tr>
<tr>
<td>ATIC</td>
<td>5-aminoimidazol-4-karboksamid ribonukleotid formiltransferaza/IMP ciklohidrolaza</td>
</tr>
<tr>
<td>BCA</td>
<td>bikinkoninična kislina</td>
</tr>
<tr>
<td>BFR</td>
<td>zmanjšan pretok krvi</td>
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<tr>
<td>CaMKKβ</td>
<td>od Ca$^{2+}$/kalmodulina odvisna kinaza kinaza β</td>
</tr>
<tr>
<td>CST</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>DEPC</td>
<td>dietil piroarbonat</td>
</tr>
<tr>
<td>DHPRA1S</td>
<td>$\alpha_{1S}$ podenota dihidropiridinskega receptorja</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbeccovo modificirano Eagleovo gojišče</td>
</tr>
<tr>
<td>DPY</td>
<td>dipiridamol</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earlova uravnotežena solna raztopina</td>
</tr>
<tr>
<td>ENT1</td>
<td>ekvilibrativni nukleozidni prenašalec 1</td>
</tr>
<tr>
<td>ENT2</td>
<td>ekvilibrativni nukleozidni prenašalec 2</td>
</tr>
<tr>
<td>EPS</td>
<td>električna pulzna stimulacija</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>z ekstracelularnim signalom regulirana kinaza (poznana tudi pod imenom p44/42 z mitogenom aktivirana protein-kinaza (p44/42 MAPK))</td>
</tr>
</tbody>
</table>
FBS  fetalni goveji serum
FXYD1  fosfoleman
FXYD5  disadherin
GART  glicinamid ribonukleotid formiltransferaza
GD  odvzem glukoze
GLUT4  glukoznii prenašalec tipa 4
GSK-3  glikogen sintaza kinaza-3
HGD  hipoksija in odvzem glukoze (umetna ishemija)
HIF-1α  s hipoksijo inducirani dejavnik-1α
HI-BFR  visokointenzivna ishemična vadba
HSMC  humane (človeške) skeletnomišične celice
HYP  hipoksija
I_{max}  maksimalna transportna aktivnost
IMP  inozin monofosfat
LKB1  jetrna kinaza B1
LL-BFR  ishemična vadba proti majhnemu uporu
MACS  magnetno pogojeno ločevanje celic
MDCK  celice Madin-Darby pasje ledvice
mTORC1  kompleks 1 mehanistične/sesalčje tarče rapamicina
MTX  metotreksat
NCAM  živčna celična pritrjevalna molekula (CD56)
NKA  Na^{+}/K^{+}-ATPaza
NOR  normoksiija
PAK  s p21-aktivirana kinaza
PBS fosfatni pufer z NaCl
PGK1 fosfoglicerat kinaza 1
PI3K fosfatidil inozitol 3-kinaza
PKCζ protein kinaza Cζ
PP2A proteinska fosfataza 2A
PRPP 5-fosforibozil pirofosfat
PVDF poliviniliden fluorid
RT-qPCR kvantitativna polimerazna verižna reakcija v realnem času
SCB Santa Cruz Biotechnology
SDS natrijev dodecil sulfat
SDS-PAGE poliakrilamidna gelska elektroforeza v prisotnosti natrijevega dodecilsulfata
SERCA Ca\(^{2+}\)-ATPaza sarkoplazemskega retikuluma
Sp1 specifičnostni protein 1
Sp3 specifičnostni protein 3
SSZ sulfasalazin
TBST fiziološka raztopina s Tris pufrom in Tween-20
VEGFA žilni endotelijski rastni dejavnik α
\(V_{\text{max}}\) maksimalna ATPazna aktivnost
ZMP AICAR monofosfat
ŽMS živčno-mišični stik
LIST OF PUBLICATIONS / SEZMAM RAZISKOVALNIH ČLANKOV


REVIEW PAPER/PREGLEDNI ČLANEK

ABSTRACT

AIMS: General purpose of this doctoral dissertation was to evaluate molecular mechanisms, which, during stressful conditions, alter Na+/K+-ATPase (NKA) activity and disturb regulation of energy metabolism in skeletal muscle. Specific aims were: (1) to establish an in vitro experimental model for the observation of NKA function and metabolism in skeletal muscle, in which physiological and biochemical characteristics would move closer to in vivo conditions compared to present experimental models; (2) to study molecular mechanisms through which hypoxia affects the functioning of NKA in skeletal muscle and (3) explore how metabolic changes affect NKA function in skeletal muscle.

HYPOTHESES: We explored three hypotheses: (H1) Myotubes innervated in vitro and mature muscle fibres in vivo have a similar expression pattern of NKA and FXYD proteins. (H2) Hypoxia alters NKA function in skeletal muscle via activation of 5' AMP-activated protein kinase (AMPK). (H3) Modulation of NKA by pharmacological activators of AMPK depends on the metabolic state of skeletal muscle.

METHODS: As an experimental model we used innervated and non-innervated human skeletal muscle cells (HSMC). We also used skeletal muscle biopsies of patients that were exposed to blood flow restriction (BFR) training. For analyses we used appropriate biomolecular methods (western blot, RT-qPCR).

RESULTS: Ad H1: Non-innervated HSMC are the most common cell model for skeletal muscle research. These cells predominantly express NKAα1 while skeletal muscles in vivo express higher amounts of NKAα2. Differentiation of HSMC increased the expression of NKA and FXYDs, which are more abundant in mature skeletal muscle, but innervation itself caused only minor additional changes. Ad H2: Knee injury patients are commonly exposed to hypoxia during surgery, which can also cause metabolic disorders or muscle damage. Energy stress activates AMPK, which has an important role in modulating energy metabolism. A study on skeletal muscle cells indicated that AMPK stimulation could lead to increased NKA activity, which is why we presumed that NKA function might increase in hypoxic conditions. We explored possible effects of low load training with blood flow restriction (LL-BFR training) of knee injury patients on NKA function in skeletal muscles. LL-BFR training increased NKAα1 content, which may have an important role in muscle hypertrophy. Increase in NKAα1 might lead to increased muscle mass of knee injury patients. We observed no changes in AMPK signalling. Ad H3: Increased activity of AMPK in skeletal muscles leads to improved energy status. Metformin, a known indirect AMPK activator, lowered mRNA expression of NKA only in ischaemic conditions, but not under normoxic or hypoxic conditions. We also evaluated sulfasalazine (SSZ), a salicylate derivative, for its potential effect on AMPK activity. SSZ increased AMPK activity in L6 cells and to a smaller extent in HSMC.

CONCLUSIONS: (1) Using cell-culture homogenates we could not detect significant effect of innervation on the expression of NKA, FXYD1 and FXYD5. Our results therefore do not support the idea that innervated human myotubes in vitro display a similar expression pattern as myofibers in vivo (2) We showed that LL-BFR training increased the expression of NKAα1, which might lead to increased muscle mass, but our results do not provide evidence that hypoxia alters NKA function via AMPK (3) We showed that effects of metformin on NKA expression were dependent on energy status of skeletal muscle cells, which supports the third hypothesis. Also, SSZ, a known antirheumatic drug, increased AMPK activity in L6 cells and to a smaller extent in HSMC.
POVZETEK

CILJI: Glavni namen doktorske disertacije je bil proučiti molekularne mehanizme, ki v stresnih razmerah spremenijo delovanje Na\(^+\)/K\(^-\)-ATPaze in negativno vplivajo na uravnavanje energijske presnove v skeletni mišici. Specifični cilji so bili: (1) vzpostavitev in vitro celičnega modela za proučevanje delovanja NKA in presnove v skeletni mišici, ki bi bil po fizioloških in biokemijskih značilnostih skeletnomišičnih celic bolj podoben in vivo mišičnim vlaknom, kot trenutno uveljavljeni modeli, (2) proučiti molekularne mehanizme, ki preko hipoksije vplivajo na delovanje NKA v skeletni mišici in (3) raziskati, kako presnovne spremembe vplivajo na delovanje NKA v skeletni mišici.

HIPOTEZE: Preverili smo tri hipoteze: (H1) Oživčene mišične cevčice v kulturi imajo podoben vzorec izražanja podenot NKA in proteinov FXDY kot zrela mišična vlakna. (H2) Hipoksija skeletne mišice vpliva na delovanje NKA prek aktivacije z AMP aktivirane protein-kinaze (AMPK). (H3) Farmakološki aktivatorji AMPK vplivajo na delovanje NKA v odvisnosti od presnovnega stanja skeletne mišice.

METODE: Kot eksperimentalni model smo uporabili inervirane in neinervirane humane skeletne mišične celice (HSMC). Uporabili smo tudi mišične biopsije pacientov, ki so bili izpostavljeni ishemični vadbi. Za analizo smo uporabili primerne biomolekularne metode (odtis western in obratna transkripcija z verižno reakcijo s polimerazo - RT-qPCR).


ZAKLJUČKI: (1) V homogenatih celičnih kultur nismo zaznali statistično značilnih učinkov inervacije na izražanje podenot NKA, FXDY1 in FXDY5. Naši rezultati ne podpirajo hipoteze, da imajo in vitro inervirane HSMC podoben profil izražanja kot mišična vlakna in vivo. (2) Pokazali smo, da LL-BFR vadba v primerjavi z NKAα1, kar bi lahko vodilo v vzbujanje mišične mase, ne moremo pa potrditi, da hipoksija spremeni delovanje NKA preko AMPK. (3) Pokazali smo, da so učinki metformina na izražanje NKA odvisni od presnovnega stanja skeletnomišičnih celic, kar potrjuje tretjo hipotezo. Poleg tega je sulfasalazin, znan antirevmatik, povišal aktivnost AMPK v L6 celicah in v manjši meri tudi v HSMC.
RAZŠIRJEN SLOVENSKI POVZETEK (EXTENDED SLOVENIAN ABSTRACT)

UVOD

Skeletne mišice so lahko izpostavljene različnim stresnim razmeram, ki lahko vključujejo različne stopnje oksidativnega in presnovnega stresa. Ali ima stres pozitivne ali negativne posledice na delovanje skeletne mišice, je odvisno od dolžine in intenzivnosti stresa, ki so mu izpostavljene skeletne mišice. Stresne razmere, ki se v mišici pojavijo med telesno vadbo lahko povzročijo pozitivne adaptacije mišice, medtem ko lahko stresne razmere v patoloških stanjih prispevajo k mišični atrofiji in sarkopeniji. Delovanje Na⁺/K⁺-ATPaze (NKA) je tesno prepleteno s presnovnim stanjem in oksigenacijo skeletne mišice, njeno delovanje pa je v stresnih razmerah lahko izrazito okrnjeno.

NKA je ATPaza tipa P, ki hkrati transportira 2 K⁺ v celico, 3 Na⁺ pa iz nje proti njej nima koncentracijskih gradientov. V živalskih celicah NKA predstavlja edini mehanizem, ki se upira izgubi znotrajceličnega K⁺ in vnosu izvenceličnega Na⁺, s tem pa celicam omogoča vzdrževanje homeostaze vode in ionov, ohranjanje membranske potenciala, in z Na⁺-sklopljeni transport različnih snovi. V skeletni mišici NKA igra pomembno vlogo pri ohranjanju mišičnih kontrakcij. NKA je sestavljena iz katalitske α-podenote in glikoproteinske β-podenote. V humanih skeletnih mišicah so izražene α1-/, α2- in α3-izooblike, od katerih je najbolj zastopana α2. NKA heterodimeri, sestavljeni iz različnih α- in β-podenot, se razlikujejo v svojih kinetičnih značilnostih, kot so encimska in transportna aktivnost, ter afiniteta za Na⁺, K⁺ in ATP. Medtem ko so α1/β-heterodimeri zadolženi predvsem za uravnavanje gradientov Na⁺ in K⁺ v mirujoči mišici, pa so α2/β-heterodimeri aktivni predvsem, ko se mišica aktivno krči.

Intrinzična aktivnost NKA je regulirana preko koncentracije substratov, kovalentnih modificacij podenot NKA in proteinskih interakcij med NKA in proteini iz družine FXYD. FXYD proteini so majhni transmembranski regulatorji ionskega transporta, ki tkivno specifično regulirajo NKA. Fosfoleman (FXYD1) je najpomembnejši regulator NKA iz družine FXYD v skeletnih mišicah. FXYD1 se veže tako na α1- kot tudi na α2-izoobliko NKA. Interakcija med nefosforiliranim FXYD1 in NKA reverzibilno zniža afiniteto NKA za Na⁺ in s tem zniža aktivnost NKA, medtem ko fosforilacija FXYD1 poveča afiniteto NKA za Na⁺ in aktivnost NKA. V skeletni mišici NKA najverjetneje regulira tudi disadherin (FXYD5), katerega ekspresija se poveča v obdobjih neaktivnosti, drugače pa je znan po tem, da ob vezavi na NKA povira njeno maksimalno ATPazno aktivnost ali V_max.

V in vitro razmerah podgajanje in humane skeletne mišične celice v večji meri izražajo α1/β-heterodimere, v primerjavi s svojim izvornim tkivom pa prav tako zelo šibko ali pa sploh ne izražajo FXYD1. Znano je, da so v in vivo podganjih in humanih skeletnih mišicah najbolj izraženi α2/β-heterodimere, ki so locirani predvsem v T-tubulih, ki pa jih neenervirane humane skeletne mišične celice (HSMC) v kulturi ne razvijejo. Znano je, da inervacija HSMC zviša membranski potencial in da se je ekspresija in aktivnost NKA povečala, ko so HSMC tretirali z nevralnim agrinom, ki je najbolj znan po svoji vlogi pri organizaciji in vzdrževanju postsinaptičnih struktur živčno-mišičnega stika (ŽMS). Zaradi razkoraka v izražanju NKA med skeletnomiščnimi celičnimi kulturami in vitro ter skeletnomiščnimi vlakni in vivo bi bil za nadaljnji razvoj znanja o delovanju NKA v skeletnih mišicah zelo pomemben razvoj in vitro modela, ki bi bil v svojem profilu izražanja podenot NKA in FXYD proteinov bolj primerljiv zrelim miščnim vlaknom.

Različni kirurški posegi vključujejo daljše zaustavitve pretoka krvi v operirano tkivo ali organ, čemur sledi reperfuzija tkiva ob ponovni vzpostavitvi dotoka krvi. Reperfuzija
ishemičnega tkiva povzroči vnetje, poškodbo tkiva in motnjo v delovanju skeletne mišice, kar je v veliki meri posledica oksidativnega in presnovnega stresa. Energijski stres, ki se vzpostavi med operativnim posegom aktivira z AMP-aktivirano protein-kinazo (AMPK). AMPK, heterotrimeren αβγ Ser/Thr kinaza, je celični energijski senzor in glavni regulator presnovnega ravnovesja med energijskim primanjkljajem v celicah. Poskusi na L6 podganji skeletnomišični liniji so pokazali, da lahko aktivacija AMPK stimulira NKA, zaradi česar predpostavljamo, da bi lahko s hipoksijo-inducirana aktivacija AMPK v skeletnomišičnih celicah prav tako povišala aktivnost NKA. Hipoksijo razmere se v skeletni mišici pojavijo tudi med ishemično motnje v okisignaciji in dostopnosti energijskih molekul prispevajo k motenem delovanju skeletne mišice.

Kot AMPK je tudi NKA tesno povezana z energijsko presnovo skeletne mišice. Mišične kontrakcije stimulirajo tako AMPK kot tudi NKA. AMPK, ki je aktivirana zaradi povečanega razmerja med znotrajceličnima koncentracijama AMP in ATP, povzroči preklop celične presnove iz anabolnega v katabolnega stanje, kar v splošnem povzroči inhibicijo procesov, ki v celici porabljajo ATP. Čeprav AMPK načeloma zavre energijske potrate in spodbudi proizvodnjo energije, pa lahko hrkati stimulira aktivnost NKA in s tem porabo ATP. Zanimivo bi bilo natančnoji proučiti, ali lahko s pomočjo farmakoloških aktivatorjev AMPK spremenimo delovanje NKA v skeletni mišici, ki je izpostavljena različnim stopnjam presnovnega stresa.

Nekatere farmakološke učinkovine aktivirajo AMPK neposredno, medtem ko druge aktivacijo AMPK povečajo posredno preko inhibicije glikolize in mitohondrijske dihalne verige. Delovanje NKA v skeletni mišici naj bi bilo odvisno predvsem od ATP, ki se ga pridobi v glikolizi, tako da bi lahko aktivatorji AMPK, ki zavrejo glikolizo, hkrati inhibirali delovanje NKA. Aktivacija AMPK vpliva tudi na občutljivost skeletnih mišic na inzulin, ki je eden najpomembnejših regulatorjev NKA v skeletni mišici. Učinek farmakoloških učinkovin na delovanje NKA v skeletni mišici zatorej ni odvisen samo od aktivacije AMPK, ampak tudi od njihovih neposrednih učinkov na energijsko presnovo skeletne mišice.

V doktorski nalogi smo si zadali tri cilje: (1) vzpostavitev in vitro celičnega modela za proučevanje delovanja NKA in presnove v skeletni mišici, ki bi bil po fizioloških in biokemijskih značilnostih skeletnomišičnih celic bolj podoben in vivo mišičnim vlakom, kot trenutno uveljavljeni modeli, (2) proučiti molekularne mehanizme, ki preko hipoksije vplivajo na delovanje NKA v skeletni mišici in (3) raziskati, kako presnovne spremembe vplivajo na delovanje NKA v skeletni mišici.

**NAMEN IN HIPOTEZE**

Glavni cilj doktorske naloge je bil proučevanje molekularnih mehanizmov, ki tekom stresnih razmer v skeletni mišici negativno vplivajo na delovanje NKA in na uravnavanje energijske presnove. Preverili smo sledeče hipoteze:

- Oživene mišične cevčice v kulturi imajo podoben vzorec izražanja podenot NKA in proteinov FXYD kot zrela mišična vlakna.
- Hipoksijske skeletne mišice vpliva na delovanje NKA prek aktivacije z AMP aktivirane protein-kinaze (AMPK).
- Farmakološki aktivatorji AMPK vplivajo na delovanje NKA v odvisnosti od presnovnega stanja skeletne mišice.
ZASNOVA ŠTUDIJE IN METODE

Etična dovoljenja za izvedene poskuse


Eksperimentalni model: kultura primarnih človeških skeletnomišičnih celic (HSMC)

V in vitro poskusih smo uporabili neinervirane diferencirane HSMC in inervirane kulture HSMC, ki so bile oživljene s segmenti podganje embrionalne hrbtenjače. V določenih poskusih smo uporabili tudi L6 podganje skeletnomiščno celično linijo. V skupini smo imeli pridobljena vsa potrebna dovoljenja za evtanazijo in izolacijo organov evtanaziranih živali (navedena pod točko Etična dovoljenja za izvedene poskuse).

Merjenje izražanja podenot NKA, FXYD1 in FXYD5

Izražanje podenot NKA, FXYD1 in FXYD5 smo določali na ravni mRNA in proteinski ravni. Za merjenje mRNA smo uporabili metodo RT-qPCR, medtem ko smo raven tarčnih proteinov določili z metodo odtis western.

Statistična analiza

Vsi podatki so prikazani kot aritmetična sredina ± standardna napaka aritmetične sredine. Za statistične analize smo uporabili program GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Za statistično značilno razliko med dvema skupinama smo šteli rezultate, kjer je bila p vrednost <0,05. Izbrane statistične metode so navedene v opisih slik posameznih poskusov.

REZULTATI

Ad H1

Diferenciacija HSMC s pomočjo gojišča z nižjo vsebnostjo (2 %) fetalnega govejega seruma (FBS) je povzročila porast številnih mišično-specifičnih proteinov kot tudi podenot NKA in proteinov FXYD, ki so visoko zastopani v zrelih mišičnih vlaknih. Od NKA α-podenot se je povišalo izražanje α2, ki je najbolj zastopana v mišičnih vlaknih in vivo, medtem ko sta NKAα1 in α3 ostali nespremenjeni. Znižanje koncentracije seruma ni imelo vpliva na izražanje NKAβ-podenot, kar nakazuje, da so NKA α- in β-podenote uravnavane preko različnih mehanizmov. Diferenciacija HSMC je povečala tudi izražanje FXYD1, medtem ko je bilo izražanje FXYD5 na ravni mRNA znižano.

Inervacija ni povzročila večjih sprememb v izražanju podenot NKA in FXYD1 ter FXYD5 na ravni mRNA. Na proteinski ravni smo zaznali porast v vsebnosti NKAα1 in α2, ter v celokupnem in fosforiliranem FXYD1, kar nakazuje, da je pomembno predvsem posttranskripcijsko oziroma posttranslacijsko uravnavanje. Obstaja pa tudi možnost, da so
zaznane spremembe na proteinski ravni posledica kontaminacije s proteini iz podganjega tkiva (ekspantatora hrbtenjače), ker uporabljena primarna protitelesa niso bila vrstno-specifična. Na ravni mRNA sicer z vrstno-nespecifičnimi PCR sondami nismo zaznali izrazite kontaminacije iz podganjega tkiva, kar bi lahko posredno ovrglo zaznavo večjih količin podganjenih proteinov iz ekspantatov embrionalnih hrbtenjač. Tako v neinerviranih kot tudi v inerviranih HSMC smo uspešno zaznali FXYD1. Po našem vedenju je to prvo poročilo o izražanju FXYD1 v kulturi HSMC.

Zanimalo nas je tudi, ali kontrakcije in živčno-mišična stimulacija igrata pomembno vlogo pri izražanju NKA in proteinov FXYD. Ustavitev kontrakcij z blocalo živčnoliščnega prenosa s pomočjo nevrotoksinov, ki inhibirajo nikotinski acetilholinski receptor, ni imela vpliva na izražanje podenot NKA, FXYD1 in FXYD5.

**Ad H2**

V drugi hipotezi nas je zanimalo, kako bi lahko ishemična predpriora skeletne mišice vplivala na delovanje NKA in ali so morebitne spremembe povezane z aktivacijo AMPK. Primerjali smo vzorce mišic vastus lateralis in semitendinosus pacientov s poškodbo ACL, ki niso opravljali nobene dodatne telesne aktivosti (kontrolna skupina) s pacienti, ki so opravljali vadbo proti majhnemu uporu brez ishemije (LL-Sham) ali z ishemijo (LL-BFR). LL-BFR vadba je povišala izražanje NKA tako proti kontrolni skupini kot tudi proti skupini LL-Sham, medtem ko drugih večjih sprememb v izražanju podenot NKA in proteinov FXYD nismo zaznali. Prav tako nismo zaznali večjih razlik v aktivnosti AMPK med skupinami.

**In vitro** poskusu, kjer smo diferencirane HSMC za 24 ur izpostavili hipoksiji (0,1 % O₂), pomanjkanju glukoze ali umetni ishemiji (pomanjkanje glukoze in 0,1 % O₂) smo pokazali, da je AMPK aktivirana le v ishemičnih razmerah, medtem ko je bilo izražanje NKAα1 in β1-3 znižano tako pri tretiranju brez glukoze kot tudi pri ishemičnem tretanju. Tako pri hipoksiji, kot tudi pri pomanjkanju glukoze in ishemiji smo zaznali padec v fosforilaciji NKAα1 na ostanku Tyr10, ki je zlasti v skeletni mišici dokaj neraziskano fosforilacijsko mesto.

**Ad H3**

V tretji hipotezi smo proučili, kako bi lahko presnovno stanje skeletnomiščnih celic vplivalo na sposobnost aktivatorjev AMPK, da uravnavajo NKA, FXYD1 in FXYD5. Uporabili smo metformin, znan neposredni aktivator AMPK in najpogostejši peroralni antidiabetik in z njim tri dni tretirali diferencirane HSMC. Na četrtni dan smo celice izpostavili normoksičnim, hipoksičnim in ishemičnim razmeram (0,1 % O₂, brez glukoze) brez ali ob prisotnosti metformina. Visoka koncentracija metformina je znižala izražanje mRNA NKAα1, α2, β1, β2 in FXYD1 v ishemičnih razmerah, medtem ko pri normoksičnih in hipoksičnih razmerah nismo zaznali signifikantnih sprememb v izražanju NKAα in β. Visoka koncentracija metformina je izražanje mRNA FXYD1 znižala v normoksičnih razmerah.

V nasprotju s podenotami NKA in FXYD1 se je izražanje mRNA FXYD5 pri tretmanu z visoko koncentracijo metformina povišalo v normoksičnih razmerah, pozitiven trend pa smo opazili v ishemičnih razmerah.
poskusih aktiviral AMPK, nismo pa opazili sinergističnega učinka z AICAR na aktivacijo AMPK. Ko smo celice tretirali s SSZ in inzulinom, smo v L6 celicah opazili upad fosforilacije proteinov, vključenih v inzulinski signalni poti, kar bi lahko nakazovalo, da SSZ zavirja privzem glukoze. Po drugi strani pa smo v HSMMC zaznali aditivne učinke SSZ na inzulinsko stimulacijo, kar bi lahko povzročilo povišan privzem glukoze.

RAZPRAVA

Ad H1

Naši rezultati ne podpirajo prve hipoteze, da de novo inervacija HSMC in vitro vodi v profil izražanja NKA, FXYD1 in FXYD5, ki bi bil bolj podoben tistemu v zrelih mišičnih vlaknih. Inervacija ni imela učinkov na izražanje mRNA podenot NKA in FXYD1, smo pa pri inerviranih HSMC zaznali manjše povišanje izražanja FXYD5. Zdi se, da je pri HSMC za diferenciacijo bolj pomembno znižanje koncentracije seruma v gojišču. To je pri diferenciaciji HSMC, ko celice fuzirajo v večjedrne celice, povišalo izražanje NKAa2 in FXYD1. Po drugi strani pa smo v HSMC zaznali aditivne učinke SSZ na inzulinsko stimulacijo, kar bi lahko povzročilo povišan privzem glukoze.

Ad H2

Rezultati naših poskusov ne podpirajo druge hipoteze, da hipoksija uravnava delovanje NKA preko aktivacije AMPK. Čeprav ne moremo zagotovo trditi, da AMPK ni vplivala na delovanje NKA, pa naši rezultati tega ne nakazujejo. Morebitni razlogi, zakaj nismo zaznali povišane aktivnosti AMPK pri pacientih, ki so izvajali LL-BFR vadbo, med drugim vključujejo neprimeren čas vzorčenja mišic, protokole vadbe in intenzitete vadbenih setov. Smo pa v HSMMC zaznali povišano izražanje NKAa1. Nedavno je bila opisana nova možna vloga NKAa1 v skeletni celici, kjer naj bi NKAa1 vplivala na rast mišic. Obstaja torej možnost, da bi povišanje v vsebnosti NKAa1 pozitivno vplivalo na mišično maso pacientov s poškodbo ACL. Naši in vitro rezultati kažejo, da akutna 24-urna hipoksija ne aktivira AMPK. Zdi se, da je pri HSMC za aktivacijo AMPK potrebno tako pomanjkanje hranil kot tudi pomanjkanje O2, kar smo v našem primeru dosegli z gojenjem celic v gojišču brez glukoze pri nizki koncentraciji O2 (0,1 %). Pomanjkanje glukoze je bil največji dejavnik pri zavrtju ekspresije NKAa1 in vseh treh NKA β-podenot.

Ad H3

Rezultati iz metforminskega poskusa podpirajo hipotezo, da so učinki aktivatorjev AMPK na delovanje NKA odvisni od presnovnega stanja HSMC. V ishemičnih razmerah je visoka koncentracija metformina značilna povišana izražanje podenot NKA in FXYD1. Zanimiv rezultat predstavlja različen učinek metformina v ishemičnih razmerah na izražanje FXYD1 in FXYD5. Medtem ko je metformin v ishemičnem zavrl izražanje FXYD1, pa smo pri FXYD5 opazili trend k povečanju izražanja. Potrdili smo tudi, da lahko SSZ aktivira AMPK. V nasprotju s pričakovanji nismo zaznali aditivnega učinka SSZ na aktivacijo AMPK, ko so bile celice tretirane s SSZ in AICAR, kar nakazuje, da v HSMMC in L6 celicah SSZ aktivira AMPK preko drugih mehanizmov, kot je inhibicija ATIC. Zanimivo je, da je imel SSZ nasprotno učinke v L6 celicah in HSMMC na inzulinsko signalizacijo.
ZAKLJUČKI

Prvič, pokazali smo, da fuzija mioblastov v večjedrne cevčice vpliva na izražanje podenot NKA, FXYD1 in FXYD5 na način, da njihov profil izražanja postane bolj podoben tistemu v zrelih mišičnih vlaknih, a so razlike še vedno izrazite, še posebej v profilu izražanja NKA β-podenot. Inervacija ni imela vpliva na izražanje podenot NKA, FXYD1 in FXYD5 na ravni mRNA. V HSMC smo uspešno zaznali FXYD1 in FXYD5, kar odpira nove možnosti za proučevanje njunih vplivov na delovanje NKA v skeletnih mišicah.

Drugič, ishemična vadba proti majhnemu uporu poveča izražanje NKAα1, kar bi lahko povečalo mišično maso pacientov s poškodbo ACL. Naši rezultati na mišičnih vzorcih pacientov s poškodbo ACL niso potrdili povezave med aktivacijo AMPK in spremembami v izražanju NKA in FXYD proteinov, tako da druge hipoteze ne moremo potrditi. V in vitro poskusih je imelo na izražanje podenot NKA največji vpliv pomanjkanje glukoze, medtem ko hipoksiija ni imela večjih učinkov.

Tretjič, učinki metformina, ki je aktivator AMPK, so bili izrazito odvisni od presnovnega stanja HSMC, kar podpira tretjo hipotezo doktorske naloge. Visoka koncentracija metformina je znižala izražanje podenot NKA in FXYD1 v kombinaciji z ishemijo, medtem ko je v ishemičnih razmerah metformin povišal izražanje FXYD5. Pokazali smo tudi, da SSZ aktivira AMPK v L6 celicah in v manjši meri tudi v HSMC. V nobenem celičnem modelu nismo zaznali aditivnih učinkov SSZ v kombinaciji z AICAR na aktivnost AMPK.
1 INTRODUCTION

Skeletal muscles can be subjected to many kinds of stressful conditions that normally involve different levels of oxidative and metabolic stress. Whether the stress is beneficial or negative for functioning of skeletal muscles depends largely on the length and intensity of the stressful stimuli (Scicchitano et al., 2018). Stressful conditions that arise in skeletal muscle during exercise training can cause beneficial adaptations in the muscle (de Freitas et al., 2017), which makes it function better and more efficiently, while the stressful conditions that arise as a result of injury or critical illness for example, can cause muscle atrophy and sarcopenia (Sutton, 1938; Marcell, 2003; Puthucheary et al., 2013). Skeletal muscles are also subjected to stressful conditions during operative procedures. Skeletal muscles of patients that undergo orthopaedic operative procedures are exposed to ischaemia-reperfusion (Appell et al., 1993; Daniel et al., 1995). Ischaemia-reperfusion injury, caused by blocking arterial blood supply to the operated tissue contributes to the muscle atrophy in the first weeks after surgery (Appell et al., 1993). It was reported that usage of tourniquet, which is needed during surgery to reduce the loss of blood, could cause oedema of muscle and occlusion of surrounding microcirculation after as little as 15 min (Appell et al., 1993), while irreversible damage on larger scale develops after 3-6 hours (Blebea et al., 1987). Here we examined how different forms of stress, including exercise and ischaemia, might affect Na⁺/K⁺-ATPase (NKA) in skeletal muscle.

1.1 Na⁺/K⁺-ATPase

NKA is a transmembrane pump, which transports two K⁺ into the cell and three Na⁺ from the cell against their concentration gradients (Skou, 1989) (Fig. 1.1). In animal cells NKA represents the only mechanism that resists the loss of intracellular K⁺ and uptake of extracellular Na⁺ and enables the cellular maintenance of water and ion homeostasis, preservation of membrane potential and Na⁺-coupled transport of various substances (Rossier
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et al., 2015). In addition, in skeletal muscle maintenance of ion gradients by NKA is important for muscle contractility (Clausen, 2003; Pirkmajer & Chibalin, 2016).

Figure 1.1: Structure of $\text{Na}^+$/K$^+$-ATPase. NKA comprises a catalytic $\alpha$-subunit (isoforms $\alpha$1-4) and a glycoprotein $\beta$-subunit (isoforms $\beta$1-3). Among $\alpha$-isoforms the $\alpha$2-subunit predominates in skeletal muscle and is especially important during contractions, while the $\alpha$1-subunit is involved in housekeeping functions under resting conditions. Among $\beta$-isoforms the $\beta$1- and $\beta$2-subunits predominate in skeletal muscle. FXYDs are a family of small transmembrane proteins that regulate NKA. In skeletal muscles phospholemman (FXYD1) is the most important regulator of NKA, while dysadherin (FXYD5) may also play a role, especially during inactivity.

1.1.1 NKA in skeletal muscle and its regulation

NKA is a heterodimer that consists of the catalytic $\alpha$-subunit (type IIC P-ATPase of 100-112-kDa size) and the glycoprotein $\beta$-subunit (35-60 kDa) (Blanco & Mercer, 1998; Garty & Karlish, 2006; Geering, 2008) (Fig. 1.1). In skeletal muscles $\alpha$1-, $\alpha$2- and $\alpha$3-isoforms can be found (Orlowski & Lingrel, 1988; He et al., 2001), of which $\alpha$2 predominates (Hansen, 2001). This is in contrast with majority of other tissues, where $\alpha$1 is the most common (Shyjan & Levenson, 1989). By estimates $\alpha$2 isoform represents between 60 and 90% of all $\alpha$-isoforms in the skeletal muscle (Orlowski & Lingrel, 1988; Hansen, 2001; He et al., 2001; Kristensen & Juel, 2010).
Three isoforms of β-subunit are found in skeletal muscle (β1-3) (Blanco & Mercer, 1998), of which β1 and β2 present a large majority (Clausen, 2003). Unlike the α-subunit, β-subunits have no catalytic activity, but play a fundamental role in assembly, maturation and function of α/β-heterodimers (Ackermann & Geering, 1990; McDonough et al., 1990; Geering, 2008). β-subunit is also involved in modulation of kinetic characteristics of α/β-heterodimers (Crambert et al., 2000) and is presumed to modify sensitivity of α/β-heterodimers to regulatory alterations like glutathionylation (Juel, 2014).

Heterodimers, built from different α- and β-isoforms, differ in their kinetic characteristics, such as enzyme and transport activity, and their affinity for Na⁺, K⁺ and ATP (Blanco & Mercer, 1998). While α1/β-heterodimers are primarily responsible for maintenance of Na⁺ and K⁺ gradients in resting skeletal muscle, α2/β-heterodimers are predominantly active when the muscle contracts and therefore have a more important role in muscle contractions (Hundal et al., 1992; Radzyukevich et al., 2013). Beside their differences in function α1/β- and α2/β-heterodimers also differ in their location within skeletal muscle cells. While α1/β-heterodimers are largely localized to sarcolemma (Hundal et al., 1994; Lavoie et al., 1997), α2/β-heterodimers are predominantly found in T-tubules (Cougnon et al., 2002; Radzyukevich et al., 2013). Although the T-tubules hold the majority of α2/β-heterodimers, α2/β-heterodimers are also found in specific domains of sarcolemma, like caveolae (Kristensen et al., 2008), costameres (Williams et al., 2001) and neuromuscular junction (NMJ) (Heiny et al., 2010; Chibalin et al., 2012a). NKAα3 has also been detected in skeletal muscle (Perry et al., 2013), but compared to α2- and α1-subunits it is expressed in far lower quantities, and it is still not clear what is its role in skeletal muscle.

NKA in skeletal muscle can be regulated by factors of local or systemic origin (Clausen, 2003; Pirkmajer & Chibalin, 2016; Pirkmajer et al., 2021) and these can affect NKA's total levels, its total membrane content, and its intrinsic activity. Intrinsic activity of NKA is
regulated by the concentrations of its substrates, $\text{Na}^+$, $\text{K}^+$, and ATP (Skou, 1957), covalent modifications of NKA subunits through phosphorylation and glutathionylation (Clausen, 2003), and protein interactions between NKA and proteins from the FXYD family (Sweedner & Rael, 2000).

1.1.2 **FXYD regulatory proteins of NKA in skeletal muscle**

FXYD proteins are small transmembrane regulators of ion transport, which regulate NKA in tissue-specific manner (Sweedner & Rael, 2000; Geering, 2008). Their name derives from a common PFxYD motif in their N-terminal extracellular part. Among FXYD proteins phospholemman (FXYD1) is the most important regulator of NKA in skeletal muscles (Benziane et al., 2011; Boon et al., 2012). FXYD1 binds both to NKA $\alpha$1- and $\alpha$2-isoforms (Crambert et al., 2002). Interaction between unphosphorylated FXYD1 and NKA reversibly reduces NKA’s affinity for $\text{Na}^+$ (increased $K_{1/2\text{Na}}$) and reduces its activity, while the phosphorylation of FXYD1 causes an increase in NKA activity (increased maximal ATPase activity or $V_{\text{max}}$; increased maximal transport activity or $I_{\text{max}}$) and affinity for $\text{Na}^+$ (reduced $K_{1/2\text{Na}}$) (Crambert et al., 2002; Bibert et al., 2008). Human FXYD1 contains phosphorylation sites in the C-terminal at Ser63, Ser68, and Thr69 (Bibert et al., 2008; Fuller et al., 2009). NKA in skeletal muscles is probably also regulated by dysadherin (FXYD5), which increases NKA's $V_{\text{max}}$ and whose expression has also been reported to increase in the periods of inactivity (Boon et al., 2012).

1.2 **Effect of oxidative and metabolic stress on NKA function in skeletal muscle**

NKA is one of the main consumers of energy in human body. It is actually reported to use somewhere between 19-28% of all systemic ATP (Rolfe & Brown, 1997), and even though percentage-wise actomyosin ATPase and sarcoplasmic reticulum Ca$^{2+}$-ATPase are bigger consumers in skeletal muscles, NKA still uses about 5-10% of all ATP in contracting muscle
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(Rolfe & Brown, 1997). Thus, it seems logical that any kind of metabolic or oxidative stress would have pronounced effect on NKA function and could in excess have disastrous consequences for cellular and whole-body homeostasis.

1.2.1 Oxidative stress and its effects on NKA function

Suppression of NKA activity by oxidative stress is achieved with reversible glutathionylation of \(\alpha\)- (Petrushanko et al., 2012) and \(\beta1\)-subunit (Figtree et al., 2009). Glutathionylation reduces NKA activity by forming covalent links between glutathione and reactive thiol residues on \(\alpha\)- and \(\beta\)-subunits (Juel, 2014). FXYD1 is another way, how glutathionylation can affect NKA function, but in this case, glutathionylation inhibits FXYD1, which leads to increased NKA function (Bibert et al., 2011). Interestingly NKA activity can also be reduced by glutathionylation during training, and has been proposed as one of the mechanisms that lead to the development of muscle fatigue (Juel et al., 2015).

1.2.2 Effects of hypoxia and ischaemia on NKA function

It has been shown that ischaemia supresses NKA activity in many different tissues, among others it supresses NKA activity in rat cardiac myocytes (Fuller et al., 2003), in basolateral membranes of human liver allografts (Benkoel et al., 2004) and in rat liver (Lee et al., 2000), and causes NKA endocytosis from the basolateral membrane of Madin-Darby canine kidney cells (MDCK) (Seo-Mayer et al., 2011). Hypoxic stress can also reduce NKA activity or induce its endocytosis. In rat alveolar epithelial cell line prolonged hypoxia supressed NKA activity (Planès et al., 1996; Dada et al., 2003; Comellas et al., 2006; Gusarova et al., 2011), while exposure to prolonged hypoxic conditions caused a decrease in NKA content in skeletal muscles of human subjects that were acclimatizing to high altitude (Green et al., 1999; Green et al., 2000).
1.2.3 Energy stress, AMPK and its effect on NKA function

Skeletal muscle is a contractile tissue and one of major energy consumers in human body (Rolfe & Brown, 1997), and thus susceptible to energy availability fluctuations (Clausen, 2003). As ATP molecules are consumed during prolonged and intensive muscle contractions, large quantities of ADP and AMP molecules accumulate in skeletal muscle tissue (Hardie & Hawley, 2001; Allen et al., 2008). Increased ADP/ATP and AMP/ATP ratios cause an activation of 5' AMP-activated protein kinase (AMPK) (Hardie & Hawley, 2001). AMPK is a heterotrimeric complex that consists of the catalytic α-subunit and the regulatory β- and γ-subunits. It acts as a cellular energy sensor that modulates energy metabolism, cell growth and cell cycle (Hardie et al., 2012b; Hardie, 2015). When the energy levels are low, AMP binds to γ-subunit, this in turn causes an allosteric activation of AMPK and increases phosphorylation of the α-subunit at Thr172 (Grahame Hardie, 2016). AMPK stimulates energy-producing processes and inhibits energy-consuming processes and thus restores energy balance. NKA uses ATP for its transporting of Na\(^{+}\) and K\(^{+}\) ions, and this makes its function inseparably intertwined with AMPK's role in the cell.

According to the available evidence the role of AMPK in regulation of NKA is unclear. Indeed, activation of AMPK was shown to stimulate or inhibit the activity of NKA. While Gusarova et al. (2011) observed that an activation of AMPK can reduce the activity of NKA through Ca\(^{2+}\)/calmodulin-dependent kinase kinase β (CaMKKβ) in rat alveolar epithelial cells, Benziane et al. (2012) report that activation of AMPK with AICAR (5-Aminoimidazole-4-carboxamide ribonucleoside), which is the most commonly used pharmacological AMPK activator, in L6 rat muscle cell line increases NKA activity (Fig. 1.2). It was also shown that metformin, an indirect activator of AMPK reduced endocytosis of NKA in MDCK cells (Seo-
Mayer et al., 2011). Thus, hypoxia might, in the same way as AICAR, cause an activation of skeletal muscle NKA through AMPK.

**Figure 1.2 Possible connections between AMPK activation and NKA activity.** Changes in energy status activate AMPK in three different ways. When ADP and/or AMP replace ATP at one or more locations on AMPKγ-subunit, this leads to conformational change in the heterotrimeric complex. Conformational changes (1) promote phosphorylation, (2) suppress dephosphorylation of Thr172, which causes up to 100-fold increase in AMPK activity. (3) Binding of AMP (but not ADP) induces additional activation of phosphorylated AMPK (up to 10-fold). Upstream liver kinase B1 (LKB1) is constitutively active but its high basal activity is necessary for the effects of AMP or ADP binding to the γ-subunit on net Thr172 phosphorylation to become evident. Increase in intracellular Ca^{2+} causes an increase in activity of Ca^{2+}/calmodulin-dependent kinase kinase β, which can also activate AMPK by Thr172 phosphorylation, but this process is independent of changes in AMP/ATP and ADP/ATP ratios. Hypoxia causes CaMKKβ-mediated AMPK activation in alveolar type II cells, which consequently leads to decreased NKA activity. When AICAR, the most widely used experimental activator of AMPK, enters the cell, it is transformed into ZMP (AICAR-monophosphate). ZMP is an AMP analogue that binds to AMPK γ-subunit and directly activates AMPK. AICAR-induced AMPK activation in L6 cell line leads to increased NKA activity. Modified from Hardie (2013), Gusarova et al. (2011) and Benziane et al. (2009).
1.3 Effects of exercise and pharmacological AMPK activators on NKA function

1.3.1 Effects of physical activity on NKA function

Physical activity or inactivity has a great effect on the expression, content and activity of NKA (Clausen, 2003). Exercise can regulate NKA in both acute and chronical way. A bout of exercise leads to higher intrinsic activity of NKA (Tsakiridis et al., 1996; Juel et al., 2000a) and also promotes NKA translocation to the sarcolemma (Tsakiridis et al., 1996; Juel et al., 2000a). The recruitment of α2/β-heterodimers is necessary for the increased NKA activity during exercise and it is important to note that hypothetical activation of only α1/β-heterodimers cannot support normal exercise performance (Radzyukevich et al., 2013). Generally chronic exercise training elevates muscle NKA content (Green et al., 1993; McKenna et al., 1993; Evertsen et al., 1997), while physical inactivity decreases it (Ditor et al., 2004; Boon et al., 2012). In some studies exercise training increased the expression of FXYD1 (Thomassen et al., 2016; Christiansen et al., 2018b), but in others FXYD1 was unchanged or even reduced (Benziane et al., 2011; Nordsborg et al., 2012; Christiansen et al., 2019). In subjects with complete spinal cord injury, FXYD1 was reduced, while ratio of phosphorylated to total FXYD1 did not change (Boon et al., 2012). Conversely, 2 weeks of inactivity decreased ratio of phosphorylated to total FXYD1 but did not reduce total abundance of FXYD1 in professional football players (Thomassen et al., 2010). A recent study proposed that α1-subunit may play a major trophic role in skeletal muscles (Kutz et al., 2018). A decreased expression of α2-subunit was also observed in vastus lateralis muscle of knee injury patients (Perry et al., 2015). The same authors proposed that the decreased abundance of NKA and content of α2 might play a role in the impaired muscle function. The question thus remains whether an increased NKA content would lead to an overall improvement in muscle fitness and health.
1.3.2 Effects of ischaemic preconditioning and training with blood flow restriction on NKA function

NKA shows remarkable plasticity in different physiological conditions and changes in its content and activity may play a role in certain pathologies as well as play a part in beneficial effects of certain interventions like ischaemic preconditioning or training with blood flow restriction (BFR). Ischaemic preconditioning is a powerful mechanism that can protect human tissue and organs against injury from ischaemic reperfusion. Ischaemic preconditioning maintains NKA activity in physiologically normal levels and by this protects cells against detrimental effects of ischaemic reperfusion (de Souza Wyse et al., 2000; Elmoselhi et al., 2003; Inserte et al., 2006). Low-load resistance training with blood-flow restriction (LL-BFR) in active muscles is emerging as a plausible effective alternative for enhancing muscle strength and hypertrophy (Takarada et al., 2000; Kubo et al., 2002) and it shows great therapeutic potential for patients with knee joint dysfunction, such as knee injury (Zargi et al., 2018), osteoarthritis (Segal et al., 2015; Bryk et al., 2016) and patellofemoral pain syndrome (Giles et al., 2017) because it substantially reduces mechanical stress of the joints. It is reported that LL-BFR training can induce hypertrophy even when it is performed at only 15-40% of training intensity, recommended when training without BFR (Loenneke et al., 2012; Yasuda et al., 2014). A recent clinical studies showed protective effects of LL-BFR exercise on endurance after anterior cruciate ligament (ACL) reconstruction (Zargi et al., 2018) and on increased leg press and knee extensor strength in women with risk factors for symptomatic osteoarthritis (Segal et al., 2015). High intensity BFR training (HI-BFR) induced isoform specific changes of NKA and FXYD1 in skeletal muscle of healthy human subjects (Christiansen et al., 2018b; Christiansen et al., 2019) and induced AMPK activation (Christiansen et al., 2018b). However, it has not been established whether alterations in NKA content and/or function contributes to skeletal muscle adaptations to LL-BFR training.
1.3.3 AMPK activators and their potential use in NKA regulation

Modulation of AMPK presents an opportunity to ameliorate or treat many pathological conditions. In type 2 diabetes pharmacological AMPK activation reduces gluconeogenesis, increases glucose disposal and lipid oxidation, which improves metabolic homeostasis (Long & Zierath, 2006; Fogarty & Hardie, 2010). Activation of AMPK also leads to reduced lipid and protein synthesis, consequently suppressing cell growth and proliferation rate. This implies that AMPK activators might be effective in cancer treatment (Fogarty & Hardie, 2010; Hardie, 2015), however in the recent years there were also reports that in some cancers AMPK actually plays an important role in tumour growth and proliferation, where inhibition of AMPK might be beneficial for cancer treatment (Rios et al., 2013; Laderoute et al., 2014). Due to this AMPK is a major target for development of new pharmacological treatments for type 2 diabetes and cancer (Hardie et al., 2012a).

Apart from type 2 diabetes and cancer, AMPK activators have been implied for treatment of heart failure (Wang et al., 2012), ischaemia (Seo-Mayer et al., 2011), atherosclerosis (Li et al., 2010; Cao et al., 2016), chronic pain (Baeza-Flores et al., 2020) and aging (Burkewitz et al., 2014) among others, while AMPK inhibitors might have a beneficial role in treating nicotine-induced insulin resistance (Nogueiras et al., 2015; Wu et al., 2015) and hereditary cardiomyopathy (Hudson et al., 2003). Some of the diseases, where AMPK could be a possible therapeutic target are listed in Fig. 1.3. As AMPK activation can increase NKA activity, we were interested, whether we can use potential pharmacological activators of AMPK to affect NKA function. In continuation a brief overview of pharmacological AMPK activators is provided first, which is followed by a discussion of modulation of NKA by AMPK in skeletal muscle.
Figure 1.3: AMPK is a possible therapeutic target in an expanding number of diseases. The left panel cites diseases that may benefit from AMPK activators; the ones in italics are more novel and require further studies. The right panel represents the disorders that could benefit from AMPK inhibitors; the ones in green letters indicate diseases that could benefit from both AMPK activators and inhibitors depending on the case. Modified from Miglianico et al. (2016).

There are many known pharmacological activators of AMPK that can work through different mechanisms. Direct AMPK activators, such as AICAR-monophosphate (ZMP); A-769662, and salicylate bind to AMPK and activate it directly (Hardie et al., 2012a). AICAR, the most widely used AMPK activator, is a non-phosphorylated precursor of ZMP (AICAR-monophosphate). Indirect AMPK activators on the other hand can activate AMPK by suppressing mitochondrial respiration, like metformin or phenformin (El-Mir et al., 2000; Owen et al., 2000; Veiga et al., 2018) by inhibiting glycolysis like 2-deoxyglucose (Kim et al., 2004) or suppress conversion of ZMP to inosine monophosphate like methotrexate (Beckers et al., 2006; Pirkmajer et al., 2015) (Fig. 1.4; as reviewed by Hawley et al. (2010)). Although some pharmacological activators might have a potential to also activate NKA, it is known that A-769662, a direct activator of AMPK also inhibits NKA activity (Benziane et al., 2009). Apart from some of them being known NKA inhibitors, the problem with available pharmacological
AMPK activators is that they are not effective in activating muscle AMPK complexes, have poor bioavailability and can cause serious side effects. Finding new effective strategies to activate AMPK in skeletal muscle is a huge challenge that is still ongoing.

Figure 1.4: Pharmacological activators of AMPK and their possible interactions with NKA activity. AMPK has a catalytic α-subunit and regulatory β- and γ-subunits. Energy shortage increase concentrations of AMP, which binds to the AMPK γ-subunit. AMP causes allosteric activation of AMPK (10-fold) and suppresses dephosphorylation of the α-subunit. This leads to further increases in AMPK activity (100-fold). Once activated, AMPK increases glucose uptake, fatty acid oxidation, and mitochondrial biogenesis in skeletal muscle. These effects improve metabolic control in type 2 diabetes. Direct AMPK activators, such as ZMP, A-769662, salicylate, and 991, bind to AMPK and activate it directly. AICAR, the most widely used AMPK activator, is a non-phosphorylated precursor of ZMP (AICAR-monophosphate). Indirect AMPK activators suppress mitochondrial respiration and increase concentrations of AMP, which then activates AMPK directly. Methotrexate promotes
AMPK activation by suppressing conversion of ZMP to inosine monophosphate. A-769662 is also known to directly bind to NKA and inhibit its activity.

Metformin, the most commonly used oral antidiabetic drug, acts as an indirect AMPK activator. It activates AMPK by inhibiting complex I of mitochondrial respiratory chain (El-Mir et al., 2000; Owen et al., 2000), this in turn leads to a decrease in ATP production, which causes an increase in AMP levels. AMP molecules can then bind directly to AMPK and cause its allosteric activation and phosphorylation. Study on a murine model of myocardial ischaemia-reperfusion injury showed cardioprotective effects of a single dose of metformin against myocardial infarction due to ischaemia-reperfusion injury, and this was done through AMPK activation (Calvert et al., 2008). It was also shown in MDCK cells that metformin-induced AMPK activation might reduce damage to kidney cells, caused by renal ischaemia (Seo-Mayer et al., 2011). Metformin also inhibited ischaemia-induced endocytosis of NKA, and thus preserved its function on the membrane and Seo-Mayer et al. (2011) proposed that this was a consequence of metformin's activation of AMPK. In this doctoral dissertation we examined whether metformin has an effect on NKA expression in cultured HSMC and whether its effects on NKA expression depend on the metabolic state of HSMC at the time of treatment.

Several non-steroidal anti-inflammatory (aka anti-rheumatic) drugs are known to inhibit AICAR transformylase (ATIC) (Baggott et al., 1992), among them salicylate, an active component of willow bark, which is most known for its allosteric activation of AMPK and suppression of AMPK dephosphorylation on its activating phosphorylation site (Hawley et al., 2012). Methotrexate, another widely used anti-rheumatic as well as anti-cancer drug, has also been shown to promote AMPK activation (Beckers et al., 2006). Methotrexate has several intracellular targets, including ATIC, an enzyme that converts ZMP, an AMP analogue into IMP. By inhibiting ATIC they increase ZMP concentrations in the cells, which can lead to
increased AMPK activation. It was already shown in cancer cell lines, L6 cells and primary human skeletal muscle cells (HSMC) that methotrexate markedly augments AMPK activation by AICAR (Beckers et al., 2006; Pirkmajer et al., 2015), while salicylate can synergize with metformin and activate AMPK in liver cells and cancer cells (Ford et al., 2015; O'Brien et al., 2015).

ATIC is also inhibited by sulfasalazine (SSZ), a widely used anti-rheumatic drug. SSZ increased intracellular concentrations of ZMP in murine splenocytes (Gadangi et al., 1996), possibly due to inhibition of ATIC (Fig. 1.5) (Baggott et al., 1992), which may lead to increased AMPK activity. It has recently been shown that SSZ increased AMPK activity in rat colonic tissue (Park et al., 2019). Notably, certain reports linked SSZ to improvements in glucose homeostasis in humans (Svenson et al., 1987; Haas et al., 2005), which might be caused by SSZ-induced AMPK activation.

Figure 1.5: Effects of sulfasalazine and methotrexate on ZMP metabolism and AMPK activation. Adenosine kinase transforms exogenous AICAR into ZMP (AICAR-monophosphate) (not shown). ZMP is an AMP analogue that binds to AMPK γ-subunit and directly activates AMPK. Methotrexate (MTX) inhibits 2 enzymes in de novo purine synthesis: 5-Aminooimidazole-4-Carboxamide Ribonucleotide Formyltransferase/IMP Cyclohydrolase (ATIC) and Glycinamide ribonucleotide formyltransferase (GART). ATIC is more sensitive to MTX than GART, which is why low doses of MTX preferentially inhibit ATIC. Sulfasalazine (SSZ), another widely used
AMPK activation can lead both to NKA inhibition or activation. Pirkmajer et al. (2021) propose two regulatory mechanism through which AMPK can affect NKA function. First involves AMPK-induced suppression of FXYD1 that can also include changes to the relative amount of the phosphorylated FXYD1, while the second affects NKA translocation to the plasma membrane via AMPK activation. Whether AMPK activation leads to increased or decreased NKA activity/content seems to depend on the signalling pathway which connects NKA and AMPK. In pulmonary alveolar cells there are several studies that show AMPK-stimulated reduction in plasma membrane levels of NKAα1 (Vadasz et al., 2008; Gusarova et al., 2011). Increase in CO2 levels stimulated AMPK through CaMKKβ, which consequentially promoted activation and translocation of protein kinase Cζ (PKCζ) (Vadasz et al., 2008). PKCζ induced NKAα1 phosphorylation on Ser18 (Dada et al., 2003), which promotes NKAα1 endocytosis (Chibalin et al., 1998). In rat L6 skeletal muscle cell line AICAR-induced AMPK activation did not promote PKCζ activation and actually led to dephosphorylation of NKAα1 at Ser18 (Benziane et al., 2012). Benziane et al. (2012) proposed that AICAR-induced AMPK activation led to an increased activity of protein phosphatase 2A (PP2A), which dephosphorylated NKAα1 at Ser18 and actually promoted NKAα1 transport to the cell membrane.

As we showed there is strong evidence that AMPK plays an important part in NKA regulation and that AMPK-induced activation of NKA might have beneficial effects on its function in skeletal muscle. The problem is that numerous known AMPK activators are being ineffective in activation of muscle AMPK heterotrimers, have poor bioavailability and can cause serious side due to their unspecific mode of action, while several are also known to inhibit anti-rheumatic drug, was also reported to inhibit ATIC, which also makes it a potential AMPK activator. Abbreviations: IMP – inosine monophosphate, PRPP – 5-phosphoribosyl pyrophosphate
NKA activity. Thus, search for new pharmacological agents that would efficiently activate AMPK in skeletal muscle and also promote NKA activity remains a popular topic of research.

1.4 In vitro cell models for NKA research in skeletal muscle

In majority in vitro experiments concerning NKA research are performed either on L6 rat skeletal muscle cell line (Richler & Yaffe, 1970), mouse C2C12 skeletal muscle cell line (Yaffe & Saxel, 1977), or primary HSMC (Blau & Webster, 1981) and various other animal species, including rat (Tesseraux et al., 1987), mouse (Metzinger et al., 1993), and chicken (Mege et al., 1992). Among others, L6, C2C12 and HSMC myoblasts can be differentiated into multinucleated myotubes by lowering serum content in culture media (Yaffe & Saxel, 1977; Lawson & Purslow, 2000; Abdelmoez et al., 2020). Normally myoblasts are kept in media, supplemented by 10% foetal bovine serum (FBS) (v/v) (Blau & Webster, 1981; Cox et al., 1990). The differentiation is induced by lowering serum concentration to 2% (v/v). The problem is that by reducing serum content we can achieve only limited differentiation of myotubes (Askanas et al., 1977) that in regard to NKA content and localization only slightly resemble mature muscle fibres (Pirkmajer et al., 2020).

As we described earlier, human skeletal muscle contains large amounts of NKA α2/β-heterodimers that are predominantly located on the inner membranes of T-tubules (Radzyukevich et al., 2013), while, to the lesser extent, they are also found on the postsynaptic membrane of NMJ (Chibalin et al., 2012a) and surface caveolae (Kristensen et al., 2008). Aneural (non-innervated) differentiated HSMC typically do not have basal lamina, cross-striations or T-tubules (Askanas et al., 1987). The expression of α2/β-heterodimers in rat primary skeletal muscle cells is low (Sharabani-Yosef et al., 1999) and the same was found for rat L6 and L8 skeletal muscle cell line (Mitsumoto, 1993; Ragolia et al., 1997). Also, the expression of FXYD1 was reported to be barely detectable (Benziane et al., 2012). Human
primary skeletal muscle cells do express both NKA α1- and α2-subunits and the expression of α2-subunit increases with the differentiation of myoblasts into multinucleated myotubes (Al-Khalili et al., 2004), but α2-subunit does not predominate as in mature muscle fibre (Pirkmajer et al., 2020). Also, to our knowledge there are no available publications that would describe successful detection of FXYD1 in cultured HSMC. When Abdelmoez et al. (2020) compared transcriptomes and metabolomes of L6 cells, C2C12 and cultured HSMC with their tissues of origin, they observed that although in vitro cell cultures were most similar to their tissue of origin regarding gene expression and metabolic profile, there were still significant differences.

Expression of α1- and α2-subunits and activity of NKA also increase when differentiated HSMC are treated with neural agrin (Jurdana et al., 2009), heparan sulphate proteoglycan, which is best known for its role in the organization and maintenance of postsynaptic structures at the NMJ (McMahan, 1990; Burden, 2002). This is in accordance with the finding that innervation of differentiated HSMC with explants of rat embryonic spinal cords increases their membrane potential compared to non-innervated myotubes (Askanas et al., 1987). To trigger contractions cultured HSMC need innervation (Martinuzzi et al., 1986), which leads to the development of T-tubules and more complex structured sarcoplasmic reticulum (Askanas et al., 1987), or electrical pulse stimulation (Aas et al., 2002; Nikolić & Aas, 2019), (Fig. 1.6). In our laboratory we use rat embryonic spinal cord explants for innervation of HSMC. Spinal cord explants provide motor neurons that in time innervate HSMC, form NMJs, induce prolonged maturation of HSMC and cause them to contract. As HSMC contract because of the stimulation through NMJ, in vitro innervation presents a model that allows muscle contractions in physiological conditions. By using chimeric co-culture model, we can also benefit from using species-specific antibodies or PCR primers and distinguish between the expression of specific proteins in neural and muscle component
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(Jevsek et al., 2004). We presume that by using innervated HSMC, we might achieve an expression pattern of NKA subunits and its FXYD regulators that would mimic the one, found in mature human muscle fibres.

**Figure 1.6: Human skeletal muscle cell cultures.** Satellite cells are isolated from the skeletal muscle biopsy. After cells are seeded, they develop into myoblasts, which proliferate and start to fuse into multinucleated myotubes either spontaneously (innervated co-cultures) or by lowering serum concentration from 10% FBS to 2% FBS. Human aneural (non-innervated) myotubes do not have neuromuscular junctions (NMJ) and do not contract spontaneously. Human skeletal muscle cells contract when exposed to electric pulse stimulation (EPS) (Aas et al., 2002) or if they are co-cultured with rat embryonic spinal cord explants (Askanas et al., 1987). Neurites from spinal cord explants innervate human myotubes and form NMJ. Innervated myotubes start to contract spontaneously after 7-10 days of co-culture due to neuromuscular transmission by rat motor neurons.
UVOD


1.1 Na⁺/K⁺-ATPaza

NKA je transmembranska črpalka, ki preko celične membrane transportira 2 K⁺ v celico, medtem ko 3 Na⁺ transportira ven iz celice proti njunima koncentracijskim gradientoma (Skou, 1989) (Slika 1.1). V živalskih celicah NKA predstavlja edini mehanizem, ki nasprotuje izgubi znotrajceličnega K⁺ in vnosu zunajceličnega Na⁺, s tem pa vzdržuje celično
homeostazo vode in ionov, membranski potencial in z Na\(^+\)-sklopljeni transport številnih snovi (Rossier \textit{in sod.}, 2015). V skeletni mišici je ohranjanje ionskih gradientov s strani NKA poleg vsega naštetega potrebno tudi za mišično krčenje (Clausen, 2003; Pirkmajer in Chibalin, 2016).

**Slika 1.1: Struktura Na\(^+\)/K\(^+\)-ATPaze.** NKA je sestavljena iz katalitske α-podenote (izooblike α1-4) in glikoproteinske β-podenote (izooblike β1-3). V skeletni mišici med α-izooblikami prevladuje α2-podenota, ki ima še posebej pomembno vlogo med mišičnim krčenjem, medtem ko ima α1-podenota vzdrževalno funkcijo v obdobjih mirovanja. V skeletni mišici izmed β-podenot prevladujeta β1- in β2-podenoti. FXYD molekule sestavljajo družino malih transmembranskih proteinov, ki regulirajo NKA. Fosfoleman (FXYD1) ima izmed FXYD proteinov v skeletni mišici najpomembnejšo regulatorno vlogo, medtem ko naj bi v regulacijo vpleten tudi disadherin (FXYD5), še posebej v obdobjih telesne neaktivnosti.

1.1.1 Regulacija NKA v skeletni mišici

NKA je heterodimer, sestavljen iz katalitske α-podenote (P-ATPaza tipa IIC velikosti od 100-112 kDa) in glikoproteinske β-podenote (35-60 kDa) (Blanco in Mercer, 1998; Garty in Karlish, 2006; Geering, 2008) (Slika 1.1). V skeletni mišici so izražene α1-, α2- in α3-izooblike (Orlowski in Lingrel, 1988; He \textit{in sod.}, 2001), od katerih prevladuje α2 (Hansen, 2001). To je v nasprotju z večino drugih tkiv, kjer je v najvišjem odstotku zastopana α1-izooblika (Shyjan in Levenson, 1989). Po ocenah naj bi α2-izooblika predstavljala med 60 in
90 % vseh α-izooblik v skeletni mišici (Orlowski in Lingrel, 1988; Hansen, 2001; He in sod., 2001; Kristensen in Juel, 2010).


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V skeletni mišici je NKA regulirana s strani lokalnih ali sistemskih dražljajev (Clausen, 2003; Pirkmajer in Chibalìn, 2016; Pirkmajer in sod., 2021), ki lahko vplivajo na njeno celokupno količino v mišičnem vlaknu, na njeno vsebnost v celični membrani, in na njeno intrinzično aktivnost. Intrinzično aktivnost NKA uravnavajo spremembe v koncentraciji njenih substratov, Na\(^+\), K\(^+\), in ATP (Skou, 1957), kovalentne modifikacije podenot NKA, ki jih povzročita fosforilacija in glutationilacija (Clausen, 2003), ter proteinske interakcije med NKA in proteini iz družine FXYD (Sweedner in Rael, 2000).

1.1.2 Uravnavanje NKA s strani FXYD proteinov v skeletni mišici

FXYD proteini so mali transmembranski regulatorji ionskega transporta, ki NKA regulirajo tkivno-specifično (Sweedner in Rael, 2000; Geering, 2008). Njihovo ime izvira iz skupnega PFxYD motiva na N-končnem zunajceličnem delu proteina. Izmed FXYD proteinov je fosfoleman (FXYD1) najpomembnejši regulator NKA v skeletni mišici (Benziane in sod., 2011; Boon in sod., 2012). FXYD1 se veže tako na α1- kot tudi na α2-izoobliko NKA (Crambert in sod., 2002). Interakcija med nefosforiliranim FXYD1 in NKA reverzibilno zniža afiniteto NKA za Na\(^+\) (zviša se K\(_{1/2\text{Na}}\)) in aktivnost NKA, medtem ko fosforilacija FXYD1 povzroči zvišanje aktivnosti NKA (povečana maksimalna ATPazna aktivnost ali V\(_{\text{max}}\); povečana maksimalna transportna aktivnost ali I\(_{\text{max}}\)) in njene afinitete za Na\(^+\) (zniža se K\(_{1/2\text{Na}}\)). (Crambert in sod., 2002; Bibert in sod., 2008). Humani FXYD1 vsebuje fosforilacijska mesta na C-koncu na ostankih Ser63, Ser68 in Thr69 (Bibert in sod., 2008; Fuller in sod., 2009). V skeletni mišici delovanje NKA verjetno uravnava tudi disadherin (FXYD5), ki ob vezavi na NKA poviša njeno V\(_{\text{max}}\). Zvečano izražanje FXYD5 v skeletni mišici so zaznali pri pacientih s popolno poškodbo vratne hrbtenjače (Boon in sod., 2012).
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1.2 Učinki oksidativnega in presnovnega stresa na delovanje NKA v skeletni mišici

NKA je ena glavnih porabnic energije v človeškem telesu. Študije poročajo, da naj bi NKA porabila nekje med 19-28 % ATP v telesu (Rolfš in Brown, 1997), in kljub temu da je ta odstotek v skeletni mišici nižji zaradi aktomiozinske ATPaze in Ca\(^{2+}\)-ATPaze sarkoplazemskega retikuluma (SERCA), naj bi NKA v skeletni mišici še vedno porabila približno 5-10 % vsega ATP v kontrahirajoči mišici (Rolfš in Brown, 1997). Zdi se torej logično, da bi imel kakršen koli presnovni ali oksidativni stres izrazite učinke na delovanje NKA, ki bi lahko v preveliki meri imeli uničujoče posledice za homeostazo celic in celotnega telesa.

1.2.1 Oksidativni stres in njegovi učinki na delovanje NKA


1.2.2 Učinki hipoksije in ishemije na delovanje NKA

Pretekle študije so pokazale, da ishemija zavre aktivnost NKA v številnih različnih tkivih, med drugim ishemija zavre aktivnost NKA v podganjih miocitih (Fuller in sod., 2003), v bazolateralnih membranah humanih transplantiranih ledvic (Benkoel in sod., 2004) in v podganji ledvici (Lee in sod., 2000). Ishemija je v celicah Madin-Darby pasje ledvice (MDCK) povzročila tudi endocitozo NKA z bazolateralnih membran (Seo-Mayer in sod., 2011).
Hipoksični stres lahko zniža aktivnost NKA (Planès in sod., 1996; Dada in sod., 2003; Comellas in sod., 2006; Gusarova in sod., 2011), medtem ko je daljša izpostavljenost hipoksičnim razmeram znižala vsebnost NKA v skeletni mišici preiskovancev, ki so bili v procesu višinske aklimatizacije (Green in sod., 1999; Green in sod., 2000).

1.2.3 Energijski stres in njegovi učinki na delovanje NKA


Vloga AMPK pri uravnvanju NKA glede na trenutne študije še ni povsem jasna. Aktivacija AMPK lahko stimulira ali inhibira aktivnost NKA. Medtem ko so Gusarova in sod. (2011) pokazali, da lahko aktivacija AMPK v podganjih alveolarnih epitelijških celicah povzroči znižanje aktivnosti NKA preko od Ca²⁺/kalmodulina odvisne kinaze kinaze β
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(CaMKKβ), pa so Benziane in sod. (2012) pokazali, da aktivacija AMPK s strani AICAR (5-aminoimidazol-4-karboksamid ribonukleozid), najbolj pogosto uporabljenega farmakološkega aktivatorja AMPK, v podganji L6 skeletnomišični celični liniji vodi v povišano aktivnost NKA (Slika 1.2). Prav tako so v ledvičnih celicah MDCK pokazali, da metformin, posredni aktivator AMPK, zmanjša endocitozo NKA (Seo-Mayer in sod., 2011). Posledično bi lahko hipoksija v skeletnih mišicah, podobno kot AICAR, povzročila aktivacijo NKA preko aktivacije AMPK.

[Slika 1.2: Možne povezave med aktivacijo AMPK in aktivnostjo NKA] Spremembe v energijskem stanju celice lahko aktivirajo AMPK na tri različne načine. Ko ADP in/ali AMP zamenjata ATP na vezavnih mestih na AMPKγ-podenoti, pride do konformacijske spremembe heterotrimernega kompleksa. Konformacijske spremembe (1) vzpodbudijo fosforilacijo, (2) zavrejo defosforilacijo na ostanku Thr172, kar povzroči do 100-kratno povišanje aktivnosti AMPK. (3) Vzave AMP ali ADP na γ-podenoto ima izrazit vpliv na celokupno fosforilacijo ostanka Thr172 samo ob hkratni visoki bazalni aktivnosti konstitutivno aktivne jetrne kinaze B1 (LKB1), ki AMPK regulira višje na signalni poti. Zvišani nivo znotrajceličnega Ca²⁺ poveča aktivnost od Ca²⁺/kalmodulina odvisne kinaze kinaze β (CaMKKβ), kar lahko prav tako vodi v aktivacijo AMPK preko fosforilacije na ostanku

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1.3 Učinki telesne vadbe in farmakoloških aktivatorjev AMPK na delovanje NKA

1.3.1 Učinki telesne aktivnosti na delovanje NKA

NKAα1 v skeletni mišici lahko igrala pomembno trofično vlogo (Kutz in sod., 2018). Znižano izražanje NKAα2 so zaznali tudi v mišicah vastus lateralis oseb s poškodbo kolena (Perry in sod., 2015). V isti študiji so predlagali, da bi zmanjšana vsebnost NKAα2 lahko prispevala k oslabljenemu delovanju mišice. Vprašanje ostaja odprto ali bi povečana vsebnost NKA lahko prispevala k izboljšanju mišične kondicije in njenem delovanju.

1.3.2 Učinki ishemične predpriprave in ishemične vadbe na delovanje NKA

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ženskah s faktorji tveganja za simptomatsko osteoartroz (Segal in sod., 2015). Visokointenzivna BFR vadba (HI-BFR) je v skeletni mišici zdravih posameznikov inducirala izoblikam-specifične spremembe v NKA in FXYD1 (Christiansen in sod., 2018b; Christiansen ins od., 2019) in hkrati povišala aktivnost AMPK (Christiansen in sod., 2018b). Ali morebitne spremembe v vsebnosti NKA in/ali njenem delovanju prispevajo k skeletnomišičnim prilagoditvam LL-BFR vadbe ni znano.

1.3.3 Aktivatorji AMPK in njihov potencial za uravnavanje NKA


 Različne študije so nakazale, da bi lahko med drugim z aktivatorji AMPK zdravili tudi srčno popuščanje (Wang in sod., 2012), ishemijo (Seo-Mayer in sod., 2011), aterosklerozo (Li in sod., 2010; Cao in sod., 2016), kronično bolečino (Baeza-Flores in sod., 2020) in staranje (Burkewitz in sod., 2014), inhibitorji AMPK pa bi lahko imeli pozitivno vlogo pri zdravljenju z nikotinom povzročene inzulinske rezistence, (Nogueiras in sod., 2015; Wu in sod., 2015) in
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dedne kardiomiopatije (Hudson in sod., 2003). Nekatere bolezni, pri katerih bi AMPK lahko predstavljala morebitno terapevtsko tarčo, so navedene na Sliki 1.3. Ker lahko aktivacija AMPK privede do povišane aktivnost NKA, nas je zanimalo, ali bi lahko z uporabo farmakoloških aktivatorjev AMPK vplivali na delovanje NKA. V nadaljevanju bodo najprej na kratko predstavljeni farmakološki aktivatorji AMPK, ki ji bo sledil kratek vpogled v mehanizme, prek katerih bi AMPK v skeletni mišici lahko regulirala NKA.


Poznamo številne farmakološke aktivatorje AMPK, ki lahko delujejo preko različnih mehanizmov. Neposredni aktivatorji AMPK, kot so AICAR-monofosfat (ZMP), A-769662 in salicilat, se vežejo na AMPK in jo aktivirajo neposredno (Hardie in sod., 2012a). AICAR, najbolj pogosto uporabljen eksperimentalni aktivator AMPK, je nefosforilirani prekurzor ZMP.
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Slika 1.4: Farmakološki aktivatorji AMPK in njihove morebitne interakcije z aktivnostjo NKA. AMPK je sestavljena iz katalitske α-podenote and regulatornih β- in γ-podenot. Pomanjkanje energijskih zalog v celici

Metformin, najbolj pogost peroralni antidiabetik, deluje kot posreden aktivator AMPK. Metformin dokazano inhibira kompleks I v mitohondrijski dihalni verigi (El-Mir in sod., 2000; Owen in sod., 2000), kar povzroči padec v proizvodnji ATP in porast v koncentraciji AMP. AMP molekule se nato lahko neposredno vežejo na AMPK in povzročijo njeno alosterično aktivacijo in fosforilacijo. Študije na mišjem modelu ishemično-reperfuzijske poškodbe srčne mišice so pokazale, da bi lahko metforminska predpriprava z enojnim nizkim odmerkom preko aktivacije AMPK zaščitila srčno mišico pred ishemično-reperfuzijsko poškodbo pri miokardnem infarktu (Calvert in sod., 2008). Prav tako je bilo v ledvičnih MDCK celicah pokazano, da lahko z metforminom-spodbušena aktivacija AMPK zmanjša poškodbo celic, ki jo povzroči ledvična ishemia (Seo-Mayer in sod., 2011). Metformin je zavrl tudi z ishemijo-spodbušeno endocitozo NKA, s čimer je bila ohranjena funkcionalnost membran in po besedah Seo-Mayer in sod. (2011) naj bi bila to posledica z metforminom povšačene aktivnosti AMPK. V doktorski nalogi smo proučili, ali metformin vpliva na izražanje NKA v kulturi humanih skeletnih mišičnih celic (HSMC) in ali so ti učinki na izražanje NKA odvisni od presnovnega stanja, v katerem se v tistem trenutku nahajajo HSMC.
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Znano je, da nekatera nesteroidna protivnetna zdravila (znana tudi kot nesteroidni antirevmatiki) inhibirajo AICAR transformilazo (ATIC) (Baggott in sod., 1992), med njimi tudi salicilat, aktivna komponenta vrbovega lubja, ki je bolj znan po svoji alosterični aktivaciji AMPK in zavrtju AMPK defosforilacije na njenem fosforilacijskem mestu (Hawley in sod., 2012). Metotreksat, še eno pogosto predpisano protirevmatisko in protirakavo zdravilo, dokazano spodbuja aktivacijo AMPK (Beckers in sod., 2006). Metotreksat ima številne znotrajcelične tarče, vključno z encimom ATIC, ki pretvara ZMP (AMP analog) v IMP. Metotreksat inhibira ATIC, kar povzroči porast koncentracije ZMP v celicah, posledica tega pa je povišana aktivnost AMPK. V rakavih celični linijah, L6 celičah in HSMC je metotreksat signifikantno povišal aktivacijo AMPK s strani AICAR (Beckers in sod., 2006; Pirkmajer in sod., 2015), sinergistični vpliv metformina pri aktivaciji AMPK pa je bil opažen tudi v kombinaciji s salicilatom v jetrnih in rakavih celičah (Ford in sod., 2015; O'Brien in sod., 2015).

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Slika 1.5: Učinki sulfasalazina in metotreksata na presnovo ZMP in aktivacijo AMPK. Adenozinska kinaza pretvori eksogeni AICAR v ZMP (AICAR monofosfat) (ni prikazano). ZMP je AMP analog, ki se veže na AMPK γ-podenoto in neposredno aktivira AMPK. Metotreksat (MTX) inhibira dva encima v de novo purinski sintezni poti: 5-aminoimidazol-4-karboksamid ribonukleotid formiltransferaza/IMP ciklohidrolaza (ATIC) in glicinamid ribonukleotid formiltransferaza (GART). ATIC je bolj občutljiv na modulacijo s strani MTX kot GART, zaradi česar nizke doze MTX preferenčno inhibirajo ATIC. Sulfasalazin (SSZ), pogosto predpisano antirevmatsko zdravilo, naj bi prav tako inhibiralo ATIC, kar pomeni, da bi lahko potencialno SSZ aktiviral AMPK v skeletni mišici. Kratice: IMP – inozin monofosfat, PRPP – 5-fosforibozil pirofosfat

Aktivacija AMPK lahko vodi tako v aktivacijo kot tudi v inhibicijo NKA. Pirkmajer in sod. (2021) so predlagali dva regulatorna mehanizma, preko katerih bi AMPK lahko vplivala na delovanje NKA. Prvi vključuje z AMPK-inducirano zavrtje FXYD1, ki lahko vključuje tudi spremembe v relativnem nivoju fosforiliranega FXYD1, medtem ko drugi vpliva na translokacijo NKA na plazemsko membrano preko aktivacije AMPK. Od signalne poti, ki povezuje NKA z AMPK je odvisno, ali bo aktivacija AMPK vodila v povišano ali znižano aktivnost/vsebnost NKA. Določene študije so pokazale, da aktivacija AMPK v pljučnih alveolarnih celicah vodi v znižanje ravni NKA\(\alpha\)1 v plazemski membrani (Vadasz in sod., 2008; Gusarova in sod., 2011). Zvišanje koncentracije CO\(_2\) stimulira AMPK preko CaMKKβ, kar posledično spodbuja aktivacijo in translokacijo proteinske kinaze C\(ζ\) (PKC\(ζ\)) (Vadasz in sod.,
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Kot je bilo predstavljeno, obstajajo močni dokazi, da igra AMPK pomembno vlogo v regulaciji NKA, z AMPK-inducirana aktivacija NKA pa bi lahko imela pozitivne učinke na delovanje NKA v skeletni mišici. Kot smo že omenili, obstaja problem, da so številni znani aktivatorji AMPK neučinkoviti pri aktivaciji mišičnih heterotrimerov AMPK, imajo slabo biološko razpoložljivost, lahko povzročijo resne stranske učinke, številni pa so tudi znani hkratni inhibitorji NKA. Zaradi vsega naštetega se iskanje novih farmakoloških spojin, ki bi v skeletni mišici uspešno aktivirala AMPK in tudi povečala aktivnost NKA, nadaljuje.

1.4 In vitro celični modeli za raziskovanje NKA v skeletni mišici

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1990), diferenciacija pa se sproži z znižanjem serumske vsebnosti na 2 % (v/v). Problem je, da lahko z znižanjem seruma dosežemo le omejen nivo diferenciacije mišičnih cevčic (Askanas in sod., 1977), ki se v zvezi z vsebnostjo in lokalizacijo NKA precej razlikujejo od zrelih mišičnih vlaken (Pirkmajer in sod., 2020).


Izražanje α1- in α2-podenot in aktivnost NKA se poveča tudi, ko so diferencirane HSMC tretirane z nevralnim agrinom (Jurdana in sod., 2009), heparan sulfatnim
Slika 1.6: Različne kulture človeških skeletnamiščnih celic. Satelitske celine so bile izolirane iz biopsije skeletne mišice. Po nasaditvi v gojitvene posodice so se satelitske celine razvile v mioblaste, ki so proliferirali in fuzirali v večjedne cevčice. Fuzija je pri cevčicah, namenjenih poskusom s kokulturami, potekla spontano, medtem ko se je pri poskusih z anevralnimi (neinerviranimi) celicami diferencija vzpodbudila z znižanjem vsebnosti FBS iz 10 % na 2 %. Humane anevralne cevčice nimajo živčno-mišičnega stika (ŽMS) in se ne krčijo spontano. Humane skeletne mišične cevčice se krčijo, če jih izpoštavimo električni pulzni stimulaciji (EPS) (Aas in sod., 2002) ali pa pa če jih gojimo skupaj z eksplantati embrionalnih podganjih hrbtenjač (Askanas in sod., 1987). Nevriti, ki izraščajo iz eksplantatov, inervirajo humane cevčice in tvorijo ŽMS. Inervirane cevčice se začnejo spontano krčiti po 7-10 dneh zaradi živčno-mišičnih prenosov s strani podganjih motoričnih nevronov.

2 AIMS AND HYPOTHESES

The basic purpose of doctoral dissertation was to study molecular mechanisms, which, during stressful conditions, contribute to the development of impaired NKA function and regulation of energy metabolism in skeletal muscle. In this doctoral dissertation we have set ourselves three aims: (1) to establish an in vitro experimental model for the observation of NKA function and metabolism in skeletal muscle, in which physiological and biochemical characteristics would move closer to in vivo conditions compared to present experimental
models; (2) to study molecular mechanisms through which hypoxia affects the functioning of NKA in skeletal muscle and (3) explore how metabolic changes affect NKA function in skeletal muscle. We tested the following hypotheses:

- Myotubes innervated in vitro and mature muscle fibres in vivo have a similar expression pattern of NKA and FXYD proteins.
- Hypoxia alters NKA function in skeletal muscle via activation of 5' AMP-activated protein kinase (AMPK).
- Modulation of NKA by pharmacological activators of AMPK depends on the metabolic state of skeletal muscle.

2.1 Argumentation of hypotheses

2.1.1 Ad H1

In immature developing skeletal muscle NKAα1 represents the majority of NKAα pool (Orlowski & Lingrel, 1988), but during postnatal development NKAα2 increasingly becomes the most expressed isoform. Although differentiation of cultured HSMC in low serum conditions increases protein expression of NKAα2 and lowers the expression of NKAα1 (Al-Khalili et al., 2004), NKAα1 still predominates after 2 weeks of differentiation (Pirkmajer et al., 2020). In skeletal muscles NKA α2/β-heterodimers are predominantly located on the inner membranes of T-tubules, while, to the lesser extent, they are also present on the postsynaptic membrane of NMJ and surface caveolae (Kristensen et al., 2008). Under standard in vitro conditions aneural human cultures of skeletal myotubes typically do not have basal lamina, cross-striations and T-tubules or they are only poorly developed (Askanas et al., 1987). To trigger contractions cultured human skeletal muscle cells need innervation, which induces the development of T-tubules and more complex structured sarcoplasmic reticulum (Askanas et al., 1987), or electrical-pulse stimulation (EPS) (Nikolić et al., 2017). Morphological
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differences between in vivo muscle fibres and cultured skeletal muscle cells seem to coincide with differences in the expression and activity of NKA heterodimers and their regulators from FXYD protein family, as innervation-induced differentiation of human myotubes that causes the development of T-tubules also leads to increased membrane potential (Askanas et al., 1987). In this doctoral dissertation we tested whether by using an in vitro model of innervated human skeletal muscles we could achieve an expression pattern of NKA and FXYD1, which would be more alike to the expression in in vivo conditions.

2.1.2 Ad H2

Reconstruction of ACL is a common surgery in patients with knee injury. Despite a high success rate in ACL reconstruction surgeries and best efforts from physiotherapist and patients in rehabilitation period, function of quadriceps femoris muscle can be impaired for long period of time (Kim et al., 2010). One of the causes of postoperative muscle atrophy and dysfunction might be ischaemia-reperfusion injury that is triggered by the arterial occlusion during surgery (Appell et al., 1993; Daniel et al., 1995). It is known that ischaemic preconditioning of patients before operative procedures could decrease the negative effects of ischaemia and reperfusion during surgery (Elmoselhi et al., 2003). It has also been shown that LL-BFR training that uses transient restrictions of blood flow, comparable to standard ischaemic preconditioning protocols, in combination with low-load resistance exercises can be an effective intervention for maintaining and increasing muscle mass and strength (Takarada et al., 2000). This is especially interesting for knee injury patients, as they cannot perform high resistance exercises, because of joint damage. Indeed, LL-BFR training limits the loss of muscle mass and function before surgery as well as prepare the muscle to better withstand ischaemia and reperfusion during surgery, thus leading to better muscle function after surgery and accelerating rehabilitation (Ohta et al., 2003; Grapar Zargi et al., 2016; Zargi et al., 2018).
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It is known that injury of ACL causes a decrease in NKA content in *vastus lateralis* muscle and this might contribute to prolonged muscle dysfunction after surgery (Perry *et al.*, 2015). It has been shown that HI-BFR increases NKA expression in healthy subjects (Christiansen *et al.*, 2018b; Christiansen *et al.*, 2019). We thus hypothesize that LL-BFR might also affect NKA expression in knee injury patients, which might lead to improved muscle function after ACL reconstruction.

AMPK is activated by energy stress (Fogarty & Hardie, 2010) and also by hypoxia (Gusarova *et al.*, 2011). Activation of AMPK could stimulate or inhibit the activity of NKA. While Gusarova *et al.* (2011) observed that an activation of AMPK can reduce the activity of NKA through CaMKKβ, Benziane *et al.* (2012) report that activation of AMPK with AICAR, which is the most commonly used pharmacological AMPK activator, in L6 rat muscle cell line increases NKA activity. Thus, hypoxia might, in the same way as AICAR, cause an activation of NKA through AMPK. It has already been implied by Christiansen *et al.* (2018b) that HI-BFR in healthy subjects might affect NKA function through AMPK signalling, but no such studies exist for LL-BFR in patients with knee injury. In this doctoral dissertation we studied whether LL-BFR of patients with ACL injury causes alterations of NKA function in skeletal muscles and whether this might happen via AMPK activation.

2.1.3 *Ad H3*

Besides blood flow restriction training, ischaemic preconditioning in combination with pharmacological agents might also protect skeletal muscle against ischaemia-reperfusion injury. One of the promising drugs that might be useful in combination with ischaemic preconditioning is the most widely used antidiabetic drug metformin. Metformin preconditioning is reported to have a neuroprotective and cardioprotective effect against cerebral ischaemia and ischaemia-reperfusion injury (Solskov *et al.*, 2008; Jiang *et al.*, 2014).
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Metformin is a known indirect activator of AMPK, which it activates by suppression of mitochondrial respiration. It was reported that metformin exerts its cardioprotective properties by protein kinase B (PKB aka Akt) and AMPK activation, and this increases cell survival during ischaemic events (El Messaoudi et al., 2011). Studies have also shown that metformin-induced AMPK activation can reduce damage of epithelial cells, caused by renal ischaemia (Seo-Mayer et al., 2011), and some of this might be due to improved NKA function. We were therefore interested in whether metformin pretreatment might cause any changes to NKA function in cultured skeletal muscle cells, exposed to hypoxic and ischaemic conditions.

Activation of AMPK with pharmacological agents can be achieved through various mechanisms. Pharmacological activation of AMPK can be achieved with compounds that bind directly to AMPK, or with substances that cause energy stress through the inhibition of glycolysis or mitochondrial respiratory chain (Fogarty & Hardie, 2010). Various antirheumatic drugs have been shown to inhibit AICAR transformylase (ATIC) (Baggott et al., 1992), e.g. salicylate and methotrexate, which is commonly used anti-rheumatic drug. ATIC is an enzyme that transforms ZMP (AMP analogue) into IMP. If ATIC is inhibited, increased number of ZMP molecules can increase AMPK activity by direct binding. In this doctoral dissertation we also explored whether SSZ, another common anti-rheumatic drug can increase AMPK activity. SSZ can increase AMPK activity in rat colonic tissue (Park et al., 2019), increase intracellular concentrations of ZMP in murine splenocytes (Gadangi et al., 1996) and certain reports have linked SSZ to improved glucose homeostasis in humans (Svenson et al., 1987; Haas et al., 2005). We were interested, whether SSZ might activate AMPK in skeletal muscle cells.
3 MATERIALS AND METHODS

3.1 Ethical approvals

3.1.1 Ethical approvals for cell cultures

Ethical approval for the experiments on primary cultures of human skeletal muscle cells (KME: 71/05/12 and 0120-698/2017/4) was obtained from Republic of Slovenia Medical Ethics Committee. The participants or their legal representatives (in case of minors) signed informed written consent before the muscle tissue for preparation of human skeletal muscle cell cultures was taken. Human skeletal muscle cell cultures were established from cells, isolated from samples of semitendinosus muscles. These were acquired during routine orthopaedic surgeries as surgical waste. No surgery was performed for sole purpose of cell isolation. They were all undertaken because of medical reasons. All performed experiments complied with the Declaration of Helsinki and Good Laboratory Practice. Co-culture experiments were performed in accordance with the 3R principles; number of rats was minimized and so was their suffering. All the procedures that included rats and their tissues were approved by the Ethics Committee for Experiments on Animals (Administration for Food Safety, Veterinary Sector and Plant Protection (permit numbers U34401-38/2015/6 and U34401-2/2020/4) All the experiments with skeletal muscle cell cultures and skeletal muscle cell lines were performed at the Laboratory for the Molecular Neurobiology at the Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana.

3.1.2 Ethical approvals for clinical trials

Surgical biopsies of semitendinosus and vastus lateralis muscles were obtained during a clinical study “Effects of preoperative conditioning of orthopaedic patients with ischaemic training on systemic and molecular mechanisms of skeletal muscles”, for which ethical permission was obtained from the Republic of Slovenia National Medical Ethics Committee.
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(KME 45/08/14, amendment no.: 62/05/12). All subjects participated in the study by their own volition and have given their informed consent before their inclusion.

### 3.2 Cell models: in vitro skeletal muscle cell cultures

#### 3.2.1 Primary HSMC

##### 3.2.1.1 Isolation of HSMC

Satellite cells were isolated from samples of *semitendinosus* muscles, which were obtained during surgical reconstructions of the ACL. Muscle samples were kept in Advanced Minimum Essential Medium (aMEM) (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA) at 4˚C for a maximum of 7 days. At the start of isolation procedure, the muscle tissue was moved to Earle’s Balanced Salt Solution (EBSS), cleaned, separated from connective tissue, capillaries and adipose tissue, and cut into small pieces of approximately 0.5 mm in diameter. The small pieces of tissue were trypsinized for 45 min during constant magnetic mixing with stir bar at 37˚C. Released muscle satellite cells were grown in petri dishes (d=10 cm; cells are labelled as passage 0 or P0 cells) in 10% (v/v) FBS-supplemented aMEM in cell incubator (New Brunswick™ Galaxy 170R CO\(_2\) Incubator, Eppendorf, Hamburg, Germany) at 37˚C, 95% relative humidity and air mixture with 5% CO\(_2\). After 2 to 3 weeks of growth and before fusion of myoblast colonies into myotubes we separated muscle satellite cells from other cell types by using magnetic-activated cell sorting (MACS). We used CD56 microbeads to isolate cells that express CD56 antigen (also called neural cell adhesion molecule (NCAM) (Miltenyi Biotec, Bergisch Gladbach, Germany). Since the early 1980s, when the CD56/NCAM expression on the surface of satellite cells was first described (Hurko & Walsh, 1983), CD56/NCAM has been most frequently utilized for the identification of satellite cells in human skeletal muscle cryosections (Studies thoroughly listed in the review article from Snijders et al. (2015) and immune-
magnetic cell sorting (Cashman et al., 1987; Mackey et al., 2009). After the isolation the myoblasts (CD56⁺ cells) were further grown in cell culture flasks (S=75 cm²).

3.2.1.2 Freezing and thawing of HSMC

When the CD56⁺ cells reached an appropriate confluence (~75%) and before they started to fuse into myotubes, we trypsinized them (P2 cells), added 10% FBS-supplemented aMEM with 10% dimethyl sulfoxide and distributed them in 2 mL cryovials (1 mL per vial). The vials were put in freezing containers, filled with isopropanol, which were kept in -80°C refrigerator overnight. After the cells were slowly cooled down to -80°C in isopropanol-filled freezing containers they were transferred to liquid nitrogen cell container. For the experiment we thawed the cells and resuspended them in aMEM with 10% FBS in 75 cm² culture flasks. To start the experiment, we seeded CD56⁺ cells to an appropriate cell culture plate (6- or 12-well or petri dish) with 10% FBS-supplemented aMEM. After 24h the medium was exchanged for 2%-supplemented aMEM, which induced the fusion of myoblasts into myotubes. Cells were differentiated for 7 to 10 days. Once differentiated, myotubes were used for experiments.

3.2.1.3 Evaluation of desmin expression

To assess the fraction of myogenic cells, we stained human CD56⁺ cells with Hoechst 33258 stain (Thermo Fisher Scientific, Waltham, MA, USA) and labelled them with mouse anti-desmin antibody (D33, DAKO, Agilent Technologies, Santa Clara, CA, USA; dilution 1:50). The fluorescence stain Hoechst 33258 (Thermo Fisher Scientific, MA, USA) was used to evaluate the total number of CD56⁺ cells in cell culture by staining cellular nuclei, while the desmin-positive cells represented the myogenic cells (Kaufman & Foster, 1988; Ono et al., 1993). The percentage of myogenic cells was calculated by dividing the number of desmin-positive cells with Hoechst-stained cells. P1 cells, which were grown on microscope cover slips, were washed with phosphate buffer saline (PBS) and fixated for 10 min with freshly
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prepared 4% paraformaldehyde at room temperature. For desmin staining we added 100 µL of mouse anti-desmin antibody diluted 1:50 in PBS to the cover slips. Cells were incubated over night at 4°C. After the incubation we washed the cover slips with PBS and added 100 µL of secondary goat anti-mouse fluorescein-conjugated IgG antibodies (dilution 1:200 in PBS, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) to each cover slip for 1 h at 37°C. Nuclei were stained with Hoechst. We mixed 1 mL of PBS and 1 µL of Hoechst stain and added 100 µL of said mixture to cover slips. After 5-min incubation at room temperature the medium was removed, and cover slips were washed with PBS. Cover slips were mounted in NF Mounting Medium and attached to microscope slides. Cells were visualized with fluorescent microscopy. The nuclei were stained in green, while desmin was stained in red. We captured the nuclei and desmin pictures separately and then merged them together. Merged pictures were used to evaluate the percentage of desmin-positive cells in cell culture. The evaluation was performed in ImageJ program.

3.2.2 Innervation of cultured myotubes: co-cultures with embryonic rat spinal cord

3.2.2.1 Isolation of rat embryonic spinal cords

To obtain embryonic spinal cords we used Wistar rats that were killed in CO\(_2\) chamber on the 14\(^{th}\) day of gestation. Embryos were removed with caesarean section and kept in EBSS. Spinal cords were isolated from the embryos under aseptic conditions under microscope and cut into 1 mm explants, which had intact meninges and anatomic connections to dorsal root ganglia.

3.2.2.2 Preparation of HSMC and rat embryonic spinal cord explant co-cultures

Procedure was adapted from Askanas et al. (1987) and is in routine use in our research group (Mars et al., 2003; Mis et al., 2005; Mars et al., 2020). For the preparation of co-cultures myoblasts were seeded on 6-well plates. Before seeding the cells, we covered the growth
surface of the plates with a mixture of 1.5% gelatine (Merck/Sigma-Aldrich, Darmstadt, Germany) and human plasma in a ratio of 1:2. HSMC myoblasts (Fig. 3.1B) were grown in 10% FBS-supplemented aMEM for 6 days with media exchange every 2 to 3 days and left to spontaneously fuse into myotubes (Fig. 3.1C). 24 h before the addition of spinal cord explants the medium was changed to 10% FBS-supplemented Ham’s F14 medium (Biowest SAS, Nuaillé, France or Thermo Fisher Scientific, Waltham, MA, USA) supplemented with fibroblast growth factor (50 ng/mL), epidermal growth factor (10 ng/mL) and insulin (10 µg/mL) (all from Merck/Sigma-Aldrich, Darmstadt, Germany). After 24 h in F14 medium with added growth factors we added 3 explants of rat embryonic spinal cord per well with preserved meninges and dorsal root ganglia. With the addition of explants to the myotube monolayer we also exchanged the culture medium for 10% FBS-supplemented F14 medium with insulin (10 µg/mL) but without the added growth factors. Maturation of myotubes and development of contractions was observed daily under the microscope. Innervated HSMC started to spontaneously contract after 7-10 days (Fig. 3.1D). The whole scheme of the procedure is shown in Fig. 3.1A.
Figure 3.1 Preparation of aneural human myotubes and co-cultures of human myotubes and rat embryonic spinal cord explants. (A) To innervate cultured human myotubes, explants of rat embryonic spinal cord were placed on a monolayer of CD56+ HSMC. In co-cultures motor neurons functionally innervate myotubes, which after 7-10 days start to contract. Microscopic pictures of (B) proliferating myoblasts with differentiating potential, (C) aneural myotubes that do not show striations, do not contract and do not have T-tubule systems, and (D) contracting innervated myotubes that are striated and develop more and more complex T-tubule systems with prolonged cultivation, are shown. Co-cultures and aneural controls were grown for 21 days from addition of rat embryonic spinal cord explants to co-culture wells. Adapted from Jan et al., 2021.

3.2.3 Rat skeletal muscle cell line L6

Rat skeletal muscle cell line L6 was from ATCC (Manassas, VA, USA). L6 cells were cultured in MEMα with nucleosides supplemented with 10% (v/v) FBS, 1% (v/v) Pen Strep (50 U/mL of penicillin and 50 μg/mL of streptomycin) and 0.3% (v/v) Fungizone (0.75 μg/mL of amphotericin B) (all from Thermo Fisher Scientific, Waltham, MA, U.S.). The culture medium was exchanged every 2-3 days. The differentiation of L6 myoblasts into myotubes was triggered by reducing FBS concentration in culture medium from 10% to 2% (MEMα (2%...
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(v/v) FBS, 1% (v/v) Pen Strep (50 U/mL of penicillin and 50 μg/mL of streptomycin) and 0.3% (v/v) Fungizone). The experiments were performed after 7-9 days of differentiation.

3.3 Experiments on primary HSMC

The descriptions of most cell culture experiments are described in figure legends of each separate experiment. Below we describe in detail those that are perhaps more complex and need wider description. Unless stated otherwise described HSMC were from CD56$^+$ fraction.

3.3.1 Differentiation of CD56$^+$ and CD56$^-$ HSMC and its effect on ion transport machinery regulation

With MACS HSMC were separated into CD56$^+$ and CD56$^-$ fractions. Both fractions were either grown only in 10% FBS-supplemented aMEM (CD56$^-$ 10% FBS and CD56$^+$ 10% FBS) or were subsequently differentiated for additional 7 days in 2% FBS-supplemented aMEM (CD56$^-$ 2% FBS and CD56$^+$ 2% FBS). Undifferentiated CD56$^+$ HSMC were collected before they reached ~75% confluence and potentially start to fuse. Non-differentiated CD56$^-$ cells were collected at the same time. Both were washed with ice-cold PBS and frozen till further analysis. As mentioned, the differentiating CD56$^+$ and CD56$^-$ HSMC were collected after 7 days of cultivation in 2% FBS-supplemented aMEM. Experiment’s layout is shown in Fig. 3.2.
Figure 3.2: Differentiation of CD56⁻ and CD56⁺ HSMC. HSMC were obtained from *semitendinosus* muscle samples and first grown in aMEM with 10% FBS. Before cells reached confluence, they were separated with MACS microbeads into 2 fractions on the basis of CD56 expression. CD56⁻ and CD56⁺ HSMC were then grown in either 10% FBS-supplemented aMEM (CD56⁻ 10% FBS and CD56⁺ 10% FBS cells) or differentiated for 7 days in 2% FBS-supplemented aMEM (CD56⁻ 10% FBS and CD56⁺ 10% FBS cells). Adapted from Jan et al., 2021.

3.3.2 Effects of acute (24-hour) exposure to hypoxia and/or glucose deprivation on cultured HSMC

HSMC were grown in the presence of 2% (v/v) FBS for 7-9 days to differentiate them into myotubes. 24h before the experiment culture medium was changed to Dulbecco's Modified Eagle Medium (DMEM with 1g/L glucose, Thermo Fisher Scientific, Waltham, MA, USA) with 2 % (v/v) FBS. During the last 24 h myotubes were kept in control conditions (NOR, serum-free DMEM with 1 g/L of glucose, at 37 °C, humidified atmosphere and 5% CO₂), glucose deprivation (GD, serum- and glucose-free DMEM), hypoxia (HYP, serum-free DMEM with 1 g/L of glucose, 0.1% O₂) or combination of hypoxia and glucose deprivation (HGD, artificial ischaemia, serum- and glucose-free DMEM, 0.1% O₂). Hypoxic conditions...
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were maintained with the help of New Brunswick Galaxy 48R CO\(_2\) incubator (Eppendorf, Hamburg, Germany).

3.3.3 Effects of intermittent exposure to hypoxia and/or glucose deprivation on cultured HSMC

HSMC were differentiated for 6 days in 2% FBS-supplemented aMEM (Fig. 3.3). On the 7\(^{th}\), 8\(^{th}\) and 9\(^{th}\) day cells were exposed to preconditioning with hypoxia, glucose deprivation, or both for 1 h/day. Before each treatment HSMC were washed with glucose- and serum-free DMEM and then incubated in serum-free DMEM with 1 g/L glucose in normoxia (NOR), serum-free DMEM with 1 g/L glucose in hypoxia (0.1% O\(_2\)) (HYP), serum-free DMEM without glucose in normoxia (GD), or serum-free DMEM without glucose in hypoxia (0.1% O\(_2\)) (HGD, artificial ischaemia) as presented in Figure 3.3. The 1-h countdown was started when 0.1% O\(_2\) concentration was achieved in the hypoxic incubator. On the 7\(^{th}\) and 8\(^{th}\) day cells were switched back to aMEM with 2% FBS after preconditioning. On the 9\(^{th}\) day cells were switched to DMEM with 1 g/L glucose and 2 % FBS for 23 h (Fig. 3.3), which was followed by 2-h exposure to HGD (serum-free DMEM without glucose, 0.1% O\(_2\)) or control normoxic conditions (NOR, serum-free DMEM with 1g/L glucose).

![Figure 3.3: Schematic representation of in vitro preconditioning with intermittent exposure to hypoxia and/or glucose deprivation. HSMC were differentiated in Advanced MEM (aMEM) with 2% FBS for 6 days.](image)
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On 7-9\(^{th}\) day, they were exposed to normoxia (NOR), glucose deficiency (glucose-free medium, NGD), hypoxia (0.1% O\(_2\), HYP), or both (HGD) in serum-free DMEM for 1 h/day. After the last preconditioning HSMC were switched to DMEM with 2% FBS for 23 h and then exposed to HGD or NOR for 2 h.

3.3.4 Effects of AMPK activator metformin preconditioning of cultured HSMC on latter hypoxic and ischaemic treatment.

HSMC were differentiated for 5 days in aMEM with 2% FBS. On the 6\(^{th}\) we started the first 24-h treatment in either 2% FBS-supplemented aMEM with vehicle, 0.3 mM metformin or 3.0 mM metformin. After 24 h on the 7\(^{th}\) we changed the medium with fresh 2% FBS-supplemented aMEM with the same treatments (vehicle, 0.3 mM and 3.0 mM metformin). On the 8\(^{th}\) day we repeated the same treatment (24 h), but this time in DMEM (2% FBS, 1g/L of glucose). On the 9\(^{th}\) day, cells were exposed to normoxia (NOR), hypoxia (0.1% O\(_2\), HYP), or artificial ischaemia (glucose-free medium, 0.1% O\(_2\), HGD) in serum-free DMEM with either vehicle (Basal), 0.3 mM metformin or 3.0 mM metformin for 24h.

3.4 Clinical study: Effects of preoperative conditioning of orthopaedic patients with ischaemic training

3.4.1 Study overview

The described clinical study was designed as a prospective, single-center, single-blinded, quasi-randomized trial. The study consisted of 3 groups of subjects. One underwent low load blood flow restriction training (LL-BFR), second one was an active control group that performed work-matched sham low load blood flow restriction training (LL-SHAM), and the third one was a negative control group that performed no intervention (Control). To reduce potential bias in training effect expectations between groups the participants of 2 active groups were blinded of the physiological effect of different pneumatic cuff pressures used during exercise. By familiarizing test subjects with training and testing protocols during preliminary
visits the effect of the initial motor learning on testing results was reduced. It was deemed especially important to teach the participants proper exercise pace and lifting techniques. During these preliminary visits the test subjects also got acquainted with pneumatic tourniquet. Test subjects were asked to avoid any caffeine or alcohol ingestion in the 48-hour period before testing. All the experimental work (tests and measurements) was done in thermoneutral environment (room temperature ranging between 20 and 22°C with 40-50% humidity)

3.4.2 Study population

Patient enrollment was performed at the Department of Orthopaedic Surgery of Ljubljana University Medical Centre between May 2015 and December 2016. From the 35 patients that were assessed for eligibility to participate in the study, 16 patients were selected and, in the beginning, introduced to the study. Of this selected group 12 patients (6 females and 6 males) finished the whole protocol and were included in the data analysis. Inclusion criteria included: participants had to be between 18 to 45 years of age; they had a total unilateral ACL rupture that was confirmed by MRI and clinical assessment; they had no history of injury or surgery to the affected knee; participants had no neurologic deficits or systemic illnesses of lower limbs; they had no prior injuries to the contralateral knee; pain intensity during exercise had to be below or equal to 2 on Visual Analogue Scale; range of motion of the affected knee had to be sufficient to allow the subjects to perform the exercises (active extension deficit ≤ 5°, active flexion ≥ 120°). Exclusion criteria included any concurrent intra-articular pathology, which would deter tolerance to exercise or safety, like substantial damage to articular cartilage, spine or other lower limb injuries, neuromuscular impairments, presence or history of any vascular disease or deep vein thrombosis.
3.4.3 Training regimen

The purpose of 3-week LL-BFR training for the injured leg was to prompt hypertrophy of muscle and to enhance muscle performance (Kacin & Strazar, 2011; Fitschen et al., 2014). To reach an adequate volume of exercise and training intensity regardless of reduced tolerance of patients to mechanical loading of joints, several sets of low workload numerous repetitions was undertaken to voluntary failure. Training regimen included 9 exercise sessions (ES) that were done three times a week. Each ES included exercising with either sham blood flow restriction (LL-Sham) or true blood flow restriction (LL-BFR). LL-BFR group sessions comprised of 4 sets of knee extension and knee flexion workouts (40RM to voluntary failures, only performed on injured leg, remained constant over the whole period of intervention). Throughout the whole period of clinical study ES were done on dynamometric system in isotonic setting (HUMAC NORM, CSMi Medical Solutions, Stoughton, MA, USA). A 13.5 cm wide double-chamber pneumatic cuff with asymmetric pressure (Ischemic Trainer, University of Ljubljana and Iskra Medical d.o.o., Ljubljana, Slovenia) was fitted to BFR patients’ proximal thigh to prompt a partial blood flow restriction in the vastus lateralis and the semitendinosus muscle. Subjects were first warmed up, performing 10-12 repetitions at minimal possible intensity and then the pneumatic cuff was inflated with air (cumulative pressure of 150 mmHg: inner cuff compartment =185 mmHg; outer cuff compartment =125 mmHg). For 30 s the inflated cuff was kept on a resting muscle and then the test subjects underwent isotonic knee extension exercises to voluntary failure. 45 s of break without reperfusion was permitted after the first and third set, but after the second one, the pneumatic cuff was deflated for 90 s, which allowed tissue reperfusion. After knee extension exercises, the same protocol was also performed for knee flexion. Test subjects from LL-Sham group performed the same exercise protocol with matched number of repetitions as LL-BFR group, but their cuffs were only inflated to 200 mmHg. Each participant from LL-Sham group was
assigned a peer in LL-BFR group. This pairing was performed on the basis of gender, Lysholm score and BMI. Participants from LL-Sham group than performed the same number of repetitions that was achieved by their peers in LL-BFR group. This type of protocol allowed for even mechanical work of muscles at different level of muscle perfusion. Each exercise session volume was computed by combining volumes (reps × workload) of extensor and flexor muscles. The sum of all session volumes equalled the total dose of training intervention. Groups were formed in a counterbalanced manner. Firstly, two patients were selected for LL-BFR group and then two to LL-Sham group. This was continued until both groups were filled. Participants of the study were told to keep to their daily routine and write a diary of their daily physical activities. The overall representation of training protocol is shown in Figure 3.4.

**Figure 3.4: Schematic representation of patient training protocol.** Training intervention comprised 9 exercise sessions (ES) that were performed with either true blood flow restriction (LL-BFR) or sham blood flow restriction (LL-Sham). Control group patients did not perform any intervention (Control). Every session included 4 sets of knee flexion and extension exercises performed at 40RM to volitional failure with injured leg only. See text for further details.

### 3.4.4 Muscle sampling

Biopsies of *vastus lateralis* and *semitendinosus* muscles from the trained leg were taken less than 48 h after the final testing. For the establishment of baseline values, we took biopsies
from an additional group of ACL deficient patient that did not perform any exercises before their surgery (Control). Samples were collected at the start of surgical ACL reconstructions, which were performed under spinal block anesthesia. One sample was surgically cut from the *semitendinosus* muscle during the harvest of autologous graft for the reconstruction of ACL, while the other was taken from the lateral portion of *vastus lateralis* muscle with a percutaneous needle biopsy technique using a 50 mm Bergstrom needle with syringe suction.

### 3.5 Analytical techniques

#### 3.5.1 Quantitative real-time polymerase chain reaction

#### 3.5.1.1 Reverse transcription

Total RNA was extracted from cells with RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) or E.Z.N.A. HP Total RNA Kit (Omega Bio-Tek, Norcross, GA, U.S.), while the RNA from muscle fibres was extracted with RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany). In the isolation procedure we used sterile demineralized water, pretreated with diethyl pyrocarbonate (DEPC). The chemical treatment with DEPC inactivates RNases that are present in the environment and might degrade RNA. DEPC inactivates RNases by covalent modifications of histidine, cysteine, lysine and tyrosine residues (Wolf *et al.*, 1970; Chirgwin *et al.*, 1979). RNA concentrations were measured spectrophotometrically with Epoch Microplate Spectrophotometer (Biotek, Winooski, VT, USA), while purity of samples was evaluated by the A260/A280 ratio. RNA concentration is determined by the reading at 260 nm and protein concentration. Pure RNA has an A260/A280 ratio of 2.1, but values above 1.8 were considered acceptable in experiments. For the reverse transcription we used “High-Capacity cDNA Reverse Transcription” kit (Thermo Fisher Scientific, Waltham, MA, USA) with random primers and MJ Research PTC-100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The composition of reaction mixture is listed in Table 3.1, while the temperature
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program for reverse transcription is listed in Table 3.2. We used approximately 1 µg of RNA for reverse transcription.

### Table 3.1: Composition of reaction mixture for reverse transcription

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-free water</td>
<td>9.5</td>
</tr>
<tr>
<td>10X RT buffer</td>
<td>5</td>
</tr>
<tr>
<td>25 dNTP</td>
<td>2</td>
</tr>
<tr>
<td>10x random primers</td>
<td>5</td>
</tr>
<tr>
<td>Inhibitors of RNase</td>
<td>1</td>
</tr>
<tr>
<td>MultiScribe reverse transcriptase</td>
<td>2.5</td>
</tr>
<tr>
<td>RNA</td>
<td>25</td>
</tr>
<tr>
<td>total volume</td>
<td>50</td>
</tr>
</tbody>
</table>

### Table 3.2: Temperature program AB-RT-4S for reverse transcription

<table>
<thead>
<tr>
<th>Phase</th>
<th>Temperature (°C)</th>
<th>Duration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Phase 2</td>
<td>37</td>
<td>120</td>
</tr>
<tr>
<td>Phase 3</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

### 3.5.1.2 Quantitative real-time polymerase chain reaction

Expression of target genes and endogenous controls (PPIA, ACTB and 18S rRNA) was determined with RT-qPCR on 96-well plates in ABI PRISM SDS 7500 machine (Thermo Fisher Scientific, Waltham, MA, USA). The composition of reaction mixture for qPCR for a single reaction is listed in Table 3.3. The temperature program for qPCR is listed in Table 3.4. Approximately 40 ng of cDNA was added to the qPCR reaction. Expression levels of target mRNAs were determined as gene expression ratios (target gene mRNA/reference mRNA or rRNA) according to the following equation: 

\[
(1 + E_{reference})^{Ct,reference} / (1 + E_{target})^{Ct,target},
\]

where E is the efficiency of PCR, while Ct is the threshold cycle for the
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endogenic control (cyclophilin mRNA, β-actin mRNA or 18S rRNA) or the target genes (Ruijter et al., 2009; Tuomi et al., 2010). Efficiency of PCR was estimated with LinReg PCR software (Ruijter et al., 2009; Tuomi et al., 2010). TaqMan gene expression assays used in the experiments are listed in Table 3.5.

Table 3.3: Composition of reaction mixture for qPCR for a single reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
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<tbody>
<tr>
<td>TaqMan ® Universal PCR Mastermix</td>
<td>10</td>
</tr>
<tr>
<td>DEPC H(_2)O</td>
<td>7</td>
</tr>
<tr>
<td>TaqMan gene expression assay</td>
<td>1</td>
</tr>
<tr>
<td>cDNA</td>
<td>2</td>
</tr>
<tr>
<td>total volume</td>
<td>20</td>
</tr>
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</table>

Table 3.4: qPCR temperature program

<table>
<thead>
<tr>
<th>1 cycle</th>
<th>40 cycles</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation of DNA polymerase</td>
<td>Denaturation of DNA</td>
<td>Elongation of DNA</td>
</tr>
<tr>
<td>95 °C, 10 min</td>
<td>95 °C, 15 seconds</td>
<td>60 °C, 60 seconds</td>
</tr>
</tbody>
</table>

Table 3.5: List of TaqMan gene expression assays

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay ID</th>
<th>Dye</th>
<th>Manufacturer</th>
</tr>
</thead>
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<tr>
<td>NCAM1 (CD56)</td>
<td>#Hs00941830_m1</td>
<td>FAM</td>
<td>all from Thermo Fisher Scientific</td>
</tr>
<tr>
<td>DES</td>
<td>#Hs00157258_m1</td>
<td>FAM</td>
<td></td>
</tr>
<tr>
<td>MYH1</td>
<td>#Hs00428600_m1</td>
<td>FAM</td>
<td></td>
</tr>
<tr>
<td>MYH2</td>
<td>#Hs00430042_m1</td>
<td>FAM</td>
<td></td>
</tr>
<tr>
<td>MYH7</td>
<td>#Hs01110632_m1</td>
<td>FAM</td>
<td></td>
</tr>
<tr>
<td>CACNA1S</td>
<td>#Hs00163885_m1</td>
<td>FAM</td>
<td></td>
</tr>
<tr>
<td>ATP2A1</td>
<td>#Hs04983082_m1</td>
<td>FAM</td>
<td></td>
</tr>
<tr>
<td>ATP2A2</td>
<td>#Hs05000494_m1</td>
<td>FAM</td>
<td></td>
</tr>
<tr>
<td>ATP2A3</td>
<td>#Hs00545433_g1</td>
<td>FAM</td>
<td></td>
</tr>
<tr>
<td>ATP1A1</td>
<td>#Hs00167556_m1</td>
<td>FAM</td>
<td></td>
</tr>
<tr>
<td>ATP1A2</td>
<td>#Hs00265131_m1</td>
<td>FAM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>#Hs01560077_m1</td>
<td>FAM</td>
<td></td>
</tr>
</tbody>
</table>
3.5.2 Immunoblotting

3.5.2.1 Preparation of lysates from muscle biopsies

Frozen muscle samples were first ground to fine powder with pestle and mortar in liquid nitrogen. Subsequently, they were homogenized using the homogenization buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1% (v/v) Triton X-100, 10% (w/v) glycerol, 20 mM Tris (pH 7.8), 10 mM NaF, 1 mM EDTA, 0.5 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1% (v/v) protease inhibitor cocktail) and mechanical homogenizer. In the next step, the samples were put on a rotating mixer for 1 h at 4 °C. After the rotating mixer incubation, the lysates were centrifuged for 15 min at 12000xg at 4 °C. We took the supernatants and performed BCA protein concentration analysis after which the samples were all diluted to the same protein concentration with Milli-Q water. In the end we mixed normalized samples with 4x Laemmlli buffer (250 mM Tris-HCl (pH 6.8), 8% (w/v) sodium dodecyl sulfate (SDS), 40% (w/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.008% (w/v) bromophenol blue). Before the loading
on electrophoresis gels, the samples were heated to 56°C for 20 min during constant shaking (600 rpm) in a Thermomixer R (Eppendorf, Hamburg, Germany).

3.5.2.2 Preparation of lysates from cell culture experiments

We prepared lysates either in Laemmli buffer or homogenization buffer. When lysates were prepared in Laemmli buffer we added 1x Laemmli buffer (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue) to each well or petri dish, which lysed the cells. We used cell scrapers to cause further degradation of cells and then transferred the lysates to 1.5 mL microcentrifuge tubes. Samples in microcentrifuge tubes were sonicated 10-15x with UP50H – Compact Lab Homogenizer (Hielscher Ultrasonics GmbH, Teltow, Germany) at medium setting to further homogenize them. After a short spin-down in a MiniSpin microcentrifuge (Eppendorf, Hamburg, Germany) the samples were heated to 56°C for 20 min during constant shaking (600 rpm) in a Thermomixer R (Eppendorf, Hamburg, Germany).

When we prepared lysates in homogenization buffer, we added ice-cold homogenization buffer to each well or petri dish and then used the cell scrapers to completely lyse the cells. After lysis the samples were transferred to microcentrifuge tubes. The lysates were centrifuged for 15 min at 12000xg at 4 °C. We took the supernatants and determine total protein concentration using the BCA assay after which the samples were all diluted to the same protein concentration with Milli-Q water. In the end we mixed normalized samples with 4x Laemmli buffer (250 mM Tris-HCl (pH 6.8), 8% (w/v) sodium dodecyl sulfate (SDS), 40% (w/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.008% (w/v) bromophenol blue; 3 parts of sample and 1 part of 4x Laemmli). Before the loading on electrophoresis gels, the samples were heated to 56°C for 20 min during constant shaking (600 rpm) in a Thermomixer R (Eppendorf, Hamburg, Germany).
3.5.2.3 Quantitation of total protein concentrations

Protein concentration in supernatants and/or sediments was measured with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) by manufacturer’s protocol. It is compatible with detergents and based on bicinchonic acid (BCA) for the quantification of total protein and colorimetric detection. In this method the Cu$^{2+}$ ions are reduced to Cu$^{1+}$ by protein in an alkaline medium (biuret reaction). In the next phase Cu$^{1+}$ ions are bound to 2 molecules of BCA. This water-soluble purple complex absorbs light at wavelengths of 550-570 nm. For the preparation of standards, we used bovine serum albumin. For the measurements we added 20 µL of standard or diluted sample (1:5 for cell culture samples, 1:25 for muscle samples) duplicate into a 96 microplate well. After the standards or samples, we added 160 µL of working solution, consisting of reagent A (Na$_2$CO$_3$, NaHCO$_3$, Na-tartrate, NaOH and BCA) and reagent B (CuSO$_4$ x 5H$_2$O in water) in a 50:1 ratio. After the addition of working solution, the plates were incubated for 30 min at 37°C in the dark. In the end of incubation period the plates were cooled to room temperature and the absorbance was measured at 550 nm with Victor 3 plate reader (PerkinElmer, Waltham, MA, USA).

3.5.2.4 Polyacrylamide gel electrophoresis

Proteins were resolved with sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Criterion™ Cell electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) and Criterion™ XT Precast polyacrylamide gels (4-12%) (Bio-Rad Laboratories, Hercules, CA, USA). We used Full Range Rainbow™ Recombinant Protein Molecular Weight Markers as standard for molecular size (RPN800E, GE Healthcare, Chicago, IL, USA). Electrophoresis was run in XT MES Running Buffer (Bio-Rad Laboratories, Hercules, CA, USA) at 200 V for 40-45 min at room temperature.
3.5.2.5 Electrotransfer to polyvinylidene fluoride membrane

After the proteins were resolved with SDS-PAGE they were transferred to Immobillon-P Transfer Polyvinylidene fluoride (PVDF) membrane (pore size 0.45 µm (Merck/Sigma-Aldrich, Darmstadt, Germany)) with wet electrotransfer. The proteins were transferred to a PVDF membrane, which was activated beforehand in 100% methanol (Alkaloid AD, Skopje, FYR Macedonia). Transfer of proteins onto a membrane was performed in 1x transfer buffer (31 mM Tris, 0.24 M glycine, 10% (v/v) methanol and 0.01 % SDS (Merck/Sigma-Aldrich, Darmstadt, Germany)) at 100 V for 75 min. After the transfer the membranes were washed with Tris-buffered saline with Tween (TBST: 20 mM Tris, 150 mM NaCl, 0.02% (v/v) Tween 20, pH 7.5). To evaluate the uniformity of sample loading and transfer we used Ponceau S staining (0.1% Ponceau S (w/v) in 5% acetic acid). Ponceau S is a sodium salt of a diazo dye that reversibly binds to proteins and is used as an unspecific detection of proteins on a PVDF or nitrocellulose membrane. Membranes were first washed with 5% acetic acid and then incubated in Ponceau S for 60 s. After the incubation the membrane were once again washed with 5% acetic acid to remove the residual stain. To remove all Ponceau S staining the membranes were washed with TBST.

3.5.2.6 Immunodetection of specific proteins

Once we removed all Ponceau S from the membranes, they were shortly incubated in methanol and then blocked for 1 h in 7.5% skimmed milk (Pomurske mlekarne, Murska Sobota, Slovenia) in TBST buffer. The blocking in milk was performed to block all unspecific binding sites on the membrane. Incubation with primary antibodies was performed at 4°C overnight. For the detection of proteins, we used rabbit or mouse primary antibodies, prepared in the buffer for primary antibodies (20 mM Tris, 150 mM NaCl, pH 7.5, 0.1% (w/v) BSA and 0.1% (w/v) sodium azide).
Antibody phospho-FXYD1$^{Ser68}$ was from Abnova (Taipei, Taiwan), phospho-AMPK$\alpha^{Thr172}$ (40H9, #2535), AMPK$\alpha$ (#2532), ACC (C83B10, #3676), phospho-ACC$^{Ser79}$ (#3661), phospho-NKAn$\alpha1^{Tyr10}$ (E1Y9C, #13566), Sp1 (D4C3, #9389), phospho-ERK1/2$^{Thr202/Tyr204}$ (D13.14.4E, #4370), ERK1/2 (137F5, #4695), phospho-Akt$^{Ser473}$ (D9E, #4060), Akt (pan)(C67E7, #4691), phospho-AS160$^{Ser588}$ (D8E4, #8730), phospho-AS160$^{Thr642}$ (D27E6, #8881), phospho-(Ser/Thr) Akt Substrate Antibody (#9611), phospho-GSK-3$\alpha/\beta^{Ser21/9}$ (#9331), phospho-PAK1$^{Thr423}$/PAK2$^{Thr402}$ (#2601), and PAK1 (#2602) from Cell Signaling Technology, Danvers, MA, USA), NKA$\alpha1$ (05-369) and NKA$\alpha2$ (AB9094) from Merck (Darmstadt, Germany), FXYD1 (13721-1-AP) from Proteintech (USA), and actin (#sc1616r) from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against FXYD5 were kindly provided by Dr. Haim Garty (Weizmann Institute of Science, Rehovot, Israel) (Lubarski et al., 2007). SC-71 antibody, which specifically detects fast MyHC-2A in rats (Schiaffino et al., 1989), binds to both MyHC-2A and 2X in human tissue. Additional details on each antibody are listed in Table 3.6.

Table 3.6: List of used primary antibodies

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Source</th>
<th>Type</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFXYD1$^{Ser68}$</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1:500</td>
<td>Abnova</td>
<td>PAB0389</td>
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<tr>
<td>pAMPK$\alpha^{Thr172}$</td>
<td>Rabbit</td>
<td>Monoclonal</td>
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<td>pACC$^{Ser79}$</td>
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<td>pERK1/2$^{Thr202/Tyr204}$</td>
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<tr>
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<td>Monoclonal</td>
<td>1:1000</td>
<td>CST</td>
<td>#4695</td>
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<td>Rabbit</td>
<td>Monoclonal</td>
<td>1:2000</td>
<td>CST</td>
<td>#4060</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Code</th>
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<tbody>
<tr>
<td>Akt</td>
<td>Rabbit</td>
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<td>CST</td>
</tr>
<tr>
<td>pAS16O(^{588})</td>
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<td>1:1000</td>
<td>CST</td>
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<td>pAS16O(^{562})</td>
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<td>CST</td>
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<tr>
<td>p(Ser/Thr) Akt Substrate Antibody</td>
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<tr>
<td>pGSK-3 (^{\alpha/\beta} Ser219)</td>
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<td>CST</td>
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<td>Merck</td>
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<td>NKA(^{\alpha2})</td>
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<td>Merck</td>
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<td>Proteintech</td>
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<tr>
<td>Actin</td>
<td>Rabbit</td>
<td>1:1500</td>
<td>SCB</td>
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<td>Mouse</td>
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<td>See text.</td>
</tr>
<tr>
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<td>See text.</td>
</tr>
<tr>
<td>BA-D5</td>
<td>Mouse</td>
<td>1:250</td>
<td>See text.</td>
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</table>

SCB: Santa Cruz Biotechnology; CST: Cell Signaling Technology

Following the incubation with primary antibodies the membranes were washed with TBST again and then incubated for 1 h with secondary antibodies in 5% skimmed milk in TBST. For the detection of primary antibodies, we used secondary antibodies Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (cat. no. #170-6515, Bio-Rad Laboratories, Hercules, CA, USA) or Goat Anti-Mouse IgG (H+L)-HRP Conjugate (cat. no. #170-6516, Bio-Rad Laboratories, Hercules, CA, USA), used according to the source of primary antibodies. After the incubation with secondary antibodies the membranes were washed in TBST. The blots were visualized with chemiluminescent Pierce ECL Western Blotting Substrate (#32106, Thermo Fisher Scientific, Waltham, MA USA). With this method we incubated the membrane with chemiluminescent substrate that contains luminol and H\(_2\)O\(_2\). Horseradish peroxidase, conjugated to secondary antibodies, catalyses the reaction of H\(_2\)O\(_2\) and luminol that produces 3-aminophthalate. As 3-aminophthalate returns to the ground state it emits light of 428 nm
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wavelength. Signal was detected on X-ray films AGFA CP-BU NEW MEDICAL X-ray film blue 18x24 with the help of AGFA Curix60 machine (both from AGFA HealthCare NV, Mortsel, Belgium). GS800 Calibrated Densitometer and Quantity One Software (both Bio-Rad Laboratories, Hercules, CA, USA) were used for the densitometric quantification of detected bands.

3.6 Statistical analysis

Data are presented as mean ± standard error (SEM). Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). The difference between two groups was considered statistically significant when \( p \) value was \(<0.05\). Chosen statistical methods for each experiment can be found in figure captions.

4 RESULTS

4.1 Hypothesis I: Myotubes innervated \textit{in vitro} and mature muscle fibres \textit{in vivo} have a similar expression pattern of NKA and FXYD proteins.

Results of experiments that addressed Hypothesis I were published in Jan et al. (2021) and Mars et al. (2020). A summary of these results is provided below.

4.1.1 Study overview

NKA\(\alpha2\) is the most expressed \(\alpha\)-isoform in human skeletal muscle and is estimated to represent somewhere between 60 and 90\% of all NKA \(\alpha\)-subunits (Pirkmajer & Chibalin, 2016). Although the protein content of NKA\(\alpha2\) in cultured HSMC increases with differentiation in low serum culture medium and NKA\(\alpha1\) decreases (Al-Khalili et al., 2004), NKA\(\alpha1\) remains the most abundant of the two after 2 weeks of differentiation (Pirkmajer et al., 2020). We first explored how differentiation in low serum affects the expression of NKA subunits, FXYD proteins, and different muscle specific proteins, such as myosin heavy chains.
MyHC-2X, MyHC-2A, and MyHC-β/slow, α1S subunit of the dihydropyridine receptor (DHPRα1S), and sarco(endo)plasmic reticulum Ca²⁺-ATPases (SERCAs) in CD56⁻ and CD56⁺ HSMC.

The main goal of Hypothesis I was to explore, whether innervated, contracting HSMC exert an expression profile of NKA subunits and FXYD1 proteins that was more alike to that, found in mature skeletal muscle. Innervation of HSMC with explants of rat embryonic spinal cords induces the further development of many crucial structural components, found in developed muscle fibres. Among these structures are also T-tubules (Askanas et al., 1987), where majority of NKAα2 pool is found in mature skeletal muscle fibres (Kristensen et al., 2008). We thus expected to see an increase in NKAα2 expression in innervated HSMC.

We also explored the importance of muscle contractions for the expression of NKA subunits and their FXYD regulators. We used irreversible and reversible antagonists of nAChR α-bungarotoxin and d-tubocurarine to block neuromuscular transmission to evaluate the importance of contractions and neurotransmissions for the regulation of NKA subunits and FXYD proteins.

4.1.2 Effects of differentiation on the regulation of ion transport machinery in CD56⁻ and CD56⁺ HSMC

Primary HSMC were first separated with MACS microbeads on the base of their expression of CD56 (neural cell adhesion molecule 1, NCAM1) (Fig. 4.2A,B). This was done to obtain a large myogenic population of cells, as CD56 is a known myogenic marker (Hurko & Walsh, 1983; Cashman et al., 1987; Mackey et al., 2009). We were interested in how the expression of NKA subunits, FXYD proteins and some other muscle-specific proteins might differ between CD56⁺ and CD56⁻ HSMC that were either kept in 10% FBS aMEM, which is a commonly used medium formulation for myoblast cultivation (Dolinar et al., 2018) or
differentiated for additional 7 days under low-serum conditions (2% FBS aMEM) to cause an efficient fusion of myoblasts into multinucleated myotubes in CD56⁺ HSMC (Fig. 4.1A-D). For normalization of gene expression, a geometric mean of 18S rRNA and cyclophilin (PPIA) was used.

Figure 4.1: Microscopic pictures of CD56⁻ and CD56⁺ HSMC. HSMC were isolated from samples of *m. semitendinosus* and cultured in 10% FBS-supplemented aMEM. Before they reached confluence, they were separated into CD56⁻ and CD56⁺ fractions with MACS microbeads. One half was only grown in 10% FBS-supplemented aMEM ((A) CD56⁻ 10% FBS and (C) CD56⁺ 10% FBS), while the other was subsequently differentiated for 7 days in aMEM with 2% FBS ((B) CD56⁻ 2% FBS and (D) CD56⁺ 2% FBS).

From microscopic pictures of CD56⁻ and CD56⁺ it is immediately evident even to the common eye that CD56⁻ and CD56⁺ HSMC differ significantly in their morphology. While CD56⁻ started to fuse in 2% FBS-supplemented medium into multinucleated myotubes, we did not observe fusion of cells in CD56⁻ fraction (Fig. 4.1A-D). To see whether differentiation of myogenic cells made them more alike to mature muscle fibres on the mRNA and protein expression levels, we first evaluated the effects of differentiation on the expression of muscle-specific proteins. Expression of desmin, which is an intermediary filament, found in myogenic cells (Lazarides & Hubbard, 1976) (Fig. 4.2C), myosin heavy chains MyHC-2X (*MYH1* gene),
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MyHC-2A (MYH2), and MyHC-β/slow (MYH7) (Fig 4.2D,J), and DHPR\(\alpha_{1S}\) (CACNA1S gene) (Fig. 4.2E), which is a voltage-gated Ca\(^{2+}\) channel that is located in T-tubules of muscle fibres (Flucher & Franzini-Armstrong, 1996), was significantly higher in CD56\(^{+}\) compared to CD56\(^{-}\) HSMC. The expression also increased in both fractions, when they were differentiated in 2% FBS-supplemented medium, but the difference was not statistically significant in CD56\(^{-}\) cells (Fig. 4.2C-E,J). We observed no difference in the expression of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase SERCA1 (ATP2A1) between CD56\(^{+}\) and CD56\(^{-}\) HSMC or serum conditions (Fig 4.2F). On the other hand, SERCA2 (ATP2A2) was markedly higher in CD56\(^{+}\) HSMC and this was even more pronounced in 2% FBS (Fig. 4.2F). SERCA3 (ATP2A3) could not be detected in any experiment group.
Figure 4.2 Effects of differentiation of CD56+ and CD56- HSMC on the regulation of ion transport machinery. HSMC were isolated from samples of *m. semitendinosus* and cultured in 10% FBS-supplemented aMEM. Before they reached confluence, they were separated into CD56+ and CD56- fractions with MACS microbeads. One half was only grown in 10% FBS-supplemented aMEM (CD56+ 10% FBS and CD56- 10% FBS), while the other was subsequently differentiated for 7 days in aMEM with 2%FBS (CD56- 2% FBS and CD56+ 2% FBS). (B-I) qPCR was used to estimate gene expression of (B) CD56 (NCAM1), (C) desmin, (D) myosin heavy chains MYH1, MYH2, and MYH7, (E) CACNA1S, (F) SERCA1 (ATP2A1) and SERCA2 (ATP2A2), (G) NKAα1-3 (ATP1A1-3), (H) NKAβ1-3 (ATP1B1-3), and (I) FXYD1 and FXYD5. Results were normalized to geometric mean of 18S rRNA and cyclophilin (PPIA). (J-N) Immunoblotting was used for evaluation of protein.
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The evaluation of mRNA expression of NKA subunits showed that NKA\(\alpha\)1 (\textit{ATP1A1}) was the most abundant NKA\(\alpha\) in both CD56\(^{-}\) and CD56\(^{+}\) HSMC (Fig. 4.2G). Protein content of NKA\(\alpha\)1 was lowered after differentiation in both CD56\(^{-}\) (\textit{P}<0.05) and CD56\(^{+}\) (\textit{P}=0.0912) HSMC, but we observed no significant changes on the mRNA level (Fig. 4.2G). The expression of NKA\(\alpha\)2 (\textit{ATP1A2}) was markedly higher in CD56\(^{+}\) HSMC and furthermore increased in differentiated cells (Fig. 4.2G,K). The expression of NKA\(\alpha\)3 (\textit{ATP1A3}) tended to decrease during differentiation in both fractions (Fig. 4.2G), but it is important to note here that the mRNA levels of NKA\(\alpha\)3 were very low and we were unable to reliably detect the NKA\(\alpha\)3 protein with immunoblot, which suggests that NKA\(\alpha\)3 does not play a major role in cultured HSMC.

Conversely to NKA \(\alpha\)-subunits, the mRNA expression of NKA \(\beta\)-subunits (\textit{ATP1B1-3}) did not much differ between CD56\(^{-}\) and CD56\(^{+}\) HSMC (Fig. 4.2H). Expression of NKA\(\beta\)1 (\textit{ATP1B1}) was reduced with differentiation in CD56\(^{+}\) cells (Fig. 4.2H), while we observed no changes in the expression of NKA\(\beta\)2 (\textit{ATP1B2}) or NKA\(\beta\)3 (\textit{ATP1B3}) (Fig. 4.2H).

Unlike NKA \(\alpha\)-subunits expression of FXYD1 was higher in CD56\(^{-}\) than in CD56\(^{+}\) HSMC (Fig. 4.2I,L). Differentiation caused a significant increase in the expression of FXYD1 in CD56\(^{-}\) HSMC and a similar trend was observed in CD56\(^{+}\) HSMC (Fig. 4.2I,L). It is important to note that despite a high level of mRNA expression FXYD1 protein could only be reliably detected in 3 out of 4 donor HSMC. FXYD5 mRNA levels were reduced with differentiation, but changes on protein level were not statistically significant (Fig. 4.2I,L).
Additionally we also evaluated the expression of Sp1 transcription factor (Fig. 4.2M) that is described to regulate NKA expression (Li & Langhans, 2015) and actin (Fig. 4.2N), but we observed no differences between cell fractions or serum conditions.

4.1.3 Species specificity validation of qPCR assays for estimation of gene expression in co-cultures of differentiated HSMC and rat embryonic spinal cord explants

HSMC were grown in presence or absence of embryonic spinal cord explants for 21-22 days (Fig. 4.4A). Before we did our experiments on innervated HSMC, we wanted to validate qPCR assays, used in our experiments. As co-cultures include both muscle tissue of human origin and neural tissue of rat origin, we wanted to make sure that our assays did not replicate any cDNA from rat tissue. We tested our selected assays for species specificity (Fig. 4.3). When choosing commercial human gene expression assays, we selected those with good target coverage and the ones that bind to regions with lowest level of sequence similarity between human and rat transcripts. Selected assays were then tested on various rat tissues (Fig. 4.3A-P). Some gene expression assays, marketed as human-specific also detected rat transcripts (Fig. 4.3N-P), but only those that did not replicate any rat transcripts or were a lot more selective for human transcripts (Fig. 4.3K) were used in our experiments.
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Figure 4.3: Species specificity validation of qPCR assays for estimation of gene expression in co-cultures of human myotubes and rat embryonic spinal cord explants. (A-P) Validation of gene expression assays (see Methods) was performed by qPCR on human skeletal muscle (huSkM, m. semitendinosus), human skeletal muscle cells (HSMC), and a panel of rat tissues (extensor digitorum longus (EDL) and soleus muscles, brain, kidney, and L6 skeletal muscle cells). All tissues and cell samples were measured in duplicates. Gene symbols are the same as in Fig. 4.1. 18S rRNA was used as endogenous control. These results were published in PLOS One (Jan et al., 2021).
4.1.4 Effects of innervation on the ion transport machinery in differentiated HSMC

After the effects of differentiation, we were also interested how innervation of HSMC affects the expression of ion transport proteins. HSMC were first differentiated into myotubes and then grown in the presence or absence of the embryonic rat spinal cord explants (Fig. 4.4A) for 21 days. Co-cultured myotubes started to contract after 7 to 10 days. Gene expression was normalized to geometric mean of β-actin (ACTB) and cyclophilin (PPIA). We observed no statistically significant difference in the mRNA expression of NKAα1-3 (Fig. 4.4B), NKAβ1-3 (Fig 4.4C), FXYD1 (Fig 4.4D), or myosin heavy chains MyHC-2X (MYH1 gene), MyHC-2A (MYH2), and MyHC-β/slow (MYH7) (Fig. 4.4E) between aneural and innervated myotubes, but innervation did cause a minor increase in the expression of FXYD5 (Fig. 4.4D). Innervation caused no difference to the mRNA expression of SERCA1 and SERCA2 (Fig. 4.4F), nor did it cause any difference to DHPRα1S (CACNA1S; Fig. 4.4G).
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Figure 4.4: Effects of innervation on the mRNA expression of ion transport machinery in HSMC. HSMC were grown aneurally or co-cultured with the embryonic rat spinal cord for 21 days. (B-G) qPCR technique was used for gene expression evaluation of (B) NKAα1-3 (ATP1A1-3), (C) NKAβ1-3 (ATP1B1-3), (D) FXYD1 and FXYD5, (E) myosin heavy chains MYH1, MYH2, and MYH7, (F) SERCA1 (ATP2A1) and SERCA2 (ATP2A2), and (G) CACNA1S. Human-specific gene expression assays were used, and the results were normalized to geometric mean of β-actin (ACTB) and cyclophilin (PPIA). Results are means ± SEM (n=3-5), *P<0.05 - Aneural cells vs. Co-cultures. Ratio paired t-test was used for statistical evaluation. These results were published in PLOS One (Jan et al., 2021) and APNM 2020 (Mars et al., 2020).

Protein content of NKAα1 and NKAα2 was higher in co-cultures (Fig 4.5A). NKAα3 could not be detected reliably with immunoblot due to many unspecific bands. Abundance of the total and phosphorylated (Ser68) FXYD1 protein increased with innervation (Fig 4.5B). Protein levels of myosin heavy chains MyHC-2X and MyHC-2A (MYH2), and MyHC-β/slow (MYH7) decreased in innervated myotubes (Fig. 4.5 D). There was no significant difference in protein content of FXYD5 (Fig. 4.5C) or actin (Fig. 4.5E) between aneural cells and co-cultures. None of the primary antibodies are specific for human tissue, which means that proteins from rat neural component could also be detected with immunoblot. Nevertheless when gene expression assays were used for NKAα2 and FXYD1 that detect both human and rat transcripts (Fig. 4.3N,P), there were no significant differences in mRNA expression between aneural cells and co-cultures (Fig. 4.5F,G). This incidentally suggests that contribution from rat neural component was not significant.
Figure 4.5: Effect of innervation on the protein expression of NKA subunits, FXYDs, and myosin heavy chains HSMC were grown aneurally or co-cultured with the embryonic rat spinal cord for 21 days. (A-E) Immunoblotting was used to evaluate protein content of (A) NKA1 and NKA2, (B) FXYD1, and pFXYD1 Ser68, (C) FXYD5, (D) myosin heavy chains MyHC-2A and -2X, and MyHC-β/slow, and (E) actin. (F,G) qPCR was used for evaluation of gene expression of (F) FXYD1 (Hs00245327_m1) and (G) NKAα2 (Hs00265131_m1) that detect human and rat transcripts. Results are means ± SEM (n=5-6), *P<0.05 - Aneural cells vs. Co-cultures. Ratio paired t-test was used for statistical evaluation. These results were published in PLOS One (Jan et al., 2021) and APNM 2020 (Mars et al., 2020).

4.1.5 Effect of suppression of neuromuscular transmission on expression of NKA subunits, FXYD1, and FXYD5 in innervated HSMC

As acetylcholine and nAChR have been reported to regulate NKA in skeletal muscles (Dlouhá et al., 1979; Heiny et al., 2010; Chibalín et al., 2012a), we were interested in how the suppression of neuromuscular transmissions with pharmacological inhibitors of nAChR might affect the expression of NKA subunits and FXYDs. 21-day-old co-cultures and their aneural controls were treated for 24 h with either irreversible nAChR inhibitor α-bungarotoxin (BTX;
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1 μg/ml or 10 μg/ml) or reversible nAChR inhibitor d-tubocurarine (DTC; 1 mM) to repress the neuromuscular transmission. We observed variable effects on contractions. In some cultures, the contractions were completely stopped, while in others some contractile activity remained. This is in accordance with previous observations from our group, where co-cultures were treated with nAChR inhibitor rocuronium (Rezonja et al., 2014). Overall, we observed no significant effect of either α-bungarotoxin (Fig. 4.6A-F) or d-tubocurarine treatment (Fig. 4.6G-I) on the mRNA expression of NKA subunits or FXYD1, and FXYD5.

Figure 4.6: Effects of α-bungarotoxin or d-tubocurarine on expression of NKA subunits, FXYD1, and FXYD5 in aneural and innervated HSMC 21-day old co-cultures and aneural controls were treated with 1 μg/mL α-bungarotoxin (BTX; A-C), 10 μg/mL α-bungarotoxin (D-F) or 1 mM d-tubocurarine (DTC; G-I) for 24 h. (A-I) qPCR was used to estimate gene expression of (A,D,G) NKAα1-3 (ATP1A1-3), (B,E,H) NKAβ1-3 (ATP1B1-3), or (C,F,I) FXYD1 and FXYD5. Results were normalized to a geometric mean of PPIA and ACTB. Human-specific gene expression assays were used. Results are presented as means ± SEM (n=3-4), #P<0.05 – Aneural cells vs. Co-cultures. Two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons was
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used for statistical evaluation. Basal results for Aneural cell and Co-culture samples for 10 μg/mL α-bungarotoxin and 1 mM d-tubocurarine are the same but were measured on different occasions. These results were published in PLOS One (Jan et al., 2021).

4.1.6 Relative mRNA expression levels of NKA subunits and FXYDs in different HSMC models

One of the 3 main aims of this doctoral dissertation was to ascertain if HSMC innervated, in vitro, have a similar expression pattern of NKA subunits and FXYD proteins to mature muscle fibres. From our PCR results from Figs. 4.2, 4.4 and 4.6 we have calculated mRNA ratios between NKA subunits and FXYDs in different differentiation stages and cell models (Fig. 4.7) and compared them to mRNA ratios of human vastus lateralis and semitendinosus muscles. Immediately it was quite clear that NKA\(\alpha_2\) was present at a very low level in CD56\(^-\) HSMC. Conversely NKA\(\alpha_2\) represented almost 50% of total NKA\(\alpha\) mRNA expression in CD56\(^+\) HSMC, cultured in aMEM with 2% FBS (Fig. 4.7A). The level of NKA\(\alpha_2\) mRNA expression in 21-22-day-old co-cultures and aneural cells that were cultured in medium with 10% FBS, was lower than in differentiated CD56\(^+\) HSMC, which were cultured in medium with 2% FBS. In co-cultures and aneural cells NKA\(\alpha_2\) represented around 21% of all NKA\(\alpha\) in co-cultures and aneural cells alike, while NKA\(\alpha_1\) represented approximately 78%. In samples from human vastus lateralis and semitendinosus NKA\(\alpha_2\) represented more than 99% of all NKA\(\alpha\) mRNA expression, while NKA\(\alpha_1\) represented less than 1%.
Figure 4.7: Relative mRNA expression levels of NKA subunits and FXYDs in different HSMC models (A-C) Relative mRNA content of (A) NKAα1-3 (ATP1A1-3), (B) NKAβ1-3 (ATP1B1-3), (C) FXYD1 and FXYD5 in different models were computed based on results in Figs. 4.2, 4.4, and 4.6. To compare different models to in vivo skeletal muscles, semitendinosus and vastus lateralis from Control subjects from Figs. 4.8 and 4.9 were used. CD56− and CD56+ HSMC were grown either in 10%FBS- or 2%FBS-supplemented aMEM as described in Fig. 4.2. Co-cultures and their aneural controls were cultured in 10% FBS-supplemented medium for 21 (Fig. 4.4) or 22 days (Fig. 4.6). We arbitrarily set the expression level of NKAα1, NKAβ1, and FXYD1 mRNA to 1. Results are means ± SEM (n=4-12 (co)cultures), *P<0.05 vs. CD56+ (2% FBS). These results were published in PLOS One (Jan et al., 2021).
Amongst NKAβ variants NKAβ3 mRNA expression was by far the highest under all experimental conditions and differentiation or de novo innervation did not have a significant effect on the ratios between NKAβ isoforms (Fig. 4.7B). We did however, observe a non-significant decrease in the ratio of NKAβ1 compared to all NKAβ between undifferentiated (10% FBS) and differentiated (2% FBS) CD56+ cells (from 43% to 25 %), while the ratio of NKAβ3 increased from 57% to 75% (Fig. 4.7B). The percentage of NKAβ3 was even higher in aneural cells and co-cultures, where NKAβ3 represented 88% and 87% of all NKAβ, respectively, while NKAβ1 represented 11.5% and 12.5%, respectively. In vastus lateralis and semitendinosus NKAβ1 was by far the most abundant NKAβ isoforms, representing approximately 87% in vastus lateralis and 92% in semitendinosus, while NKAβ2 was the second most abundant, with 12.5% in vastus lateralis and 7% in semitendinosus, respectively. Unlike in cultured HSMC NKAβ3 was barely detectable in mature muscle samples.

Reducing serum content in medium from 10% FBS to 2% FBS significantly changed the FXYD1-to-FXYD5 ratio. By lowering serum content in medium FXYD1 ratio increased in both CD56− (25% to 76%) and CD56+ (18% to 66%) cells, while the ratio of FXYD5 dropped (75% to 24% for CD56−, 82% to 34% for CD56+) (Fig. 4.7C), but we observed no significant effects of innervation on the FXYD1-to-FXYD5 ratio. Again, there were big differences in mRNA expression between mature muscle samples and cell culture. In both vastus lateralis and semitendinosus, FXYD1 represented more than 99% of all FXYD mRNA expression.

4.2 Hypothesis II: Hypoxia alters NKA function in skeletal muscle via activation of 5' AMP-activated protein kinase (AMPK).

Results of experiments that addressed Hypothesis II are being prepared for the submission (manuscript in preparation).
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4.2.1 Study overview

Experiments on skeletal muscle cells show that AMPK activation can stimulate NKA activity (Benziane et al., 2012), which is why we hypothesized that hypoxia-induced AMPK activation could modulate NKA in skeletal muscle exposed to blood flow restricted exercise. We tested whether ischaemic preconditioning of skeletal muscles causes changes in NKA function and whether these changes are connected with AMPK activity. We compared vastus lateralis and semitendinosus samples from patients with ACL injury that performed no pre-operative exercising (Control), performed standard low-load knee exercises (LL-Sham) or performed low-load exercises with blood-flow restriction (LL-BFR). We evaluated effects of different training regimens on mRNA expression and protein content of NKA α-subunits and FXYDs, and phosphorylation and protein content of AMPK and its target Acetyl-CoA carboxylase (ACC). We were especially interested whether BFR training would have any effect on NKA α-subunits, as Kutz et al. (2018) recently proposed that α1-subunit might have an important role in muscle hypertrophy. Any possible increases in the expression of this subunit might add an explanation to possible positive effects of BFR intervention for muscle mass of patients with knee injury. After performing analysis on muscle samples, we also evaluated the effects of glucose starvation (NGD), hypoxia (HYP) and combination of both (HGD) on mRNA expression and protein content of NKA α-subunits and FXYDs in human primary skeletal muscle cells. We also checked for any changes in AMPK signalling.

4.2.1 Effects of different training interventions on AMPK signalling in vastus lateralis and semitendinosus muscles

Since there is strong evidence that AMPK plays a role in regulation of NKA activity (Pirkmajer et al., 2021), we evaluated, whether LL-Sham and LL-BFR training (Fig. 4.8A) caused any changes to protein content of AMPK and its target ACC, and to their respective phosphorylation of Thr172 and Ser79 residues. There were no changes in protein content of
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AMPK (Figure 4.8B) and ACC (Fig. 4.8E) or in their phosphorylation (Fig. 4.8C,F, respectively) in either vastus lateralis or semitendinosus muscle samples. We also did not observe any changes in ratios between phosphorylated and total forms of AMPK (Fig. 4.8D) or ACC (Fig. 4.8G).

![Graph showing effect of LL-BFR and LL-Sham on AMPK signalling in vastus lateralis and semitendinosus muscles.](image)

Figure 4.8: Effect of LL-BFR and LL-Sham on AMPK signalling in vastus lateralis and semitendinosus muscles. (A) Training intervention consisted of 9 exercise sessions (ES) that were performed with either true blood flow restriction (LL-BFR) or sham blood flow restriction (LL-Sham). Control group patients did not perform any interventions (Control). Every session included 4 sets of knee flexion and extension exercises performed at 40RM to volitional failure with injured leg only. See text for further details. (B,C,E,F)
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Immunoblotting was used to estimate protein abundance of (B) AMPK, (C) pAMPK\textsuperscript{Thr172}, (E) ACC, and (F) pACC\textsuperscript{Ser79}. Presented are also (D) ratios of phosphorylated AMPK to total AMPK and (G) phosphorylated ACC, relative to total ACC. Results are means ± SEM (n=3-6); * P<0.05 vs. Control; #P<0.05 vs LL-Sham. Kruskal-Wallis with Dunn’s post hoc test was used for statistical evaluation. Unpublished data, manuscript ready for submission.

4.2.2 Effect of LL-BFR and LL-Sham exercise on the expression of the NKA subunits, FXYD1, and FXYD5 in vastus lateralis and semitendinosus muscles

The mRNA levels of NKA\textsubscript{α1} were elevated in vastus lateralis muscles of subjects that performed LL-BFR training compared to Control and to LL-Sham (Fig. 4.9A). Protein content of NKA\textsubscript{α1} seemed to be higher in LL-BFR group compared to Control (Fig. 4.9I), but the difference did not reach statistical significance (P=0.335). In semitendinosus muscle NKA\textsubscript{α1} mRNA expression seemed to be higher in LL-BFR group compared to Control (P=0.118) and it was also higher on protein level (P=0.054). It is known that NKA activity can be modulated by Tyr10 phosphorylation of NKA\textsubscript{α1} in kidney cells (Feraille et al., 1999) and probably also in skeletal muscle tissue (Breitenbach et al., 2016), so we were interested whether the described training interventions might cause a change in phosphorylation of NKA\textsubscript{α1} on Tyr10. We observed no differences in Tyr10 phosphorylation between interventions in vastus lateralis muscle, but there was a negative trend in Tyr10 phosphorylation in semitendinosus muscle of subjects, who performed LL-BFR (P=0.174) (Fig. 4.9J).
Figure 4.9: Effect of LL-BFR and LL-Sham on the expression of NKA subunits, FXYD1, FXYD5 and Sp1 in the vastus lateralis and the semitendinosus muscles. qPCR was used to evaluate gene expression of (A) NKAα1 (ATP1A1), (B) NKAα2 (ATP1A2), (C) NKAα3 (ATP1A3), (D) NKAβ1 (ATP1B1), (E) NKAβ2 (ATP1B2), (F) NKAβ3 (ATP1B3), (G) FXYD1, and (H) FXYD5. 18S rRNA was used as endogenous control. Immunoblotting was used to estimate protein abundance of (I) NKAα1, (J) pNKAα1 Tyr10, (K) NKAα2, (L) FXYD1, (M) pFXYD1 Ser68, (N) FXYD5, and (O) Sp1. Results are means ± SEM (n=3-6); * P<0.05 vs. Control; #P<0.05 vs LL-Sham. Kruskal-Wallis with Dunn’s post hoc test was used for statistical evaluation. Unpublished data, manuscript ready for submission.

There were no significant differences between the three groups in vastus lateralis muscle concerning mRNA and protein expression of NKAα2 (Fig. 4.9B,K) or mRNA
expression NKAα3 (Fig. 4.9C), but there seemed to be an increase in NKAα2 protein content in the LL-BFR group vs. Control (P=0.197), and a possible increase in mRNA expression NKAα3 in the LL-BFR group vs. Control (P=0.075). In semitendinosus muscle there seemed to be a decrease in mRNA expression of NKAα2 in LL-Sham (P=0.156) and LL-BFR (0.079) groups vs. Control, but the same trends were not seen on protein level (Fig. 4.9B,K).

NKAα3 mRNA was decreased in semitendinosus muscle of LL-SHAM and LL-BFR groups vs. Control (Fig. 4.9C), but the importance of these changes is questionable as the detected levels of cDNA for NKAα3 were extremely low and we could not effectively detect NKAα3 protein on immunoblot.

We observed no differences in levels of FXYD1 (Fig. 4.9G,L), or in its phosphorylation of Ser68 residue (Fig. 4.9M) between the three groups in either muscle. FXYD5 was unchanged between all three groups in vastus lateralis muscle, both on mRNA (Fig. 4.9H) and protein level (Fig. 4.9N), but there was a decrease in protein abundance of FXYD5 in semitendinosus muscle of subjects, who performed LL-BFR (Fig. 4.9N). We also evaluated the protein levels of Sp1, which is a transcription factor, involved in regulation of NKA subunits (Li & Langhans, 2015) and responsive to hypoxia (Scaringi et al., 2013). There were no significant differences between groups in either evaluated muscle (Fig. 4.9O).

4.2.3 Effect of hypoxia and/or glucose deprivation cellular response to hypoxic stress and AMPK signalling in HSMC

We were interested to see, whether the consequences of LL-BFR intervention could be replicated in vitro, by exposing HSMC for 24 h to hypoxia (0.1% O₂), glucose deprivation, or both (HGD) (Fig. 3.4). Responses of cells to hypoxic stress were evaluated by mRNA and protein measurements of hypoxia-inducible factor-1α (HIF-1α), which is a transcription factor that is major actor in cell response to hypoxia (Samanta & Semenza, 2017). We also measured
mRNA expression of HIF-1α target genes vascular endothelial factor A (VEGFA) and phosphoglycerate kinase 1 (PGK1). The mRNA expression of HIF-1α decreased in hypoxic conditions and in glucose deprivation, but it reached the lowest level in combination of both (HGD) (Fig. 4.10A). Hypoxic treatment caused a significant upregulation of HIF-1α protein (Fig. 4.10B), and it also increased mRNA levels of VEGFA (Fig. 4.10C) and PGK1 (Fig. 4.10D). HGD treatment did not increase protein levels of HIF-1α and it caused the most significant decrease in HIF-1α mRNA expression (Fig. 4.10A,B). Levels of energy stress were evaluated by estimating AMPK activity. We measured protein content of AMPK and of its target ACC and we also looked at their phosphorylation levels on Thr172 and Ser79 residues, respectively. AMPK and ACC phosphorylation and therefore activity was increased with HGD treatment, but not with hypoxia or glucose deprivation alone (Fig. 4.10E-H). Glucose deprivation and HGD also caused an increase in total protein levels of ACC (Fig. 4.10G).
4.2.4 Effects of hypoxia and/or glucose deprivation on the mRNA expression of NKA subunits and FXYDs in HSMC

Hypoxia by itself did not have any significant effect on the mRNA expression of NKA subunits (Fig. 4.11A-F). On the other hand, glucose deprivation and HGD caused a significant decrease in mRNA levels of NKAα1 (Fig. 4.11A), NKAβ1 (Fig. 4.11D) and NKAβ3 (Fig. 4.11F). The mRNA expression of NKAα2 (Fig. 4.11B), NKAα3 (Fig. 4.11C), NKAβ2 (Fig. 4.11E), and FXYD1 (Fig. 4.11G) were also significantly decreased under HGD conditions. Unpublished data, manuscript ready for submission.
4.11E), FXYD1 (Fig.4.11G) and FXYD5 (Fig. 4.11H) remained unchanged in all treatments, although there seemed to be a decrease in the expression of NKAβ2 with glucose deprivation that was not significant (Fig. 4.11E).

Figure 4.11: Effect of hypoxia and/or glucose deprivation on the expression of NKA subunits, FXYD1, and FXYD5 in HSMC. For the last 24 h differentiated HSMC were exposed to normoxia (NOR), glucose deficiency (glucose-free medium, NGD), hypoxia (0.1% O₂, HYP), or both (HGD) in serum-free DMEM. See text for details. qPCR was used to estimate gene expression of (A) NKAα1 (ATP1A1), (B) NKAα2 (ATP1A2), (C) NKAα3 (ATP1A3), (D) NKAβ1 (ATP1B1), (E) NKAβ2 (ATP1B2), (F) NKAβ3 (ATP1B3), (G) FXYD1, and (H) FXYD5. Results are means ± SEM for n=6; *P<0.05 vs. NOR. Ordinary one-way ANOVA with Dunnett’s post hoc test was used for statistical evaluation. Unpublished data, manuscript ready for submission.

4.2.5 Effects of hypoxia and/or glucose deprivation on the protein levels of NKA subunits, FXYDs, and Sp1 in HSMC

Similarly to what was observed on mRNA level, hypoxia did not by itself cause any change to protein content of NKAα subunits (Fig. 4.12A,C). Glucose deprivation and HGD both suppressed protein content of NKAα1 (Fig. 4.12A) but had no effect on protein content
of NKA\(\alpha\)2 (Fig. 4.12C). Tyr10 phosphorylation of NKA\(\alpha\)1 was markedly reduced in HGD (Fig. 4.12B). Although FXYD5 was unaffected by all 3 treatment on mRNA level, glucose deprivation increased its protein content, and a similar trend could be seen with HGD (Fig. 4.12D). Protein levels of Sp1 transcription factor were suppressed under all three treatments and were lowest with HGD (Fig. 4.12E).

**Figure 4.12**: Effect of hypoxia and/or glucose deprivation on the protein content of NKA subunits, FXYD5, and Sp1 in HSMC. For the last 24 h differentiated HSMC were exposed to normoxia (NOR), glucose deficiency (glucose-free medium, NGD), hypoxia (0.1% \(O_2\), HYP), or both (HGD) in serum-free DMEM. See text for details. Immunoblotting was used to estimate protein abundance of (A) NKA\(\alpha\)1, (B) pNKA\(\alpha\)1 Tyr10, (C) NKA\(\alpha\)2, (D) FXYD5, and (E) Sp1. Results are means ± SEM for \(n=5-6\); *\(P<0.05\) vs. NOR. In case of unspecific bands arrowheads mark the specific bands that were used in densitometric analysis. Ordinary one-way ANOVA with Dunnett’s *post hoc* test was used for statistical evaluation. Unpublished data, manuscript ready for submission.

4.2.6  **Effect of in vitro preconditioning and acute HGD on cellular response to hypoxia and AMPK signalling in cultured HSMC**

LL-BFR intervention exposed training subject to short bouts of ischaemia. To mimic the intervention protocol, we kept HSMC in hypoxic (0.1% \(O_2\), HYP) or glucose-deprived
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(GD) conditions, or in conditions that combined both (HGD) for 1 h per day for 3 days (preconditioning). 23 h after the last preconditioning HSMC were exposed for 2 hours to either normal (NOR) conditions or to acute HGD. The last 2-h exposure to quasi-ischaemic conditions was there to mimic the conditions that arise during orthopaedic surgery, where complete blood flow restriction is performed on the skeletal muscle to prevent blood loss. The whole experimental protocol is presented in Fig. 4.13A.

Acute HGD (ischaemic) seemed to slightly reduce HIF-1α mRNA expression (Fig. 4.13B), but VEGFA mRNA was markedly increased (Fig. 4.13C). PGK mRNA expression was only slightly upregulated (Fig. 4.13D).

Figure 4.13: Effect of hypoxia and/or glucose deprivation on cellular response to hypoxia and AMPK signalling in HSMC. (A) Scheme of experimental protocol of preconditioning. See text for details. qPCR was used to evaluate gene expression of (B) HIF-1α (HIF1A), (C) VEGFA, and (D) PGK1. 18S rRNA was used as endogenous control. Results are means ± SEM for n=5; *P<0.05 vs. NOR (+2h NOR or 2h HGD); #P<0.05 vs.
4.2.7 Effect of in vitro preconditioning on mRNA expression of NKA subunits, FXYD1, and FXYD5 in cultured HSMC.

In vitro preconditioning treatments had no effect on mRNA expression of NKA subunits, FXYD1, or FXYD5 (Fig. 4.14A-H).

![Graphs showing mRNA expression of NKA subunits and FXYD1/FXYD5](image)

Figure 4.14: Effect of in vitro preconditioning on mRNA expression of NKA subunits, FXYD1, and FXYD5 in HSMC. qPCR was used to estimate gene expression of (A) NKAα1 (*ATP1A1*), (B) NKAα2 (*ATP1A2*), (C) NKAα3 (*ATP1A3*), (D) NKAβ1 (*ATP1B1*), (E) NKAβ2 (*ATP1B2*), (F) NKAβ3 (*ATP1B3*), (G) *FXYD1*, and (H) *FXYD5*. Results are means ± SEM for n=5; *P<0.05 vs. NOR. Ordinary one-way ANOVA with Dunnett’s post hoc test was used. Unpublished data, manuscript ready for submission.
4.3 Hypothesis III: Modulation of NKA by pharmacological activators of AMPK depends on the metabolic state of skeletal muscle.

Some of the results from Hypothesis III were published in *American Journal of Physiology-Cell Physiology* (Dolinar et al., 2018) while the majority of the remaining results are in preparation for submission (manuscript in preparation).

4.2.1 Study overview

Metformin, the most widely used antidiabetic drug and a known indirect activator of AMPK, has been widely studied for its cardio- and neuro-protective effects during ischaemia-reperfusion injury and cerebral ischaemia (Solskov et al., 2008; Jiang et al., 2014). Metformin pretreatment has also been reported to improve NKA function through AMPK activation in energy-depleted epithelial cells (Seo-Mayer et al., 2011). We explored whether metformin pretreatment would affect the mRNA expression of NKA subunits and FXYD proteins in differentiated HSMC, which are exposed to different degrees of metabolic stress (deprivation of oxygen or glucose or both).

Afterwards we also evaluated SSZ, a commonly used drug in the clinic, as a potential AMPK activator. SSZ has been reported to inhibit ATIC (Baggott et al., 1992), which we hypothesized would lead to an indirect AMPK activation via increased level of intracellular ZMP. We tested whether SSZ's potential AMPK activation would have an additive effect, when used in combination with AICAR, in the same way as methotrexate (Pirkmajer et al., 2015), another ATIC inhibitor. We were also interested, whether SSZ might have an effect on Akt signalling and how its effects might differ in presence of different insulin concentrations. SSZ's effects were evaluated in differentiated L6 rat skeletal muscle cell line and in differentiated HSMC.
4.3.1 Effects of AMPK activator metformin treatment in normoxic, hypoxic and ischaemic conditions on the mRNA expression of NKA subunits, FXYD1, and FXYD5 in HSMC

To see how AMPK activation might affect the mRNA expression of different NKA subunits in different conditions, which induce metabolic stress, we treated HSMC with metformin. Metformin preconditioning was reported to protect MDCK cells against NKA endocytosis during ischaemic conditions via AMPK activation (Seo-Mayer et al., 2011). We treated differentiated HSMC with 2 different concentrations of metformin in normoxic, hypoxic and ischaemic (glucose deprivation with 0.1% O₂, HGD) conditions. As seen in previous experiments not all subunits were susceptible to changes in oxygen and glucose content. Both mRNA expression of NKA α₁- and β₁-subunits was reduced in HGD conditions and 3.0 mM metformin further exacerbated that effect (Fig. 4.15A,C). NKA α₂-subunit was not affected by hypoxic and HGD conditions, but 3.0 mM metformin in HGD conditions suppressed its mRNA expression (Fig. 4.15B). NKA β₂-subunit mRNA expression was increased in hypoxic conditions and HGD conditions, but 3.0 mM metformin concentration caused a return of mRNA expression to basal normoxic levels (Fig. 4.15D).

High concentration of metformin seemed to have an opposite effect on the mRNA expression of FXYD1 and FXYD5 in normoxic conditions. While FXYD1 was decreased by metformin (Fig. 4.15E), we observed an increased expression of FXYD5 (Fig. 4.15F). We also observed a decreased FXYD1 expression in HGD conditions and further reduction, when the cells were treated with 3.0 mM metformin (Fig. 4.15E). On the other hand, FXYD5 expression seemed to be only slightly reduced in HGD and treatment with metformin showed a positive trend in expression with 3.0 mM metformin. The efficacy of our hypoxic and HGD treatments was evaluated by checking possible changes in VEGFA mRNA expression. As seen in Fig. 4.15G hypoxic and HGD treatment caused an increased expression of VEGFA, but high
concentration of metformin (3.0 mM) decreased its expression to almost basal normoxic levels in HGD treatment.

Figure 4.15: Effects of AMPK activator metformin treatment in normoxic, hypoxic and ischaemic conditions on the mRNA expression of NKA subunits, FXYD1, FXYD5, and VEGFA in HSMC. HSMC were differentiated for 5 days in aMEM with 2% FBS, after which we started the first 24-h treatment in either 2% FBS-supplemented aMEM with vehicle, 0.3 mM metformin or 3.0 mM metformin. After 24 h we changed the medium with fresh 2% FBS-supplemented aMEM with the same treatments (vehicle, 0.3 mM and 3.0 mM metformin). On the 3rd day we repeated the same treatment (24 h), but this time in DMEM (2% FBS, 1g/L of
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glucose). On the 4th day, cells were exposed to normoxia (NOR), hypoxia (0.1% O\(_2\), HYP), or artificial ischaemia (glucose-free medium, 0.1% O\(_2\), HGD) in serum-free DMEM with either vehicle (Basal), 0.3 mM metformin or 3.0 mM metformin for 24h. qPCR was used to estimate gene expression of (A) NKAα1 (ATP1A1), (B) NKAα2 (ATP1A2), (C) NKAβ1 (ATP1B1), (D) NKAβ2 (ATP1B2), (E) FXYD1, (F) FXYD5, and (G) VEGFA. Results are mean ± SEM for n=4; *P<0.05, RM one-way ANOVA with Dunnett’s post hoc test was used for statistical evaluation of results.

4.3.2 The role of nucleoside transporters in AMPK activation in L6 cells and HSMC

Ischaemia disrupts synthesis of ATP and leads to loss of adenine nucleotides from skeletal muscle (Lindsay et al., 1990; Tullson et al., 1990; Arabadjis et al., 1993). Resynthesis of purine nucleotides can occur via de novo pathway, which involves synthesis of ATP from phosphoribosyl pyrophosphate via IMP, or via salvage pathway, which involves synthesis of ATP from adenosine taken up from the extracellular fluid (Tullson & Terjung, 1991; Brault & Terjung, 2001). Equilibrative nucleoside transporters 1 and 2 (ENT1 and ENT2) have been shown to mediate uptake of AICAR, an adenosine analogue and AMPK activator, in many different cells and tissue (Gadalla et al., 2004; Ceschin et al., 2014). To evaluate the role of nucleoside transporters in AICAR action, L6 cells were treated with 10 µM dipyridamole, a widely used ENT1/2 inhibitor (Molina-Arcas et al., 2009) and 1 µM NBTI, a selective ENT1 inhibitor (Molina-Arcas et al., 2009). In the presence of dipyridamole AICAR did not increase phosphorylation of AMPK and ACC in L6 cells (Fig. 4.16A,B), while NBTI treatment lowered AICAR-stimulated increase in ACC phosphorylation (Fig. 4.16A,B).

We showed that ENT1 mRNA was markedly more expressed in cultured HSMC than ENT2 mRNA (Dolinar et al., 2018). Treatment with 10 µM dipyridamole (DPY) or 1 µM NBTI, a selective ENT1 inhibitor, abolished AICAR-stimulated phosphorylation of AMPK and ACC (Fig. 4.16C,D). Presence or absence of nucleosides did not have a major impact on expression of nucleoside transporters, adenosine kinase or ATIC in either L6 cells or human
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primary skeletal muscle cells (Dolinar et al., 2018). These results demonstrate that inhibition of nucleoside transporters ENT1 and ENT2 blocks AICAR-stimulated AMPK activation.

![Figure 4.16](image)

Figure 4.16: The role of nucleoside transporters in AICAR activation in L6 cells and HSMC. Phosphorylation of AMPK\(\alpha\) at Thr172 (pAMPK) and ACC at Ser79 (pACC) was determined with immunoblotting. (A,B) L6 cells were cultured in MEM\(\alpha\)- for 24 h and then treated for 1 h with 1 mM AICAR and/or 10 \(\mu\)M dipyridamole (DPY) or 1 \(\mu\)M NBTI. (C,D) HSMC were cultured in MEM\(\alpha\)- for 24 h and then treated for 1 h with 1 mM AICAR and/or 10 \(\mu\)M DPY or 1 \(\mu\)M NBTI. Results are means ± SEM. Inhibitors were added 5 min before AICAR; n = 4-8; *p < 0.05 AICAR vs. Basal under the same conditions, unpaired t-test. Results were published in AJP-Cell (Dolinar et al., 2018).

4.3.3 Effects of adenosine and 2'-deoxyadenosine on AMPK activation in L6 cells and HSMC

Total concentration of nucleosides in MEM\(\alpha\)+ is ~314 \(\mu\)M and concentration of adenosine is 37.5 \(\mu\)M, which might modulate AMPK activation. We treated L6 cells in MEM\(\alpha\)- with AICAR (1 mM) and/or different concentrations of adenosine (3-300 \(\mu\)M). In the presence of adenosine (≥10 \(\mu\)M) AICAR did not increase phosphorylation of AMPK and ACC in L6
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cells (Fig. 4.17A,B). A similar result was obtained in HSMC (Fig. 4.17C,D). In these cells basal phosphorylation of AMPK was reduced by 300 µM adenosine.

To determine whether 2’-deoxyadenosine, whose concentration in MEMα+ is 39.8 µM, also blocks AICAR action, we treated L6 cells with AICAR (1 mM) and with or without 2’-deoxyadenosine (10 or 300 µM). Basal phosphorylation of AMPK was reduced by 2’-deoxyadenosine. AICAR stimulated AMPK and ACC phosphorylation in the presence of 10 µM 2’-deoxyadenosine, but its action was blocked almost completely by 300 µM 2’-deoxyadenosine (Fig. 4.17E,F). Similar result was obtained in AICAR-treated HSMC (Fig. 4.17G,H). These results show that adenosine is more effective blocker of AICAR action than 2’-deoxyadenosine.
Figure 4.17: Adenosine and 2'-deoxyadenosine block AICAR-stimulated AMPK and ACC phosphorylation in L6 cells and HSMC. L6 cells and HSMC were cultured for 24 h without nucleosides (MEMα) and then treated with 1 mM AICAR or vehicle (Basal) for 1 h in the presence or absence of different concentrations of (A-D) adenosine or (E-H) 2'-deoxyadenosine. Adenosine and 2'-deoxyadenosine were added 5 min before AICAR. Phosphorylation of AMPKα at Thr172 (pAMPK) and ACC at Ser79 (pACC) was determined with
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immunoblotting. Results are means ± SEM (n = 3-4). *p<0.05 AICAR vs. Basal in the absence of adenosine or deoxyadenosine, under the same conditions; #p <0.05 vs. Basal under the same conditions, ordinary one-way ANOVA with Bonferroni's post hoc test was used for statistical evaluation. Results were published in AJP-Cell (Dolinar et al., 2018).

4.3.4 Effects of short (45-min) and long (8-hour) SSZ and salicylate treatment on AMPK, ACC and ERK1/2 phosphorylation in L6 cells

It is known that methotrexate inhibits ATIC, an enzyme of the de novo purine synthesis pathway, which catalyses conversion of ZMP to IMP, and thus promotes AMPK activity (Pirkmajer et al., 2015). We were interested whether SSZ, also known to inhibit ATIC (Baggott et al., 1992), might also present a possible pharmacological activator of AMPK. In our first experiment we treated differentiation L6 cells for either 45 min (Fig. 4.1A-C) or 8 hours (Fig. 4.1D-F) with 1.0 mM SSZ, 1.0 mM (Fig. 4.1A-C) or 3.0 mM (Fig. 4.1D-F) salicylate (a known direct AMPK activator) or vehicle (Basal) in MEMα with or without nucleosides. A short SSZ treatment caused an increase in AMPK (Fig. 4.1A) and ACC (Fig. 4.1B) phosphorylation in medium without nucleosides, while we only observed an increase in AMPK phosphorylation in medium with nucleosides (Fig 4.1A) and not ACC (Fig. 4.1B). Used concentration of salicylate caused no change in either AMPK or ACC phosphorylation (Fig. 4.1A,B). We also checked for possible changes in extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. None of the treatments caused any change (Fig. 4.1C).

After performing a 45-min SSZ and salicylate treatment we also performed a longer 8-h treatment in both MEMα culture medium with and without nucleosides. SSZ increased phosphorylation of AMPK and ACC in both medium without and with nucleosides (Fig. 4.1D,E). Salicylate actually caused a decrease in AMPK phosphorylation in both formulations of MEMα (Fig 4.1D), while it caused an increase in ACC phosphorylation, but only in medium without nucleosides (Fig. 4.1E): SSZ treatment caused increase in ERK1/2 phosphorylation.
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phosphorylation in both medium with and without nucleosides, while salicylate lowered it (Fig. 4.18F).

![Figure 4.18: Effects of short (45-min) and long (8-hour) sulfasalazine and salicylate treatment on AMPK, ACC and ERK1/2 phosphorylation in L6 cells. (A-C) L6 cells were cultured for 4 h in MEM\(\alpha\) with or without nucleosides (MEM\(\alpha\)-) and without serum and then treated with 1 mM sulfasalazine (SSZ), 1 mM salicylate (SA) or vehicle (Basal) for 45 min within 4 h of serum starvation. (D-F) L6 cells were cultured for 24 h in MEM\(\alpha\) with or without nucleosides (MEM\(\alpha\)-) and without serum and then treated with 1 mM SSZ, 3 mM SA or vehicle (Basal) for 8 h within 24 h of serum starvation. Immunoblot was used to evaluate phosphorylations of (A,D) AMPK\(\alpha\) at Thr172, (B,E) ACC at Ser79 (pACC), and (C,D) ERK1/2 at Thr202 and Tyr204. Results are means ± SEM (n = 8), *P<0.05 vs. Basal +/- nucleosides, ordinary one-way ANOVA with Bonferroni’s post hoc test was used for statistical evaluation. Unpublished data, manuscript in preparation]
4.3.5 Effects of SSZ and methotrexate with or without AICAR on AMPK, ACC and ERK1/2 phosphorylation in L6 cells and HSMC

After determining that SSZ can activate AMPK, we were interested whether it has the additive effect with AICAR, as seen in methotrexate (Pirkmajer et al., 2015), which would confirm our hypothesis that SSZ activates AMPK indirectly through ATIC inhibition. We performed 8-h SSZ treatment with or without additional 45min AICAR treatment.

In HSMC AMPK phosphorylation was actually reduced at 1.0 mM SSZ concentration and we actually observed an inhibitory effect of SSZ, when cells were treated both with SSZ and AICAR (Fig. 4.19A). ACC phosphorylation showed a positive trend when increasing SSZ concentration, but when cells were treated with both SSZ and AICAR, we again observed a decrease in phosphorylation (Fig. 4.19B). ERK1/2 phosphorylation was decreased both when treated with SSZ alone or in combination with AICAR (Fig. 4.19C).

In L6 cells SSZ again effectively increased both AMPK and ACC phosphorylation, but we observed no additive effect, when cells were treated with AICAR (Fig. 4.19D,E). We observed no effect on ERK1/2 phosphorylation (Fig. 4.19F). As SSZ did not seem to have an additive effect in co-treatment with AICAR we compared it side to side with methotrexate that does act additively with AICAR (Pirkmajer et al., 2015). As both SSZ and methotrexate are proposed to inhibit ATIC and so cause an accumulation of ZMP or of its precursor AICAR, it was surprising that SSZ actually completely diminished AICAR effect of AMPK and ACC phosphorylation (Fig. 4.19G,H). On the other hand methotrexate’s additive effect is evidently seen on both AMPK and ACC (Fig. 4.19G,H). SSZ treatment in combination with AICAR decreased ERK1/2 phosphorylation, while methotrexate and AICAR combination increased it (Fig. 4.19I).
Figure 4.19: Effects of SSZ and methotrexate treatment with or without AICAR on the phosphorylation of AMPK, ACC and ERK1/2 in L6 cells and HSMC. Changes in phosphorylation of AMPK, ACC and ERK1/2 were measured by immunoblotting. (A-I) For the last 24 h L6 cells were grown in MEMα without nucleosides and FBS. 48 h before the end of the experiment the medium for HSMC was changed to DMEM (1g/L glucose) with 2%FBS. For the last 24 h of the experiment cells were grown in serum-free DMEM. (A-F) Vehicle (Basal), 0.3 mM and 1.0 mM SSZ treatments lasted for the last 8 h within 24 h in serum-free cultivation for both L6 cells and HSMC. AICAR treatment was done for the last 45 min. (G-I) L6 cells were treated with vehicle (Basal), 1.0 mM SSZ or 5 µM methotrexate (MTX) treatments for last 16 h within 24 h of serum-free cultivation. AICAR treatment was done for the last 45 min. Immunoblotting was used for the evaluation of (A,D,G) pAMPKThr172.
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(B,E,H) \(p\text{ACC}^{\text{Ser79}}\) and (C,F,I) \(p\text{ERK1/2}^{\text{Thr202/Tyr204}}\). Results are mean ± SEM for \(n=8\) (L6 cells), \(n=4-6\) (HSMC); *\(p<0.05\) vs. Basal (+PBS), #\(p<0.05\) vs Basal (+AICAR); ordinary one-way ANOVA with Bonferroni's post hoc test was used for statistical evaluation. Unpublished data, manuscript in preparation.

### 4.3.6 Possible role of nucleoside transporters in SSZ action on AMPK

As we have shown in Fig. 4.16 both nucleoside transporters DPY and NBTI inhibit AICAR action in L6 cells and HSMC. We were interested whether SSZ might enter cells with the same transporters as AICAR and whether nucleoside transporters would also inhibit its effects on AMPK and ACC phosphorylation. L6 cells and HSMC were treated with 1.0 mM AICAR, 1.0 mM SSZ or vehicle (Basal) in combination with DPY and NBTI (10 μM and 1 μM, respectively). As seen in Fig. 4.16 inhibitors of nucleoside transporters completely blocked the effects of AICAR on AMPK and ACC phosphorylation in both L6 cells and HSMC (Fig. 4.20A,B,D,E). On the other hand DPY and NBTI treatment had no effect on SSZ action on AMPK and ACC phosphorylation in L6 cells (Fig. 4.20D,E), while the results in HSMC remain inconclusive as we did not observe a significant effect of SSZ on AMPK and ACC phosphorylation (Fig. 4.20A,B). DPY and NBTI reduced ERK1/2 phosphorylation in Basal and AICAR-treated L6 cells (Fig. 4.20F) and they seemed to decrease ERK1/2 in SSZ-treated HSMC.

To evaluate the hypothesis that AICAR and SSZ might compete for the same membrane transporters we added a washout step before AICAR treatment. L6 cells were treated with SSZ for 8 h inside 24-h serum starvation period. Before addition of AICAR, 45 min before the end of experiment, we washed the delegated wells twice with PBS and added fresh medium without SSZ. With a washout step SSZ-induced increase in phosphorylation of AMPK and ACC was diminished (Fig. 4.20G,H). Phosphorylation of AMPK and ACC was not higher in SSZ and AICAR washed wells compared to unwashed (Fig. 4.20G,H). ERK1/2 phosphorylation was higher in all washed wells (Fig. 4.20I).
Figure 4.20: Possible role of nucleoside transporters in SSZ action. (A-C) For the last 48h before the end of the experiment the medium for HSMC was changed from aMEM to DMEM (1g/L glucose) with 2% FBS. (A-I) For the last 24 h of the experiment cells were grown in serum-free medium (MEMα without nucleosides for L6 cells, DMEM for HSMC). (A-F) SSZ treatment lasted for 8 h within 24 h in serum-free cultivation, AICAR or PBS was added 45 min before the end of experiments. Inhibitors of nucleoside transporters DPY and NBTI were added to appropriate wells 20 min before SSZ treatment. (G-I) SSZ treatment lasted for 8 h within 24 h in serum-free cultivation. AICAR or PBS was added 45 min before the end of experiments. Half of the wells were washed twice with PBS and fresh medium was added before AICAR treatment. Immunoblotting was used to evaluate the abundance of pAMPKThr172, ACCSer69 and ERKThr202/Tyr204. Results are mean ± SEM for n=4; (A-F) *p<0.05 vs
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Basal Control/DPY+NBTI, #p<0.05 vs. DPY+NBTI; (G-I) *p<0.05 vs Basal no washout, #p<0.05 vs. Basal washout, ordinary one-way ANOVA with Bonferroni's post hoc test was used for statistical evaluation of results. Unpublished data, manuscript in preparation.

4.3.7 Effects of SSZ treatment in presence or absence of insulin on AMPK and Akt signalling in L6 cells

As our results show that SSZ increases AMPK phosphorylation we were interested in how it might affect Akt signalling. In cells, sensitive to insulin, glucose uptake can be induced via phosphatidylinositol-3-kinase (PI3K)/Akt and AMPK signalling pathways. These pathways can increase glucose uptake independently of each other (Bertrand et al., 2006), which is why AMPK activation can increase glucose uptake also in insulin-resistant subjects (Mihaylova & Shaw, 2011) and is explored as a possible way to treat type 2 diabetes (Hardie, 2013). Insulin's induction of (PI3K)/Akt signalling pathway and AMPK activation can both increase phosphorylation of Akt also known as Ser/Thr protein kinase B (PKB) (Bertrand et al., 2006), which is a crucial kinase in the cascade of reactions that cause glucose uptake.

Increased Akt phosphorylation on both Ser473 and Thr308 sites induces phosphorylation of Akt substrate of 160 kDa (AS160 or TBC1D4). AS160 inhibits glucose transporter type 4 (GLUT4) translocation in its unphosphorylated form, but when it is phosphorylated by Akt on Ser588 or Thr642 it helps with GTP binding to Rab proteins, which results in the release of GLUT4 vesicles from intracellular compartments. GLUT4 vesicles can than dock into plasma membrane and increase glucose uptake (Hutagalung & Novick, 2011). We evaluated SSZ's and/or insulin's effects on the phosphorylation of Akt at Ser473, and of its targets AS160 at Ser588 and Thr642, p21-activated kinase (PAK) 1/2 at Thr423/Thr402 (King et al., 2000), and glycogen synthase kinase-3 (GSK-3) at Ser21/9 (Welsh et al., 1996). We also evaluated how SSZ and insulin co-treatment would affect AMPK action. L6 cells were treated with vehicle.
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50 µM, 100 µM or 300 µM SSZ for 8 h and then co-treated with either PBS, 12 nM insulin or 120 nM insulin for the final 20 min of experiment.

Firstly, we evaluated how insulin co-treatments might affect SSZ action on AMPK action. While we observed a positive trend in AMPK phosphorylation when treating with 300 µM SSZ without insulin treatment, the effects were diminished when we also added either 12 or 120 nM insulin (Fig. 4.21A). 100 µM and 300 µM SSZ in combination with 120 nM insulin actually caused a significant reduction in AMPK phosphorylation. 300 µM SSZ caused a significant increase in ACC phosphorylation when cells were not treated with insulin, but insulin-treatment negated that effect (Fig. 4.21B). ERK1/2 phosphorylation was significantly increased with both 12 and 120 nM insulin treatment and none of SSZ treatments caused any changes in its phosphorylation (Fig. 4.21C).

Akt phosphorylation on Ser473 was increased with both insulin treatments (Fig. 4.20D); however, when cells were pretreated with SSZ we observed a dose-dependent decrease in phosphorylation in 120 nM insulin-treated cells (Fig. 4.21D). In insulin-treated cells SSZ also decreased phosphorylation of Akt substrates on Ser and Thr residues (Fig. 4.21E), phosphorylation of AS160 on Ser588 (Fig. 4.21F) and GSK-3α/β on Ser21 and 9 (Fig. 4.21I). Phosphorylation of AS160 on Thr642 was not affected by SSZ treatment (Fig. 4.21G). While Akt (Fig. 4.21D), Akt substrates (Fig. 4.21E), AS160Thr642 (Fig. 4.21G), and GSK-3α/β (Fig. 4.21I) phosphorylations were all highly increased with insulin treatments, AS160 Ser588 phosphorylation site seemed to be completely unresponsive to insulin presence (Fig. 4.21F). Phosphorylation of PAK 1/2 at Thr423/Thr402 was slightly increased with high insulin treatment, but SSZ had no effect on it (Fig. 4.21H).
Figure 4.21: Effects of SSZ and/or insulin on AMPK and Akt signalling in L6 cells. L6 cells were cultured for 24 h in MEMα without nucleosides (MEMα-) and without serum. After 16 h of serum starvation cells were treated for 8 h with vehicle (Control), 50 µM SSZ, 100 µM SSZ or 300 µM SSZ. For the last 20 min during 8-h SSZ treatment cells were also treated with PBS (Basal), 12 nM insulin (12 nM INS) or 120 nM insulin (120 nM INS). Immunoblotting was used to evaluate levels of (A) pAMPK<sup>Thr172</sup>, (B) pACC<sup>Ser79</sup>, (C) pERK1/2<sup>Thr202/Tyr204</sup>, (D) pAkt<sup>Ser473</sup>, (E) PAS, (F) pAS160<sup>Ser588</sup>, (G) pAS160<sup>Thr642</sup>, (H) pPAK1/2<sup>Thr423/403</sup>, and (I) pGSK-3α/β<sup>Ser21/9</sup>. Results are means ± SEM (n=4-7). *P<0.05 vs. Control (within Basal, 12 nM INS or 120 nM INS subgroups), #P<0.05 Basal vs. 12 nM and 120 nM treatments (e.g. Basal Control vs. 12nM or 120 nM INS Control), ordinary
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One-way ANOVA with Bonferroni's post hoc was used for statistical evaluation. Unpublished data, manuscript in preparation.

4.3.8 Effect of washout on SSZ and insulin treatment in L6 cells

After SSZ and insulin co-treatment of L6 cells, we were interested in whether the removal of SSZ before insulin treatment would diminish SSZ's suppressive effects on Akt signalling. L6 cells were first treated with 1mM SSZ for 8 h. After 8 h, medium was removed, cells were washed twice with PBS and fresh medium without SSZ was added. After 1 additional hour, insulin or PBS was added for 20 min.

When SSZ was removed before insulin treatment of L6 cells, we saw no effect of SSZ on ACC phosphorylation (Fig. 4.22A), but ERK1/2 phosphorylation was still increased with 120 nM insulin (Fig. 4.22B). Phosphorylations of Akt at Ser473 (Fig. 4.22C), AS160 at Thr642 (Fig. 4.22F) and GSK-3\(\alpha/\beta\) at Ser21/9 (Fig. 4.22H) were not reduced. We even saw an increase in Akt phosphorylation when cells were co-treated with 12 nM insulin and SSZ (Fig. 4.22C). Conversely AS160 phosphorylation at Ser588 (Fig. 4.22E) and phosphorylation of Akt substrates (Fig. 4.22D) were still reduced, when cells were treated with SSZ and 120 nM insulin.
Figure 4.22: SSZ washout in SSZ and/or insulin-treated L6 cells. L6 cells were cultured for 24 h in MEMα without nucleosides (MEMα-) and without serum. After 16 h of serum starvation cells were treated for 8 h with...
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either vehicle (Control or 1mM SSZ). After 8 h cells were washed twice with PBS and fresh serum-free DMEM without SSZ was added. After additional 1 h insulin or PBS was then added for 20 min. Immunoblotting was used to evaluate levels of (A) pACC\(^{\text{Ser79}}\), (B) pERK1/2\(^{\text{Thr202/Tyr204}}\), (C) pAkt\(^{\text{Ser473}}\), (D) PAS, (E) pAS160\(^{\text{Ser588}}\), (F) pAS160\(^{\text{Thr642}}\), (G) pPAK1/2\(^{\text{Thr423/403}}\), and (H) pGSK-3\(\alpha/\beta\)\(^{\text{Ser21/9}}\). Results are means ± SEM (n=8). *P<0.05 vs. Control Basal, #P<0.05 Control vs. SSZ pair, ordinary one-way ANOVA with Bonferroni's post hoc was used for statistical evaluation. Unpublished data, manuscript in preparation.

4.3.9 Effects of SSZ treatment in combination or absence of insulin on AMPK and Akt signalling in HSMC

HSMC were treated just like L6 cells with vehicle 50 µM, 100 µM or 300 µM SSZ for 8 h and then co-treated with either PBS, 12 nM insulin or 120 nM insulin for the final 20 min of experiment.

We observed a positive trend in AMPK phosphorylation when treating HSMC with 50 µM SSZ without insulin treatment, but there was no statistically significant supressing effect of insulin on SSZ's action (Fig. 4.23A). Treatment with 50 and 300 µM SSZ increased phosphorylation of ACC and we saw no such effect in co-treatments with 12 or 120 nM insulin (4.23B). Only significant difference in ERK1/2 was an increase in phosphorylation in 100 µM SSZ and 120 nM insulin co-treated cells, compared to 100 µM SSZ-treated cells without insulin (4.23C).

Akt phosphorylation on Ser473 was increased with both 12 and 120 nM insulin treatments (Fig. 4.23D). Unlike in L6 cells, SSZ did not supress insulin's effect on Akt signalling. In cells that were co-treated with 100 µM SSZ and 120 nM insulin we actually observed an increase in AS160's phosphorylation of Ser588 (Fig. 4.23E) and Thr642 (Fig. 4.23F), and in phosphorylation of GSK-3\(\alpha/\beta\) (Fig. 4.23G).
Figure 4.23: Effects of SSZ in combination with insulin on AMPK and Akt signalling in HSMC. For the last 48 h of the experiment the medium for HSMC was changed from aMEM to DMEM (1g/L glucose) with 2% FBS. For the last 24h of the experiment cells were grown in serum-free DMEM. After 16 h of serum starvation cells
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were treated for 8 h with vehicle (Control), 50 µM SSZ, 100 µM SSZ or 300 µM SSZ. For the last 20 min during 8-h SSZ treatment cells were also treated with PBS (Basal), 12 nM insulin (12 nM INS) or 120 nM insulin (120 nM INS). Immunoblotting was used to evaluate levels of (A) pAMPK\(^{Thr172}\), (B) pACC\(^{Ser79}\), (C) pERK1/2\(^{Thr202/Tyr204}\), (D) pAkt\(^{Ser473}\), (E) pAS160\(^{Ser588}\), (F) pAS160\(^{Thr642}\), and (G) pGSK-3\(^{β/α}\)\(^{Ser21/9}\). Results are means ± SEM (n=4). \(^{*}\)P<0.05 vs. Control (within Basal, 12 nM INS or 120 nM INS subgroups), \(^{#}\)P<0.05 Basal vs. 12 nM and 120 nM treatments (e.g. Basal Control vs. 12nM or 120 nM INS Control), ordinary one-way ANOVA with Bonferroni's post hoc was used for statistical evaluation. Unpublished data, manuscript in preparation.

5 DISCUSSION

The main goal of this doctoral dissertation was to evaluate the effects of stress on NKA function and on regulation of energy metabolism via AMPK. As available in vitro models of skeletal muscle leave much to be desired due to their divergent expression profiles of NKA subunits and FXYDs compared to mature fibres, we first explored, whether innervated HSMC may represent a more suitable cell culture model compared to the more commonly used non-innervated HSMC, L6 and C2C12 cell culture models for evaluation of NKA and FXYD function in skeletal muscle. Secondly, we explored whether hypoxia affects NKA function via AMPK activation. We evaluated this hypothesis in muscle samples from a clinical trial, where patients with ACL injury performed training with low load training with or without blood flow restriction and in in vitro experiments, where cells were exposed to glucose deprivation and/or hypoxia. Thirdly, we were interested in whether AMPK pharmacological activators' effect on NKA function is dependent on the metabolic state of the treated cells. Metformin, a most widely orally used antidiabetic drug, which was reported to both activate AMPK and regulate NKA was used in in vitro experiments, where cells were pretreated with different concentrations of metformin and then exposed to hypoxia or artificial ischaemia (HGD). We explored whether metformin preconditioning had different effects on NKA expression if the cells were exposed to normoxic, hypoxic or ischaemic conditions at the end of experiment. We
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also explored SSZ's potential effects on AMPK action. Like methotrexate, SSZ has also been reported to inhibit ATIC. ATIC is an enzyme, involved in de novo purine synthesis pathway that promotes conversion of ZMP to IMP. By inhibiting ATIC methotrexate causes a build-up in ZMP concentration, which can increase AMPK activity by directly binding to it. We explored whether SSZ may activate AMPK in the same way as methotrexate.

5.1 **Hypothesis I: Myotubes innervated in vitro and mature muscle fibres in vivo have a similar expression pattern of NKA and FXYD proteins.**

We showed that the differentiation of HSMC into myotubes in cultured medium with low serum supplementation (2% FBS) increased expression of NKA \(\alpha_2\)-subunit and of several muscle-specific markers, such as myosin heavy chains-2A, -2X and \(-\beta/\text{slow}, \alpha_{1S}\) subunit of the dihydropyridine receptor, SERCA2 and desmin. Progressive increase in the expression of NKA \(\alpha_2\)-subunit with differentiation is in accordance with what Al-Khalili et al. (2004) and Higham et al. (1993) have shown in HSMC and C2C12 mouse skeletal muscle cell line, respectively. We also observed a negative trend in the expression of NKA \(\alpha_1\)-subunit with differentiation, which has also been reported in both HSMC and C2C12 cells (Higham et al., 1993; Al-Khalili et al., 2004). Conversely differentiation did not affect the expression of NKA \(\beta\)-subunits in either CD56\(^-\) or CD56\(^+\) fraction, which might mean that NKA \(\alpha\)- and \(\beta\)-subunits are regulated through different mechanisms

Differentiation of HSMC also increased FXYD1 content and reduced FXYD5, but downregulation of FXYD5 expression was only significant on mRNA level. Interestingly expression of FXYD1 was higher in CD56\(^-\) compared to CD56\(^+\) fraction before and after differentiation. CD56\(^-\) fraction of HSMC is reported to comprise of mostly fibroblasts, positive for TE-7, collagen VI, PDGFR\(\alpha\), vimentin, and fibronectin (Agley et al., 2013), which can undergo adipogenic differentiation in low serum (2% FBS) medium. FXYD1 is expressed both
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in fibrinogenic cells (https://www.proteinatlas.org/ENSG00000266964-FXYD1/celltype) and adipogenic cells, so this might explain high expression in CD56\(^-\) fraction (Walaas et al., 1991). To our knowledge there are no available publications, where FXYD1 would be successfully detected in cultured HSMC, and FXYD1 expression in L6 rat skeletal muscle cells, which are one of the most commonly used in vitro model of skeletal muscle, was reported to be below detection (Benziane et al., 2012). One of the major results of this dissertation is that we were able to detect FXYD1 in cultured HSMC and to show that differentiation in low serum upregulated FXYD1 both on mRNA and on protein level. Our results show that it is possible to explore FXYD1 function in cultured HSMC, which enables further research of its regulation and function in skeletal muscle.

When we innervated HSMC by co-culturing them with rat embryonic spinal cord explants, we obtained cultures with visible cross-striation and continuous contractions, stimulated by rat motor neurons. However, we observed only a slight increase in mRNA expression of FXYD5, while mRNA expression of NKA subunits, FXYD1, or myosin heavy chains-2A, -2X, or -β/slow, and SERCAs remained unchanged. On the other hand, we observed an increase in protein content of NKA α1- and NKA α2-subunit, and in protein content and phosphorylation (Ser68) of FXYD1. One explanation of increased levels of phosphorylated FXYD1 would be that NKA activity is higher in contracting innervated HSMC compared to their aneural controls. Phosphorylation of FXYD1 on Ser68 increases NKA's affinity for intracellular Na\(^+\), which leads to its increased activity (Crambert et al., 2002; Despa et al., 2005; Geering, 2008). As one of NKA's functions in skeletal muscle is to maintain Na\(^+\) and K\(^+\) gradients during contractions, it seems logical that its activity would be higher in contracting HSMC. However, it has been shown that the contractions of ex vivo muscles can increase or decrease FXYD1 phosphorylation (Thomassen et al., 2011) and that FXYD1 is not essential
for the maintenance of skeletal muscle contractions (Manoharan et al., 2015). It is still not completely clear how contractions of innervated HSMC lead to increased FXYD1 phosphorylation, but there is some evidence that supports a role for AMPK, which was shown to affect FXYD1 phosphorylation and its protein content in skeletal muscles (Ingwersen et al., 2011), while also being activated by contractions in skeletal muscle (Winder & Hardie, 1996; Pirkmajer et al., 2021). Surprisingly protein content of MyHC-2A, -2X, and β/slow was reduced, but we have shown in a recently published article that although mRNA expression and protein content of MyHC does not increase or even decrease in innervated HSMC, the localization in innervated myotubes does, as the cytoskeleton rearranges (Mars et al., 2020), so this might have implications for possible maturation of contracting myotubes.

As we have already mentioned skeletal muscles show distinct intracellular distribution of NKA α1/β-heterodimers. NKA α2/β-heterodimers are predominantly located on the inner membranes of T-tubules (Radzyukevich et al., 2013), while, to a smaller amount, they are also localized to postsynaptic membrane of NMJ (Heiny et al., 2010; Chibalin et al., 2012b) and surface caveolae (Kristensen et al., 2008). Aneural HSMC do not have basal lamina, cross-striations and T-tubules (Askanas et al., 1987). This is why we hypothesized that by innervating HSMC we could achieve a more advanced maturation, where cells would be able to develop visible cross-striation and complex T-tubule systems. By this, we expected to find much higher concentrations of both α2/β-heterodimers and FXYD1 in innervated HSMC compared to their aneural controls. It was already shown that the expression of NKA α1- and α2- subunits and activity of NKA increase when differentiated HSMC are treated with neural agrin (Jurdana et al., 2009), which is best known for its role in the organization and maintenance of postsynaptic structures at the NMJ (McMahan, 1990; Burden, 2002), and that innervation of differentiated HSMC with explants of rat embryonic spinal cords increases their membrane potential
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compared to non-innervated myotubes (Askanas et al., 1987), which might be caused by increased NKA activity. The fact that inactivity decreases NKA abundance (Leivseth & Reikerås, 1994) and physical activity increases it (Clausen, 2003; Wyckelsma et al., 2015) seemed to strengthen our hypothesis that contractions and innervation would increase NKA expression. We cultured innervated and aneural (non-innervated) HSMC for 21-22 days. In this period HSMC that were co-cultured with explants of rat embryonic spinal cords got innervated by the neurites that grew out of rat explants and contracted for 10-14 days. We have chosen the 21-day period, because it was reported that more complex T-tubules become evident at that time point (Askanas et al., 1987). T-tubule system, however, continues to develop for several weeks, and it has also been reported that myogenic isoforms of creatine kinase and phosphorylase continue to increase up to 42th day of co-culture (Martinuzzi et al., 1986). It is possible that 21-day period was too short to see more pronounced increases in mRNA expression of NKAα2, which is primarily localized to T-tubules. This would be in accordance with the result that DHPRα1S, which is also found in T-tubules (Flucher et al., 1993), was also not upregulated in innervated 3-week-old co-cultures.

There is a possibility that the observed increases in NKAα1, NKAα2, and FXYD1 protein content and an increased phosphorylation might be caused by co-detection of both proteins from muscle (human) and neural (rat) components, as the antibodies we used were not human-specific. Nevertheless, we obtained the same results in gene expression of NKAα2 and FXYD1 when we used human-specific assays and the ones that also detected rat transcripts, which suggests that contamination from rat neural tissue was negligible. This could be verified by immunocytochemical evaluation to determine the location of NKA subunits in co-cultures or by ouabain treatment. NKA activity in rats can be measured by using different concentrations of ouabain. Rat NKAα2 and α3 are significantly more susceptible to ouabain
than NKAα1, which enables us to tell apart the contribution of NKAα1 from NKAα2 and NKAα3 (Chibalin et al., 2012b; Smolyaninova et al., 2019). On the other hand all human NKA α-subunits are equally highly sensitive to ouabain suppression (Wang et al., 2001). We could use this fact to our advantage and selectively inhibit human NKA α-subunits and distinguish their contribution from that of ouabain-resistant rat NKA α1-subunit from spinal cord explant. It is possible that innervation enhances translation and suppresses degradation of NKA subunits and FXYDs, which would explain why there are differences in protein levels between aneural and innervated HSMC, while mRNA is not affected. It is known that actinomycin, a known inhibitor of RNA synthesis, does not affect NKA increase in rat soleus muscle after birth, even though it inhibits the growth of muscles (Kjeldsen et al., 1984)

Irreversible inhibitor of nAChR α-bungarotoxin suppresses NKA activation by acetylcholine (Dlouhá et al., 1979). We were interested if inhibition of contractions through neuromuscular blockade by α-bungarotoxin or d-tubocurarine would affect mRNA expression of NKA subunits, FXYD1 and FXYD5. We observed no differences in mRNA expression between untreated and treated samples. It is possible that potential changes in mRNA expression might be localized to perisynaptic regions and were not big enough to be discerned in whole homogenate evaluation. It is also possible that 24 h neuromuscular blockade was not long enough to induce changes in mRNA expression of NKA subunits and FXYDs.

On mRNA level human and rat muscle are known to express NKAα2 in significant majority (Perry et al., 2013). In differentiated CD56+ HSMC NKAα2 represented approximately 48% of all NKAα mRNA transcripts, but NKAα1 was still more abundant representing more than 50% of all NKA α-subunits in samples from most donors. This was in accordance with past results from Pirkmajer et al. (2020). Innervation did not cause any additional increase in NKAα2 expression; in fact, relative expression of NKAα2 compared to
NKAα1 was far lower in both co-cultures and their aneural controls, when compared to differentiated CD56⁺ HSMC. It is important to point out that innervated myotubes and their aneural controls were not subjected to differentiation in low serum-supplemented medium, which is a standard protocol for differentiation of CD56⁺ HSMC. They were kept in medium with 10% FBS for the whole experiment. This underscores the importance of serum deprivation for the upregulation of NKAα2 and FXYD1, whose mRNA expression was both increased during differentiation in 2% FBS-supplemented medium.

On the other hand, FXYD5 was downregulated. Notably the most expressed NKA β-subunit in all cell models was β3, which is expressed at low levels in mature skeletal muscles of rat, but its expression is much higher in skeletal muscles of 7-day-old rats (Arystarkhova & Sweadner, 1997). Expression of NKA β3-subunit was also higher in brain and heart of 7-day-old rats compared to adult ones, which suggests that β3-subunit might be regulated by postnatal development (Arystarkhova & Sweadner, 1997). This is in accordance with our results that show that NKA β3-subunit expression in mature human muscle is minimal compared to NKA β1- and β2-subunits. Interestingly, we observed a non-significant increase in ratio of NKA β3-subunit compared to total NKAβ with serum depletion and NKA β3-subunit represented even higher percentage of all NKA β-subunits in aneural and innervated HSMC that were kept in 10% FBS-supplemented medium for a prolonged period of time, which proposes that there are other important factors besides serum content that affect the expression of NKA β-subunits in early development of HSMC in vitro.

It is important to note that even though NKAα2 and FXYD1 expression increased in CD56⁺ HSMC that were differentiated in 2% FBS, they still differed greatly from expression profile of mature human vastus lateralis and semitendinosus samples. In our muscle samples NKAα2 represented more than 99% of all NKA α-subunits transcripts, which was even more
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than was reported in previous studies, where NKA\(\alpha\)2 represented 60-90% of all NKA \(\alpha\)-subunits (Orlowski & Lingrel, 1988; Hansen, 2001; He et al., 2001). FXYD1 represented approximately 99% of FXYDs, while FXYD5 represented the remaining 1%. The biggest difference regarding expression profiles was seen in NKA \(\beta\)-subunits. Conversely to cell culture models NKA\(\beta\)1 mRNA expression represented the highest share of all NKA \(\beta\) transcripts, with 87% in \textit{vastus lateralis} and 92% in \textit{semitendinosus}. NKA\(\beta\)2 represented 12.5 and 7% of total NKA\(\beta\) expression in \textit{vastus lateralis} and \textit{semitendinosus}, respectively, while NKA\(\beta\)3 was barely detectable. As we mentioned, cell culture models that we explored seem to present NKA \(\beta\)-subunit expression profile that is more alike to non-developed muscle before it goes through postnatal development.

It is also possible to study NKA function in isolated adult skeletal muscle fibres, such as rat or mouse \textit{flexor digitorum brevis, soleus} or \textit{extensor digitorum longus} muscles (Bekoff & Betz, 1977; DiFranco et al., 2015). Isolated animal muscle fibres represent one of the models that is morphologically most alike to mature \textit{in vivo} skeletal muscles. They are clearly cross-striated (Bekoff & Betz, 1977), have completely developed T-tubules and sarcoplasmic reticulum (Calderon et al., 2014) and have been used effectively in different electrophysiological studies (Bekoff & Betz, 1977; Gonoï & Hasegawa, 1988; Calderon et al., 2014). The disadvantages of isolated animal muscle fibre models include the removal of acetylcholinesterase by collagenase treatment from the end-plate region (Hall & Kelly, 1971; Betz & Sakmann, 1973), disconnection from motor neurons, caused by collagenase and Trituration damage (Cheng & Westerblad, 2017), spontaneous contractions, and importantly, species-specific differences in the expression of NKA subunits between rodents and humans (Pirkmajer & Chibalin, 2016).
In conclusion, our results do not support the statement of the first hypothesis that \textit{in vitro de novo} innervation of myotubes would result in their expression pattern of NKA and FXYDs to become more alike to that in mature muscle fibres. Innervation of up to 21 days had no pronounced effect on the mRNA expression of NKA subunits or FXYD1, while innervation caused a small increase in FXYD5 expression. It seems that serum deprivation during differentiation of CD56$^+$ HSMC is the biggest factor that leads to increased expression of NKA$\alpha_2$ and FXYD1. On the other hand, the expression of NKA $\beta$-subunits was not affected by serum deprivation or innervation and expression profile remained more similar to that found in immature muscle fibres that had not undergone their postnatal development. Our results suggest that the mRNA expression of NKA $\alpha$-subunits and $\beta$-subunits is regulated through different regulatory pathways. A positive perspective of our results is that we were able to successfully detect FXYD1 in HSMC, which has not been reported before. This opens new vistas for exploration of FXYD1 function in skeletal muscle that was hampered for a long time by its reportedly low abundance in cell culture models.

5.2 Hypothesis II: Hypoxia alters NKA function in skeletal muscle via activation of 5' AMP-activated protein kinase (AMPK).

In the orthopedic clinical trial, we showed that LL-BFR training in patients induces long lasting changes in NKA mRNA and protein expression. Major new result of the study was that LL-BFR training increased the expression NKA$\alpha_1$ in \textit{semitendinosus} and \textit{vastus lateralis} muscles of patients with ACL injury. We also observed a decrease in FXYD5 protein content in \textit{semitendinosus} muscle. This is partly in accordance with a previous study where healthy subjects performed 6-weeks of BFR cycling, which lead to an increase in protein content of NKA$\alpha_1$ in the \textit{vastus lateralis} type II fibres of the leg that performed BFR cycling compared to the control leg that performed cycling without BFR (Christiansen \textit{et al.}, 2019). Conversely,
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ey they did not find any difference in the mRNA expression of NKA\(\alpha\)1 (Christiansen et al., 2019) and they also reported of no effects of BFR on the mRNA expression of NKA\(\alpha\)1 after a single running session with BFR (Christiansen et al., 2018b).

A recent study by Kutz et al. (2018) propose that \(\alpha1/\beta\)-heterodimers play a role in skeletal muscle growth that is independent of NKA’s pumping activity. This suggests that an increase in the expression of NKA \(\alpha\)1-subunit in exercised muscles of LL-BFR patients might have a trophic effect. It has been shown in women with osteoarthritis and rheumatoid arthritis that LL-BFR training increased cross-sectional area of quadriceps muscles to a similar extent as high intensity resistance training without blood flow restriction (Ferraz et al., 2018; Rodrigues et al., 2020). Additionally, Christiansen (2019) proposes that to achieve an increase in NKA\(\alpha\)1, bouts of exercise at near maximal level and significant duration (more than 30 seconds) have to be performed. This can be achieved for example with sprint interval trainings that include sprints that last at least 30 seconds (Iaia et al., 2008; Christiansen et al., 2018a), high intensity resistance training (Dela et al., 2004), or HI-BFR training (Christiansen et al., 2019). It was shown that resistance training promotes higher levels of hypertrophy than aerobic training (Grgic et al., 2019), which strengthens our hypothesis that increase of NKA\(\alpha\)1 in muscles of LL-BFR patients would coalesce with an increase in muscle mass, as aerobic training did not affect NKA\(\alpha\)1 expression (Wyckelsma et al., 2017).

NKA \(\alpha2\)-subunit constitutes from 60-90% of total NKA\(\alpha\) pool in skeletal muscle (Orlowski & Lingrel, 1988; Sweadner et al., 1992; Hansen, 2001) and is a limiting factor for muscle’s contractile performance (Radzyukevich et al., 2013). Surprisingly, we observed a slight decrease in the mRNA expression of \(\alpha2\)-subunit in semitendinosus muscle of LL-Sham and LL-BFR groups compared to Control, while the expression on protein level remained
unchanged. This is similar to what was observed in healthy subject that performed 6 weeks of interval cycling with BFR, where NKAα2 remained unchanged (Christiansen et al., 2019)

Elevated FXYD1 content was proposed to protect skeletal muscle against ROS since Christiansen et al. (2018b) observed an increased FXYD1 mRNA expression combined with increases in indicators of response to oxidative stress. We have not observed any differences between Control, LL-Sham and LL-BFR groups in mRNA or protein expression of FXYD1. There also was no change in phosphorylation of FXYD1. We did however observe a decrease in FXYD5 protein content in semitendinosus muscles of LL-BFR group. To our knowledge our group was the first to evaluate FXYD5 in skeletal muscles of subjects who performed BFR training, but our results do seem to be compatible with the results, obtained in muscles of patients with spinal cord injury, who are inactive due to their injury, where the expression of FXYD5 is increased (Boon et al., 2012). Differences in the results from our BFR study and those performed on healthy subjects (Christiansen et al., 2018b; Christiansen et al., 2019) could be caused by different experimental designs, such as type of exercise, intensity, training volume and of course timing of sample collection. We evaluated whole homogenates, which might obscure possible changes in specific skeletal muscle fibres (Christiansen et al., 2018b; Christiansen, 2019; Christiansen et al., 2019).

In differentiated HSMC we evaluated the separate effects of glucose deprivation, hypoxia, and artificial ischaemia (i.e., combination of hypoxia and glucose deprivation, HGD) on mRNA and protein expression of NKA subunits and its FXYD regulators. We found that acute 24 h artificial ischaemia and glucose deprivation decreased mRNA and protein expression of NKAα1, which is the opposite of what we observed in LL-BFR subjects. Glucose deprivation and artificial ischaemia also decreased expression of NKA β-subunits and increased FXYD5. From the differences in in vivo and in vitro results we can
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speculate that glucose deprivation and/or hypoxia do not by itself affect the expression of NKA\(\alpha\)1 and FXYD5 in skeletal muscles of subjects, who performed LL-BFR training. It is possible that hypoxia and glucose deprivation only increase NKA\(\alpha\)1 expression when combined with the effects of muscle contractions. This would agree with the results that while aerobic training by itself does not upregulate NKA\(\alpha\)1, it does increase its expression when combined with BFR (Christiansen, 2019). As cultured HSMC do not contract without innervation or electric stimulation (Mis et al., 2017; Nikolić et al., 2017), we did not explore this hypothesis any further.

Surprisingly while NKA\(\alpha\)1 was the only \(\alpha\) subunit of the three to be affected by glucose deprivation all three NKA \(\beta\)-subunits were downregulated. Connection between glucose and NKA regulation are not precisely defined, but there are some publications on the matter. It was shown that expression of NKA\(\alpha\)1 is upregulated in choroid plexus of hyperglycaemic diabetic rats when compared to controls (Egleton et al., 2003). \(V_{\text{max}}\) of NKA in skeletal muscles is enhanced in skeletal muscle by glucose intake during training by an unknown mechanism after (Green et al., 2007). It was also shown on transcription level of hearts from NKA\(\alpha\)1\(^+/+\)mice that haploinsufficiency might cause downregulated oxidation of glucose and upregulated oxidation of fatty acids (Moseley et al., 2005). Altogether it seems that NKA\(\alpha\)1 might represent an important link between glucose metabolism and ion transport in skeletal muscle.

FXYD5 was increased in glucose deprivation and artificial ischaemia. FXYD5 plays an important role in inflammation during lung injury (Brazee et al., 2017) and this might explain its increased expression during nutrient shortage or ischaemic conditions. Also, FXYD5 is known to increase NKA's \(V_{\text{max}}\) (Lubarski et al., 2005; Lubarski et al., 2007), which might mean that FXYD5 gets upregulated as a countermeasure of reduced NKA expression to maintain ion transport of K\(^+\) and Na\(^+\). In cultured 4T1 cancer cells NKA\(\beta\)1
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expression might be suppressed by FXYD5 (Lubarski-Gotliv et al., 2017), so the observed decrease in NKAβ1 in glucose deprived and ischaemic HSMC might be explained by increased levels of FXYD5.

Even though hypoxic treatment of HSMC caused a pronounced increase in HIF-1α and its downstream targets, it had no effect on the expression of NKA subunits or FXYDs in differentiated HSMC. In vivo studies that explored the effects of hypoxia on NKA in skeletal muscle produced varying results. While some studies showed no effect of hypoxia on NKA expression in vastus lateralis (Aughey et al., 2006), or just on particular ones (increased NKAβ1) (Christiansen et al., 2018b), which correlates with our results, some studies showed reduction of NKA content in vastus lateralis muscle of healthy subjects that performed hypoxic training at altitude or sea-level (Green et al., 1999; Green et al., 2000). Effects of hypoxia on NKA and FXYD regulation are very complex and results from different studies may also, among others, vary due to different training protocols, cell used, and different timing of sampling.

Since there is strong evidence that AMPK plays a role in regulation of NKA activity in skeletal muscle (Pirkmajer et al., 2021), and it has been proposed that AMPK activation upregulates transcription of NKAβ1 and FXYD1 in vitro (Nilsson et al., 2006), we expected to see upregulation of AMPK activity in LL-BFR. Unlike Christiansen et al. (2018b) we saw no significant difference between Control, LL-Sham, and LL-BFR groups. Differences in results might be explained by different timing of muscle sampling as our samples were not collected immediately after training sessions but during ACL reconstruction surgery and the increases in phosphorylation of AMPK and ACC might be transient. AMPK and ACC phosphorylation were already dropping considerably 150 min after mild and moderate training in lean, obese and type 2 diabetes subjects (Sriwijitkamol et al., 2007). Conversely, we
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observed a very significant increase in AMPK and ACC phosphorylation in \textit{in vitro} HSMC. In case of \textit{in vitro} acute artificial ischaemia (HGD) increased phosphorylation of AMPK and ACC correlated with lower mRNA expression and protein content of NKA\(\alpha_1\) and \(\beta_1-3\). But in glucose deprivation we observed no increase in AMPK and ACC phosphorylation, even though we detected similar decrease in mRNA and protein expression of NKA \(\alpha_1\)-subunit. This suggests that AMPK is not involved in decrease of NKA content in glucose-free treatment, while it might cause some effect in ischaemic conditions.

We decided to evaluate possible regulation of NKA by phosphorylation of Tyr10 residue on \(\alpha_1\)-subunit. Functional significance of Tyr10 residue was first described by Feraille \textit{et al.} (1999); Feraille and Doucet (2001), who showed that it is susceptible to insulin stimulation, which increases NKA activity. Banday (2016) proposed that acute insulin treatment in renal proximal tubular cells increases NKA activity through Tyr10 phosphorylation, while chronic insulin treatment decreases NKA activity through Ser16 and Ser18 phosphorylations in opossum kidney cells. In recent years however, some studies also observed that increased Tyr10 phosphorylation does not increase NKA activity, but actually lowers it in porcine nonpigmented ciliary epithelium and mouse C2C12 skeletal muscle cell line (Shahidullah \textit{et al.}, 2014; Breitenbach \textit{et al.}, 2016). Our results show that LL-BFR treatment caused a relative decrease in NKA\(\alpha_1\) Tyr10 phosphorylation compared to a significant increase in total \(\alpha_1\) protein content. This was opposite to what we observed in \textit{in vitro} experiment, where decrease in mRNA and protein content of NKA\(\alpha_1\) coincided with a decrease in Tyr10 phosphorylation. It seems that different treatments do not necessarily lead to correlated increases or decreases in protein content of NKA\(\alpha_1\) and its phosphorylation on Tyr10 residue.
Experiments on cell culture models have shown that GC box-binding transcription factors, specificity protein 1 and 3 (Sp1 and Sp3) mediate the transcription of NKA α1- and β1-subunits (Wendt et al., 2000; Wang et al., 2007). Sp1 and Sp3 activation by transcriptional and post-transcriptional modification seems to be a cellular response to ROS-mediated oxidative stress (Ryu et al., 2003) or perturbations in cellular redox state (Wendt et al., 2000; Wang et al., 2007). From our results we could not determine any change in protein content of Sp1 between Control, LL-Sham and LL-BFR patients. Conversely, we have observed a decrease in Sp1 protein content in acute glucose deprivation, hypoxia and artificial ischaemia compared to normoxic cultivation, which indirectly points to the importance of Sp1 in NKA regulation in glucose-free, hypoxic and ischaemic conditions in cultured HSMC.

After acute in vitro treatments we performed in vitro preconditioning of HSCMs with glucose deprivation, hypoxia or artificial ischaemia to see, whether short exposures to glucose-free medium and/or hypoxic conditions would affect the expression of NKA subunits and FXYDs, when the cells would in the end be exposed to longer hypoxic treatment. As it turned out in vitro preconditioning did not replicate the effects of LL-BFR training.

In brief, 3 weeks of LL-BFR training caused an increase in NKA α1-subunit in both vastus lateralis and semitendinosus muscles. The change in NKAα1 have trophic effects for both skeletal muscles in LL-BFR group. Our results add new evidence to the hypothesis that LL-BFR training could be used as a plausible pre-operational intervention for patients with ACL rupture. Intervention might improve post-operational recovery and prevent muscle unloading. The described intervention might also be useful for other patients that have difficulty performing high intensity training, for example patients with osteoarthritis. To our
knowledge our study is the first where the effects of LL-BFR training on NKA and FXYD expression was evaluated in skeletal muscles of ACL injury patients. We also detected NKA subunits in FXYDs in both flexor (semitendinosus) and extensor (vastus lateralis) muscles, which is rarely described due to inaccessibility. The study could be improved if we would have baseline samples from before the patients started training intervention and/or samples from both injured and healthy leg, but this was not possible due to avoidance of procedures that are not necessary for medical treatment of ACL injury. Some of semitendinosus muscle biopsies were also mostly tendinous and had to be excluded from evaluation due to low muscle tissue, which lowered statistical power of our results.

In conclusion, our results from clinical trial and in vitro experiments do not support our hypothesis that hypoxia alters NKA function in skeletal muscle via activation of AMPK. While we cannot certainly say that AMPK did not affect NKA function in skeletal muscles of clinical trial patients, our results do not confirm it. This may be down to several factors, such as timing of sample collection, volume of muscle loading and the intensity of exercise sessions. We did however observe an upregulation of NKAα1 in patients who performed LL-BFR training. As NKAα1 was recently implied to play a trophic role in skeletal muscle, this suggests that LL-BFR training may positively affect muscle mass in ACL injury patients. Our in vitro results suggest that acute hypoxia does not affect AMPK activation. It seems that two-pronged attack on cell's energy status is needed to actually activate AMPK, which in our case was achieved by glucose deprivation and low oxygen (0.1%) cultivation. Glucose deprivation was the biggest factor in suppression of NKAα1 and all three NKA β-subunits. We also observed a reduction in NKAα1 Tyr10 phosphorylation in glucose-deprived, hypoxic and ischaemic cells. Tyr10 phosphorylation site of NKA α1-subunit has not been explored in many studies before, so this result merits further inquiry.
5.3 Hypothesis III: Modulation of NKA by pharmacological activators of AMPK depends on the metabolic state of skeletal muscle.

Metformin is the most commonly used oral antidiabetic drug, which can indirectly activate AMPK through inhibition of mitochondrial respiratory complex I (El-Mir et al., 2000; Owen et al., 2000). This leads to an increase in AMP/ATP ratio due to a decrease in mitochondrial ATP production, which enhances AMPK activity due to increased AMP levels. Acute metformin treatment increased glucose uptake in cultured HSMC (Sarabia et al., 1992), it was also reported to activate AMPK in skeletal muscles of type 2 diabetes patients (Musi et al., 2002), and its chronic treatment led to increased glucose uptake and AMPK activation in mouse soleus muscle (Kristensen et al., 2014).

As mentioned, there are several studies available that examined potentials of different pharmacological agents for their protective capabilities against ischaemia-reperfusion injury and metformin preconditioning was reported to protect neural and cardiac tissue from ischaemia-reperfusion and cerebral ischaemia (Solskov et al., 2008; Jiang et al., 2014). Calvert et al. (2008) reported that metformin preconditioning protected heart tissue against infarction via AMPK activation, while Seo-Mayer et al. (2011) observed that AMPK activation by metformin and AICAR lead to both reduced ischaemic cell damage and inhibited NKA endocytosis in MDCK cells, which would improve cellular membrane function. We were interested in how metformin preconditioning would affect mRNA expression of NKA subunits and FXYDs after acute exposure to normoxia, hypoxia or artificial ischaemia (hypoxia with glucose deprivation, HGD), which expose HSMC to different levels of metabolic and hypoxic stress.

In cultured HSMC that were not exposed to hypoxia or HGD for final 24 h, we observed no changes in the mRNA expression of NKA subunits. This seems to correlate with results...
from metformin-treated L6 cells, where metformin did not affect NKA activity in normoxic conditions (Benziane et al., 2009). We show that NKA mRNA expression is susceptible to metformin treatment only in ischaemic conditions. Surprisingly, NKAα1, α2, β1 and β2 mRNA expression was reduced when cells were treated with metformin in ischaemic conditions, which seems to negate what was reported in metformin-preconditioned MDCK cells, where NKA activity was increased after ischaemic treatment (Seo-Mayer et al., 2011). It seems that all examined NKA subunits were downregulated with metformin in HGD conditions. In hypoxic and normoxic conditions metformin did not seem to have any effect. To our knowledge there are no available publications on metformin's effects on FXYDs in skeletal muscle. Interestingly FXYD1 and FXYD1 were also affected in normoxic conditions. While FXYD1 mRNA expression was reduced with metformin treatment, FXYD5 expression increased.

It is evident that our results show just a minor part of a bigger picture, how metformin-preconditioning might affect NKA function, as we only explored its effect on mRNA level. There are several ways in which we could further elucidate how metformin preconditioning affects NKA function. Firstly, results should be also evaluated on protein level by western blotting to confirm, whether observed effects of metformin on the expression of NKA subunit and FXYDs in ischaemic conditions translate to the protein level. In experiments, presented in Hypothesis II, we found out that in vitro ischaemic treatment of HSMC does not always exert the same effects on mRNA and protein content of NKA subunits and FXYDs. With western blotting we could also confirm whether metformin preconditioning affected AMPK activation in hypoxic and HGD conditions. Secondly, glucose uptake could be evaluated, to see whether metformin increased transport of glucose into HSMC. Finally, besides AMPK signalling pathway, we should also further explore metformin's possible effects on HIF-1α signalling pathway. Both signalling pathways play a role in cell's adaptation to metabolic and hypoxic
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stress (Semenza, 2001; Loor & Schumacker, 2008; Taie et al., 2009). Expression of HIF-1\(\alpha\) is increased via activation of phosphatidyl inositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin complex 1 (mTORC1) pathway (Skinner et al., 2004; Karar & Maity, 2011). Metformin was reported to reduce the expression of HIF1-\(\alpha\) via inhibition of mTORC1 pathway (Tadakawa et al., 2015), while in another study metformin inhibited mTORC1 by inhibition of AMPK (McCarty, 2011). Our results show that 3mM metformin decreased VEGF-A mRNA expression in HGD-treated cells, which is in agreement with former studies. In our metformin experiments VEGF-A expression increased in hypoxic and ischaemic conditions, which correlated with what we showed in results from Hypothesis II. Further exploration of HIF-1\(\alpha\) signalling in metformin preconditioning might bring further elucidation of processes how metformin might ameliorate effects of ischaemia on HSMC.

Unlike in previous studies where metformin preconditioning protected the tissue or cells against ischaemia also via improved NKA function (Seo-Mayer et al., 2011), it seems that metformin preconditioning only caused a further decrease in mRNA expression of all NKA subunits in HGD conditions. Our results do however support our hypothesis that actions of AMPK activator, in this case metformin's, depend on the metabolic state of HSMC as significant decreases in mRNA expression of NKA subunits were only observed in HGD conditions. We also showed that metformin causes divergent effects on mRNA expression of FXYD1 and FXYD5 in both normoxia and HGD.

As we mentioned in the introduction available AMPK activators have several limitations when it comes to activating AMPK in skeletal muscle. They can cause serious side effects, they are not effective in activating muscle-specific AMPK complexes, and due to their poor bioavailability high doses have to be used to overcome minimum effective concentration
threshold. Finding new AMPK activators that would successfully increase AMPK activity in skeletal muscle remain a major challenge.

SSZ was shown to increase intracellular concentrations of ZMP, a direct AMPK activator, in murine splenocytes (Gadangi et al., 1996), possibly due to inhibition of ATIC (Fig. 1.4) (Baggott et al., 1992). It was already shown in L6 cells and primary human skeletal muscle cells that methotrexate, another widely used anti-rheumatic drug, markedly augments AMPK activation by AICAR via ATIC inhibition (Pirkmajer et al., 2015) and Park et al. (2019) recently showed that SSZ activates AMPK in rat colonic tissue. Importantly, there are studies that showed that SSZ improved glucose levels in human (Svenson et al., 1987; Haas et al., 2005). We proposed that SSZ might act as an AMPK activator in skeletal muscle cells, that it might cause additive effect to AICAR-induced AMPK activation in the same way as methotrexate, and that it may enhance glucose uptake through Akt signalling.

Our results show that SSZ does activate AMPK in concentrations between 0.3 and 1.0 mM. Conversely to AICAR (Dolinar et al., 2018), whose effects on AMPK activation are blunted by nucleoside presence in culture medium, SSZ effect does not seem to be effected by nucleoside presence or inhibition of nucleoside transporters. This suggests that SSZ does not enter cells with the help of nucleoside transporters. SSZ activated AMPK even after 45 min, while its direct substrate ACC was more profoundly phosphorylated after 8 h.

We also evaluated possible additive effects of SSZ and AICAR co-treatment. SSZ was proposed to inhibit ATIC (Baggott et al., 1992). By suppressing ATIC it inhibits metabolism of ZMP, which lead to increased AMPK activity. Methotrexate is also a drug that inhibits ATIC and is known to have additive effect when used in combination with AICAR (Pirkmajer et al., 2015). SSZ has no additive effect in L6 cells on AMPK activation, which suggest that ATIC inhibition is not the main mechanism through which SSZ activates AMPK. In human cells SSZ
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actually inhibits AICAR’s effects on AMPK, which might suggest that SSZ affects AICAR’s uptake or its metabolism. Removal of SSZ from cell culture medium diminished its induction of AMPK activity in L6 cells, so it is possible that SSZ's action on cell membrane led to indirect AMPK activation. Whether SSZ's action on cell membrane would explain its inhibitory effect on AICAR should be explored in HSMC, where SSZ's suppression of AICAR action was more pronounced than in L6 cells.

It is possible that SSZ might activate AMPK through different mechanisms. One possibility is that SSZ inhibits mitochondria functions, which would increase AMP:ATP ratio and consequently increase AMPK activation. This has been shown for some of the salicylates (Fleischman et al., 2008).

Pharmacological activation of AMPK is considered as a possible target for treating diabetes type 2 as it can lead to increased glucose uptake by skeletal muscles (Long & Zierath, 2006). We evaluated how SSZ treatment affects Akt signalling in combination with physiological (12 nM) and supraphysiological (120 nM) concentrations of insulin. Our results show that SSZ decreased both Akt phosphorylation, when cells were co-treated with insulin, and phosphorylation of its substrates AS160 and GSK-3α/β in L6 cells. This suggests that SSZ actually inhibits glucose uptake in L6 cells. However, we found articles that report SSZ can inhibit Akt phosphorylation in hepatocellular carcinoma cells (Shamaa, 2021) and in HSC-4 cell line (Human oral squamous cell carcinoma) (Han et al., 2014), which correlates with the results in L6 cells. Conversely, 100 μM SSZ treatment enhanced action of supraphysiological insulin on the phosphorylation of some proteins from the Akt signalling pathway. While Akt Ser473 remained unchanged, 100 μM SSZ had an additive effect on phosphorylation of AS160 at Ser588 and Thr642 residues, and also on pGSK-3α/β. This suggest that SSZ treatment in combination with insulin may increase glucose uptake and that SSZ action affects Akt pathway...
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differently in L6 cells than in HSMC. One of the explanations why we obtained different results in L6 cells and HSMC is their metabolic regulation. Abdelmoez et al. (2020) reported that compared to HSMC L6 cells had higher number of genes related to glucose transport, which correlated with higher capacity of L6 cells for glucose uptake and oxidative respiration. This correlates with reports that insulin's effects on glucose uptake in HSMC is relatively small (Ciaraldi et al., 1995; Ciaraldi et al., 1999). Also, it is evident from our results that insulin caused higher increases in phosphorylation of proteins from Akt signalling pathways in L6 cells compared to HSMC, which is in accordance with these reports. Perriott et al. (2001) reported that preincubating HSMC with 5-10 mM pyruvate lowers basal and increases insulin-induced glucose transport, which might make it easier to evaluate effects of potential AMPK activator on glucose uptake. In future glucose uptake should also be evaluated to confirm our results in western blot analysis.

To conclude, our results concerning effects of metformin on NKA subunit expression under different metabolic conditions support Hypothesis III. We observed distinct reduction in mRNA expression of NKA subunits in HGD treatment, while hypoxic and normoxic conditions had no effect on metformin's effects. Interesting result is also that metformin caused divergent effects on FXYD1 and FXYD5 expression. While FXYD1 expression was suppressed, FXYD5 expression was enhanced. We also confirmed that SSZ activates AMPK in L6 cells and HSMC, does not have additive effect on AICAR action and its effects on glucose uptake have to be explored further. There are several salicylate derivates, like for example diflunisal that might also activate AMPK (Hawley et al., 2012; Marrone et al., 2018) and be effective in glucose intake regulation, so our results open several perspectives for future research, including the possibility that SSZ modulates NKA function via AMPK like metformin.
6 CONCLUSIONS

Major conclusions of this dissertation are: First, we showed that fusion of myoblasts into myotubes does affect mRNA expression of NKA subunits and its FXYD regulators in a way that makes them more alike to mature muscle fibres regarding their expression pattern. Innervation of myotubes did not bring the expected additional upregulation of mRNA, coding for NKA subunits and FXYDs. We can conclude that co-culturing of HSMC with explants of rat embryonic spinal cords up to 21 days does not lead to changes in NKA and FXYD expression that would make them more similar to mature skeletal muscle fibres. It seems that in the early phase of de novo innervation serum concentration in cell medium plays the most significant role in gene expression regulation of NKA subunits and FXYDs. In this regard our results do not support our first hypothesis. On the other hand, we showed that FXYD1 and FXYD5 can be studied in cultured HSMC, which opens new options for evaluation of their role in skeletal muscle.

Second, we showed that LL-BFR training in patients with knee injury does cause changes in mRNA and protein expression of NKAα1, which might cause hypertrophic effects in the skeletal muscle. In muscle samples we could not determine whether AMPK activation might cause these changes. In our in vitro model of acute hypoxia, we showed that hypoxia by itself does not cause significant changes to any NKA subunit, but that ischaemia and glucose deprivation do. Comparing the effects, we concluded that the lack of glucose is the main regulator of NKA content in cultured HSMC model and that AMPK is only activated in combination of hypoxic and glucose-free conditions. While we cannot exclude the role of AMPK in regulation of NKA under hypoxic condition, our results do not support this hypothesis.
Finally, we showed that metformin decreased mRNA expression of all NKA subunits only in ischaemic conditions, while in normoxia and hypoxia it did not have significant effects. These results support our third hypothesis that metabolic state does affect the ability of AMPK activator to affect NKA function. In addition, we also discovered that SSZ can activate AMPK in L6 cells and to a smaller extent in HSMC, which opens new opportunities in research of new potential AMPK activator. As we have obtained divergent results in L6 cells and HSMC regarding SSZ’s action on insulin signalling pathway, further exploration of this topic should be undertaken. Whether SSZ also affects NKA function remains an unexplored question. Several salicylate derivate can also activate AMPK, but their effects on AMPK action in skeletal muscle has not been explored yet, which maintains hope that new potential AMPK activators in skeletal muscle will be discovered in future years.

**ZAKLJUČKI**

Glavni izsledki doktorske naloge so: Prvič, pokazali smo, da fuzija mioblastov v večjedrne cevčice vpliva na izražanje mRNA podenot NKA, FXYD1 in FXYD5 v smeri, s čimer njihovi ekspresijski profil postane bolj podobni zrelim mišičnim vlaknom. Inervacija cevčic ni povzročila pričakovanega porasta v izražanju mRNA podenot NKA, FXYD1 in FXYD5. Zaključimo lahko, da 21 dni gojenja HSMC v prisotnosti eksplantatov podganjih embrionalnih hrbtenjač ne spremeni izražanja NKA in FXYD1 na način, da bi te postale bolj podobne zrelim skeletnomišičnim vlaknom. Zdi se, da v zgodnji fazi de novo inervacije koncentracija seruma igra bolj pomembno vlogo v regulaciji genskega izražanja podenot NKA, FXYD1 in FXYD5. V zvezi s tem naši rezultati ne podpirajo prve hipoteze. Po drugi strani pa smo pokazali, da lahko FXYD1 in FXYD5 proučujemo v gojenih HSMC, kar odpira nove možnosti za proučevanje njunih vlog v skeletnih mišicah.

Tretjič, pokazali smo, da metformin zniža izražanje mRNA vseh podenot NKA le v ishemičnih razmerah, medtem ko normoksiija in hipoksija nista imeli signifikantnih učinkov. S tem lahko potrdimo našo tretjo hipotezo, da presnovno stanje celice vpliva na zmožnost aktivatorja AMPK, da povzroči spremembe v delovanju NKA. Ugotovili smo tudi, da lahko SSZ aktivira AMPK v L6 celicah in v manjši meri tudi v HSMC, kar odpira nove možnosti za odkritje novih potencialnih aktivatorjev AMPK. Glede na to, da smo dobili različne rezultate med L6 celicami in HSMC v zvezi z učinki SSZ na inzulinsko signalizacijo, bi bilo treba opraviti dodatne raziskave, kako, med drugim, SSZ vpliva na privzem glukoze. Ali SSZ vpliva tudi na delovanje NKA ostaja odprto vprašanje. Številni salicili bi lahko aktivirali AMPK, vendar njihovi učinki na delovanje AMPK v skeletni mišici ostajajo neraziskani. To ohranja upanje, da bi lahko v prihodnjih letih prišlo do odkritja novih aktivatorjev AMPK, ki bi delovali tudi v skeletni mišici.
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