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YUNIBESITHI YA PRETORIA

SUBCELLULAR EFFECTS OF PAVETAMINE ON RAT CARDIOMYOCYTES

By

CHARLOTTE ELIZABETH ELLIS

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria

Date submitted: April 2010

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SUMMARY

SUBCELLULAR EFFECTS OF PAVETAMINE ON RAT CARDIOMYOCYTES

By

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The aim of this study was to investigate the mode of action of pavetamine on rat cardiomyocytes. Pavetamine is the causative agent of gousiekte ("quick-disease"), a disease of ruminants characterized by acute heart failure following ingestion of certain rubiaceous plants. Two *in vitro* rat cardiomyocyte models were utilized in this study, namely the rat embryonic cardiac cell line, H9c2, and primary neonatal rat cardiomyocytes.

Cytotoxicity of pavetamine was evaluated in H9c2 cells using the MTT and LDH release assays. The eventual cell death of H9c2 cells was due to necrosis, with LDH release into the culture medium after exposure to pavetamine for 72 h. Pavetamine did not induce apoptosis, as the typical features of apoptosis were not observed. Electron microscopy was employed to study ultrastructural alterations caused by pavetamine in H9c2 cells. The mitochondria and sarcoplasmic reticula showed abnormalities after 48 h exposure of the cells to pavetamine. Abundant secondary lysosomes with electron dense material were present in treated cells.

Numerous vacuoles were also present in treated cells, indicative of autophagy. During this exposure time, the nuclei appeared normal, with no chromatin condensation as would be expected for apoptosis. Abnormalities in the morphology of the nuclei were only evident after 72 h exposure. The nuclei became fragmented and plasma membrane blebbing occurred. The mitochondrial membrane potential was investigated with a fluorescent probe, which demonstrated that pavetamine caused significant hyperpolarization of the mitochondrial membrane, in contrast to the depolarization caused by apoptotic inducers. Pavetamine did not cause opening of the mitochondrial permeability transition pore, because cyclosporine A, which is an inhibitor of the mitochondrial permeability transition pore, did not reduce the cytotoxicity of pavetamine significantly.

Fluorescent probes were used to investigate subcellular changes induced by pavetamine in H9c2 cells. The mitochondria and sarcoplasmic reticula showed abnormal features compared to the control cells, which is consistent with the electron microscopy studies. The lysosomes of treated cells were more abundant and enlarged. The activity of cytosolic hexosaminidase was nearly three times higher in the treated cells than in the control cells, which suggested increased lysosomal membrane permeability. The activity of acid phosphatase was also increased in comparison to the control cells. In addition, the organization of the cytoskeletal F-actin of treated cells was severely affected by pavetamine.

Rat neonatal cardiomyocytes were labelled with antibodies to detect the three major contractile proteins (titin, actin and myosin) and cytoskeletal proteins (F-actin, desmin and β-tubulin). Cells treated with pavetamine had degraded myosin and titin, with altered morphology of sarcomeric actin. Vacuoles appeared in the β-tubulin network, but the appearance of desmin was normal. F-actin was severely disrupted in cardiomyocytes treated with pavetamine and was degraded or even absent in treated cells. Ultrastructurally, the sarcomeres of rat neonatal cardiomyocytes exposed to pavetamine were disorganized and disengaged from the Z-lines, which can also be observed in the hearts of ruminants that have died of gousiekte

It is concluded that the pathological alteration to the major contractile and cytoskeleton proteins caused by pavetamine could explain the cardiac dysfunction that characterizes gousiekte. F-actin is involved in protein synthesis and therefore can play a role in the inhibition of protein synthesis in the myocardium of ruminants suffering from gousiekte. Apart from inhibition of protein synthesis in the heart, there is also increased degradation of cardiac proteins in an animal with gousiekte. The mitochondrial damage will lead to an energy deficiency and possibly to generation of reactive oxygen species. The sarcoplasmic reticula are involved in protein synthesis and any damage to them will affect protein synthesis, folding and post-translational modifications. This will activate the unfolded protein response (UPR) and sarcoplasmic reticula-associated protein degradation (ERAD). If the oxidizing environment of the sarcoplasmic reticula is disturbed, it will activate the ubiquitin-proteasome pathway (UPP) to clear aggregated and misfolded proteins. Lastly, the mitochondria, sarcoplasmic reticula and F-actin are involved in calcium homeostasis. Any damage to these organelles will have a profound influence on calcium flux in the heart and will further contribute to the contractile dysfunction that characterizes gousiekte.

Keywords

Actin, cardiotoxicity, cytoskeleton, F-actin, gousiekte, H9c2 cell line, lysosome, mitochondria, myosin, necrosis, pavetamine, polyamine, protein synthesis, rat neonatal cardiomyocytes, sarcoplasmic reticula, titin.

ACKNOWLEDGEMENTS

I wish to thank the following people and institutions that helped me to peep into the fascinating world of God's cell:

- Ms Anitra Schultz and Dr Dharmarai Naicker, project team members of the Division of Toxicology (ARC-OVI).
- Prof Christo Botha (Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria) as promoter and Prof Roy Meintjes (Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria) as co-promoter of this study.
- Mr Alan Hall (Laboratory for Microscopy and Microanalysis, University of Pretoria) for the laser scanning confocal microscopy analyses.
- The South African National Biodiversity Institute (SANBI) for the distribution maps of gousiekte plants in South Africa.
- Ms Erna van Wilpe and Ms Lizette du Plessis for the electron microscopy analyses (Electron Microscopy Unit, Faculty of Veterinary Science, University of Pretoria).
- The computer centre at ARC-OVI.
- Funding provided by the Gauteng Province (Department of Agriculture, Conservation and Environment) and the North-West Province (Department of Agriculture, Conservation, Environment and Tourism).
- Family, friends and colleagues.
- Ms Nettie Engelbrecht for proof-reading this thesis.
- Lastly, a special friend, adv. David le Roux.

DECLARATION

I hereby declare that this study was my own work, except that pavetamine was purified by Ms Karen Basson.

Candidate: C Ellis

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LIST OF ABBREVIATIONS

ACTN	Actinin
AIF	Apoptosis-inducing factor
AJ	Adhering junction
AMP	Adenosine monophosphate
ANT	Adenine nucleotide transporter
ARs	Adrenergic receptors
ATF	Activating transcription factors
ATG	Autophagy-related protein
ATP	Adenosine triphosphate
ATPase	ATP hydrolysing enzyme
β-MHC	β-Myosin heavy chain
BECN1	Beclin-1
BCl-1/2	B-cell leukemia/lymphoma ½
BSA	Bovine serum albumin
Ca^{2+}	Calcium

cAMP	3',5'-Cyclic adenosine monophosphate
CapZ	Protein that caps the barbed end of actin to the Z-band
CARP	Cardiac ankyrin-repeat protein
Cas	Crk-associated substrate
Caspase	Cytosolic aspartate residue-specific cysteine protease
CaR	Calcium-sensing receptor
CH	Cardiac hypertrophy
CHAPS	3[(3-Cholamidopropyl)dimethylammonio]-propanesulphonic acid
CHO	Chinese hamster ovary
CICR	Ca^{2+}-induced Ca^{2+} release
CLSM	Confocal laser scanning microscopy
CMA	Chaperone-mediated autophagy
CrP	Creatine phosphate
CsA	Cyclosporin A
CytoD	Cytochalasin D
DAG	Diacylglycerol
DAPI	4',6-Diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EC_{50}	Half maximum effective concentration
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FHL	Four and a half LIM domain
FITC	Fluorescein isothiocyanate
G-actin	Globular actin
GDP	Guanosine diphosphate
GJ	Gap junctions
GPCRs	G protein-coupled receptors

GTP	Guanosine triphosphate
HBSS	Hank's balanced salt solution
H9c2	A clonal cell line derived from embryonic rat ventricle
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HSP	Heat shock protein
I_{Ca-L}	L-type calcium channel
ID	Intercalated disk
IF	Intermediate filament
IκB	NF-κB inhibitor
IP_3	Inositol 1,4,5-triphosphate
I/R	Ischaemia/reperfusion
IRE	Inositol-requiring enzyme-1
JC-1	5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide
JNK	c-Jun NH_2-terminal protein kinase
K^+	Potassium
kDa	Kilo dalton
LC3	Light chain 3
LDH	Lactate dehydrogenase
LSCM	Laser scanning confocal microscopy
LVEDP	Left ventricular end diastolic pressure
MADS	Consists of genes with a conserved region of approximately 182 bp that codes for a DNA binding domain-the MADS-box
MAPK	Mitogen-activated protein kinase
MAPKKKs	MAP kinase kinases
MARP	Muscle ankyrin-repeat protein
mDa	Mega dalton
$\Delta\Psi_m$	Mitochondrial membrane potential
MHC	Myosin heavy chain
MLC1	Myosin light chain 1
MLP	Muscle LIM protein
3MA	3-Methyladenine

MPTP	Mitochondrial permeability transition pore
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MURF	Muscle-specific ring finger protein
MyBP-C	Myosin-binding protein C
NAD^+	Nicotinamide adenine dinucleotide
NEC-1	Necrostatin 1
NCX	Na^+/Ca^{2+} exchanger
NF-κB	Nuclear factor kappa beta
NO	Nitric oxide
OXPHOS	Oxidative phosphorylation
PAK1	p21-Activated kinase
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PERK	Protein kinase R-like ER kinase
PEVK	Proline (P), glutamate (E), valine (V) and lysine (K) region
Pi	Inorganic phosphate
PIK3	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB/Akt	Serine/threonine protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
PQC	Protein quality control
PSV	Polyamine-sequestering vesicles
RIP1	Receptor-interacting protein 1
RNCM	Rat neonatal cardiomyocytes
ROCK	Rho-dependent kinase
ROS	Reactive oxygen species
RYR	Ryanodine receptor
S100A1	S100 calcium binding protein A1
SER	Serine

SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
siRNA	Silencing RNA
SR	Sarcoplasmic reticulum
SRF	Serum response factor
T-cap	Telethonin
TEM	Transmission electron microscopy
THR	Threonine
TMRM	Tetramethylrhodamine methyl ester perchlorate
TN	Troponin
TNF	Tumor necrosis factor
TNT	Troponin T
TPM	Tropomyosin
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system