FUNCTION OF TRANSIENT RECEPTOR POTENTIAL CANONICAL 3 - NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE OXIDASE 2 INTERACTION IN ATROPHY OF CARDIAC AND SKELETAL MUSCLE CELLS

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, summaries and references which have been duly acknowledged.

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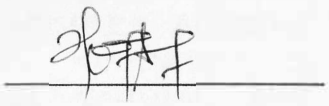

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ABSTRACT

Muscle atrophy develops after a long period of inactivity caused by malnutrition, ageing, genetic disorders, and cancer. High protein degradation rate is a hallmark in the muscle atrophy-related diseases that showed increase in reactive oxygen species (ROS) production and severe muscle wasting. The signaling pathways involving the activation of protein degradation systems are complex and regulated by many different mediators, therefore finding a specific target is a major challenge for muscle atrophy. In general, the current study aimed to reveal new key components of the protein degradation pathway involved in muscle cells atrophy. The first part of the study was to determine the involvement of transient receptor potential canonical 3 (TRPC3) and NADPH oxidase 2 (Nox2) complex in cardiac atrophy on a primary culture of neonatal rat cardiomyocytes (NRCMs) using immunostaining, western blot and luciferase assay. High concentration of adenosine triphosphate (ATP) significantly induces NRCMs atrophy through ROS-mediated up-regulation of atrophy marker, muscle atrophy F-box (MAFbx) and reduction in cell size ($p<0.05$). Gene knockdowns of TRPC3 and Nox2 significantly suppressed ATP-induced NRCM atrophy and ROS production ($p<0.05$). The study further revealed that TRPC3 and Nox2 formed an interaction in the presence of ATP through the $P2Y_2$ receptor in NRCMs atrophy. Furthermore, nutrient depletion (glucose starvation, hypoxia, and amino acid deprivation) displayed a significant increase in extracellular ATP levels that promoted NRCMs shrinkage ($p<0.05$). The second part of the study designed to provide direct evidence of TRPC3-Nox2 complex formation in *in vivo* setting incorporating with human disease models of skeletal muscle atrophy using immunohistology and quantitative polymerase chain reaction (qPCR). Denervation surgery was conducted in the hind limb of wild type (WT) and TRPC3 gene knockout (C3KO) mice to evaluate the effect of immobilization-induced skeletal muscle atrophy on TRPC3-Nox2 complex. Expectedly, 14 days post denervation significantly induces muscle atrophy and ROS overproduction in soleus, gastrocnemius, and *tibialis anterior* tissue sections. However, the deletion of TRPC3 prevented denervation-induced atrophy only in C3KO soleus. A significant up-regulation of Nox2 protein promotes interaction with TRPC3 protein in denervation-induced soleus atrophy. Finally, transgenic mice carrying a mutant superoxide dismutase 1 gene (SOD1) that mimic Amyotrophic Lateral Sclerosis disease displayed a significant decrease in fibre sizes associated with overproduction of ROS in gastrocnemius and *tibialis anterior* ($p<0.05$) but not in soleus ($p>0.05$). Nevertheless, atrophied fibres from transgenic mice failed to demonstrate a significant increase in Nox2 protein up-regulation, which suggests the SOD1-induced atrophy pathway is most likely independent to TRPC3-Nox2 complex-mediated ROS production in soleus atrophy induced by denervation. This study demonstrated the function of TRPC3 and Nox2 complex formation in cardiomyocytes atrophy and skeletal muscle atrophy, specifically in slow oxidative soleus muscle. Furthermore, this study may provide potential therapeutic targets that can delay or counteract muscle atrophy in a specific condition.

ABSTRAK

FUNGSI INTERAKSI TRANSIENT RECEPTOR POTENTIAL CANONICAL 3 - NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE OXIDASE 2 *DALAM ATROPI SEL OTOT JANTUNG DAN RANGKA*

Atrofi otot berkembang setelah lama tidak aktif berpunca dari malnutrisi, penuaan, gangguan genetik, dan kanser. Kadar degradasi protein yang tinggi adalah ciri khas penyakit berkait atrofi otot yang ditunjukkan dengan peningkatan penghasilan spesies oksigen reaktif (ROS) dan pembaziran otot. Laluan isyarat kompleks dalam pengaktifan sistem degradasi protein dikawal atur oleh perantara berbeza menjadi cabaran utama bagi mencari sasaran khusus untuk merawat atrofi otot. Secara umum, kajian ini bertujuan mendedahkan komponen baru pada tapak jalan degradasi protein dalam atrofi sel otot. Bahagian pertama kajian menentukan penglibatan kompleks transient receptor potential canonical 3 *(TRPC3) dan* NADPH oxidase 2 *(Nox2) dalam atrofi jantung kultur primer kardiomiosit tikus neonatal (NRCM) menggunakan* immunostaing, *pemblotan* western *dan pengasaian luciferase. Kepekatan tinggi adenosin trifosfat (ATP) secara signifikan mengaruh atrofi NRCMs melalui peningkatan ROS-perantara kenaikan penanda atrofi,* muscle atrophy F-box *(MAFbx) dan pengurangan saiz sel ($p<0.05$). Penyahaktifan gen TRPC3 dan Nox2 secara signifikan merencat atrofi NRCM dan penghasilan ROS disebabkan oleh ATP ($p<0.05$). Kajian seterusnya menunjukkan TRPC3 dan Nox2 membentuk interaksi dengan kehadiran ATP melalui pengaktifan reseptor $P2Y_2$ pada atrofi NRCM. Kekurangan nutrien (hipoksia, kekurangan glukosa dan asid amino) menunjukkan peningkatan signifikan tahap ATP ekstraselular mendorong pengecutan NRCM ($p<0,05$). Bahagian kedua kajian dijalankan bagi membuktikan pembentukan kompleks TRPC3-Nox2 dalam persekitaran in vivo yang menggabungkan model penyakit atrofi otot rangka manusia menggunakan* immunohistology *dan tindak balas berantai polymerase kuantitatif (qPCR). Pembedahan penyahsaraf dilakukan pada mencit liar (WT) dan terhapus gen TRPC3 (C3KO) untuk menilai kesan kompleks TRPC3-Nox2 terhadap atrofi otot rangka yang disebabkan imobilisasi. Selepas 14 hari, penyahsaraf mendorong atrofi otot dan penghasilan berlebihan ROS signifikan pada bahagian tisu* soleus, gastrocnemius *dan tibialis anterior. Tetapi, penghapusan gen TRPC3 dapat mencegah atrofi disebabkan oleh penyahsaraf hanya pada* soleus *C3KO. Pengaktifan protein Nox2 mendorong interaksi dengan protein TRPC3 dalam atrofi soleus ternyahsaraf. Seterusnya, mencit transgenik bermutasi gen* superoxide dismutase 1 *(SOD1) digunakan bagi menggambarkan penyakit* Amyotrophic Lateral Sclerosis *menunjukkan penurunan ketara dalam saiz fiber yang disebabkan oleh pengeluaran ROS berlebihan pada* gastrocnemius *dan* tibialis anterior *($p<0.05$) tetapi tidak pada* soleus *($p>0.05$). Akan tetapi, atrofi fiber dari mencit transgenik gagal menunjukkan peningkatan signifikan dalam pengaktifan protein Nox2, menunjukkan bahawa tapak jalan atrofi disebabkan oleh mutasi SOD1 kemungkinan besar bukan melalui pembentukan kompleks TRPC3-Nox2 seperti yang ditunjukkan pada atrofi* soleus *ternyahsaraf. Kajian ini menjelaskan fungsi pembentukan kompleks TRPC3 dan Nox2 dalam atrofi kardiomiosit dan otot rangka, khususnya pada otot* slow oxidative soleus. *Selanjutnya, kajian ini memberikan potensi sasaran terapi yang dapat melambatkan atau mengatasi atrofi otot dalam kondisi tertentu.*

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β-ME	-	β-mercaptoethanol
μCL	-	Ubiquitous μ-calpain
ACE	-	Angiotensin-converting enzyme
ActRIIB	-	Activin A via activin A receptor type IIB
ADP	-	Adenosine diphosphate
AKT	-	Protein kinase B
ALS	-	Amyotrophic lateral sclerosis
AMP	-	Adenosine monophosphate
AMPK	-	Adenosine monophosphate-activated protein kinase
AngII	-	Angiotensin II
Apaf-1	-	Apoptosis protease-activating factor-1
ARB	-	Angiotensin II receptor blockers
Atg	-	Autophagy-related protein
ATP	-	Adenosine triphosphate
BAX	-	Bcl-2-associated X
Bcl-2	-	B-cell lymphoma 2
BSA	-	Bovine serine albumin
C3KO	-	TRPC3 knock out
Ca^{2+}	-	Calcium
CaMK	-	Ca^{2+}/calmodulin-dependent kinase
cAMP	-	Cyclic adenosine monophosphate
CAPS	-	N-cyclohexyl-3-aminopropanesulfonic acid
cDNA	-	Complementary deoxyribonucleic acid
cGMP	-	cyclic guanosine monophosphate
CPNS1	-	Calpain small subunit 1
CSA	-	Cross-section area
Cu, Zn-SOD		Copper-zinc superoxide dismutase
DAG	-	Diacylglycerol
DAMPS	-	Danger-associated molecular patterns
DAPC	-	Dystrophin-associated protein complex
DAPI	-	4′,6-diamidino-2-phenylindole
DEPTOR	-	DEP domain-containing mTOR-interacting protein
DHE	-	Dihydroethidium
DHPR	-	Dihydropyridine receptors
DMD	-	Duchenne muscular dystrophy
DMEM	-	Dulbecco's Modified Eagle Media
DNA	-	Deoxyribonucleic acid
DOX	-	Doxorubicin
DUOX	-	Dual oxidase

E214K	-	14-kDa ubiquitin-conjugating E2 enzyme
ECL	-	enhanced chemiluminescence
EDTA	-	Ethylenediaminetetraacetic acid
EGFP	-	Enhanced green fluorescent protein
eNOS	-	Endothelial nitric oxide synthase
ERK1/2	-	Extracellular signal-regulated kinase 1/2
ERR	-	Estrogen-related receptor
FBS	-	Fetal bovine serum
Fn14	-	Fibroblast growth inducible 14
FoxO	-	Forkhead box protein
FUS	-	Fused in sarcoma (protein)
GAPDH	-	Glyceraldehyde 3-phosphate dehydrogenase
GEF-H1	-	Guanine nucleotide exchange factor
GFP	-	Green fluorescent protein
GPCR	-	G protein-coupled receptor
H_2O_2	-	Hydrogen peroxide
HIF1α	-	Hypoxia-inducible factor 1-alpha
HRP	-	Horseradish peroxidase
IEGs	-	Immediate-early genes
IFN-γ	-	Interferon gamma
IGF1	-	Insulin-like growth factor 1
IgG	-	Immunoglobulin G
IL-1	-	Interleukin 1
iNOS	-	inducible nitric oxide synthase
IP_3	-	Inositol triphosphate
IP_3R	-	Inositol triphosphate receptor
IRS1	-	Insulin receptor substrate 1
IκB	-	Inhibitor of κB
K^+	-	Potassium
LDH	-	Lactate dehydrogenase
LVAD	-	Left ventricular assist device
MAFbx	-	Muscle atrophy F box
MAPK	-	Mitogen-activated protein kinase
mCL	-	Ubiquitous m-calpain
MG29	-	Mitsugumin 29
MgCl	-	Magnesium chloride
MLST8	-	Mammalian lethal with SEC13 protein 8
Mn-SOD	-	Manganese superoxide dismutase
mRNA	-	Messenger ribonucleic acid
mTOR	-	mammalian target of rapamycin
MuRF1	-	Muscle Ring Finger 1
MyoD	-	Myoblast determination protein 1

Na^+	-	Sodium
NaCl	-	Sodium chloride
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NEMO	-	NF-kappa-B essential modulator
NFAT	-	Nuclear factor of activated T
NF-κB	-	Nuclear factor κB
NO	-	Nitric oxide
Nox	-	Nicotinamide adenine dinucleotide phosphate oxidase
NRCF	-	Neonatal rat fibroblast
NRCM	-	Neonatal rat cardiomyocyte
NRF	-	Nuclear receptor factor
Nrf2	-	Nuclear factor erythroid 2-related factor
NRROS	-	Negative Regulator of Reactive Oxygen Species
OCT	-	Optimal Cutting Temperature
P120	-	Postnatal 120 days
PBS	-	Phosphate-buffered saline
PDE	-	Phosphodiesterase
PFA	-	Paraformaldehyde
PGC1α	-	Peroxisome-proliferator-activated receptor ɣ coactivator 1 α
PI3K	-	Phosphoinositide 3-kinase
PIP_2	-	Phosphatidylinositol-4, 5-bisphosphate
PKA	-	Protein kinase A
PKC	-	Protein kinase C
PLA	-	Proximity ligation assay
PLC	-	Phospholipase C
PPAR	-	Peroxisome-proliferator-activated receptor
PRAS	-	Proline-rich Akt1 substrate 1
PVDF	-	Polyvinylidene difluoride
Pyr3	-	Pyrazole compound
Rac	-	Rho family of GTPase enzyme
Raptor	-	Regulatory-associated protein of mTOR
RNS	-	Reactive nitrogen species
ROCE	-	Receptor-operated Ca^{2+} entry
ROS	-	Reactive oxygen species
RT-qPCR	-	Real-time quantitative Polymerase Chain Reaction
RyR	-	Ryanodine receptor
SDS-PAGE	-	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	-	Standard error
SERCA	-	Sarco/endoplasmic reticulum ATPase
SH3	-	Src-homology 3
siRNA	-	short interfering ribonucleic acid
SMAD	-	Small Mothers Against Decapentaplegic

SOCE - Store-operated Ca^{2+} entry
SOD - Superoxide dismutase
$SOD1^{G93A}$ - SOD1 gene point mutation located at position 93, glycine to alanine
Src - Proto-oncogene tyrosine-protein kinase
TBST - Tris-buffered saline, 0.1% Tween 20
TDP43 - TAR DNA binding protein 43
Tfam - Mitochondrial transcription factor A
TGFβ - Transforming growth factor β
TNFα - Tumour necrosis factor α
TNFR1 - Tumour necrosis factor receptor 1
TRAF6 - TNF-associated factor 6
TRAIL - TNF-related apoptosis-inducing ligand
TRAIL-R1 - TNF-related apoptosis-inducing ligand receptor 1
TRAIL-R2 - TNF-related apoptosis-inducing ligand receptor 2
TRIM32 - Tripartite motif-containing protein 32
Tris-HCl - Tris hydrochloride
TRPC - Transient receptor potential canonical
TWEAK - TNF-like weak inducer of apoptosis
ULK1 - Unc-51 like autophagy activating kinase 1
VEGF - Vascular endothelial growth factor
WGA - Wheat germ agglutinin
WT - Wild type
XO - Xanthine oxide

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

The muscular system is an organ system that enables humans to move, maintain posture, and circulate blood throughout the body. The system consists mainly of muscle cells or myocytes, a highly specialised cell which makes up the muscle tissue that produces tension for the generation of force. Cardiac and skeletal muscles are two important muscle cells that share many physiological similarities and are the major cell that made up the heart and musculoskeletal system. The primary function of both types of cell is a contraction which is triggered by a typical rush of ions across the sarcolemma. The action potential then activates muscle contraction by increasing the calcium (Ca^{2+}) concentration inside the cytosol (Stehle *et al.,* 2009). Action potential involves an influx of both sodium (Na^{+}) and Ca^{2+} ions.

Transient receptor potential canonical (TRPC) channels are widely recognised as a critical Ca^{2+} entry regulator in excitation-contraction coupling in muscle cells (Falcon *et al.,* 2019; Numaga-Tomita *et al.,* 2019). TRPC channel mediates the increase of intracellular Ca^{2+} concentration, which is induced following physical and chemical stimulations by directly conducting Ca^{2+} entry or prompting Ca^{2+} entry secondary to membrane depolarisation (Wu *et al.,* 2010). The