Investigating Novel Caveolar Protein Interactions in Cardiovascular Redox Signalling

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, ***Seyed Mojtaba Moosavi,*** declare that this thesis is submitted in fulfilment of the requirements for the award of ***Doctor of Philosophy*** in the *School of Life Sciences/of the Faculty of Science* at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

I certify that the work in this thesis has not previously been submitted for a degree, nor has it been submitted as part of the requirements for a degree at any other academic institution except as fully acknowledged within the text. This thesis is the result of a Collaborative Doctoral Research Degree program with Kolling Medical Research Institute, University of Sydney.

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ABSTRACT

Background: Cardiovascular disease (CVD) is a chronic disorder developing perniciously during life and usually progressing to an advanced stage by the time symptoms occur. CVD has been listed as the most common non-communicable disease globally. Notwithstanding some decline, CVD remains the principal cause of death in both developing and developed countries. Despite some recent success, current therapeutic methods are not efficient enough to prevent CVD, so it is essential to look for a novel therapeutic approach to preclude mortality and morbidity caused by CVD. FXYD1 protein is abundant in the heart and is known to protect cardiac sodium-potassium ATPase from oxidative stress. Nevertheless, little is known about the interaction of FXYD1, which is localised in caveolae, with other caveolae resident proteins in the heart or the role of the FXYD1 in other cardiovascular tissues. Our lab has recently demonstrated that FXYD1 protein, which is located in the invaginations of the plasma membrane called caveolae, protects eNOS from dysregulated redox signalling in the vasculature, making it a potential therapeutic target for vascular diseases.

Methods and Results: In this project, we first aimed to investigate the role of FXYD1 in cardiac and vascular redox signalling in several models of cardiovascular disease, including atherosclerosis, diabetes, and hypertension. For this project's aim, FXYD1 knock out mice, which exhibit enhanced oxidative stress and are prone to subtle increased cardiac dysfunction under normal conditions, were used. In the first instance, I examined the cardiac and vascular expression of redox signalling proteins by immunoblotting. Overall, there appeared to be some protection from oxidative stress by the presence of FXYD1. Heart and vascular tissues were obtained from atherosclerosis-prone apolipoprotein knockout (ApoE KO) mice crossed with FXYD1 wildtype and knockout mice to examine the role of FXYD1. ApoE KO / FXYD1KO males had lower NOX2 protein expression, while females had higher eNOS. In hypertensive mice, which was induced by chronic angiotensin 2 infusion, the expression level of Prdx6 in mesentery vascular tissues in FXYD-1 KO mice was significantly decreased. In the diabetic mice, which was induced by injection of pancreatic beta-cell toxin, streptozotocin and a long-term high-fat diet, the expression level of glutaredoxin 1 (GLRX-1) and eNOS in heart tissues in FXYD-1 KO mice was significantly increased. The only pattern emerging from these three models was a propensity for modified eNOS expression. Taken together with findings from a parallel study in the laboratory (in appendix) indicating a functional

interaction of FXYD1 with eNOS, I proceeded to focus on the caveolae subcellular region, a known hotspot for both eNOS regulation and oxidative signalling.

Firstly, I examined the impact of FXYD1 on caveolae morphology using electron microscopy analysis of sections of the heart from FXYD1 +/+ and -/- mice. The results of electron microscopic images showed the caveolae were denser in FXYD1 KO heart tissue, and the diameter and circumferences significantly decreased. I next aimed to determine the interaction of FXYD1 with other caveolae resident proteins and compared this to whole heart preparations using proteomics analysis. The results of cell and molecular biology studies showed that the protein expression of FXYD1 in mouse hearts was highest in caveolae subfractions (4-6) compared with other sub-fractions, which agrees with those studies that demonstrated the FXYD1 protein localized in caveolae. The results of whole heart unbiased proteomics showed that 11 proteins were considerably upregulated, although none of these were typical redox signalling proteins; rather they fall mainly within the haemostasis, immune system, metabolism, and transportation of small molecules groups. In addition, 61 proteins were significantly down-regulated in whole hearts of FXYD1 KO mice, including peroxiredoxin 5 (Prdx5), which acts as a cytoprotective antioxidant enzyme in inflammation and Phospholamban (Pln) which has a vital role in calcium homeostasis in the heart muscle.

Remarkably, from the isolated caveolae sub-fractions (fractions 4 and 5 combined), 139 proteins were upregulated, and 39 proteins were significantly downregulated in FXYD1 KO mice compared with WT. That these 139 proteins were upregulated in the caveolae fractions suggests a potential accumulation or translocation of these proteins to the caveolae. Of these, the most common signalling pathway affected were complex I biogenesis and respiratory electron transport. On the other hand, 39 proteins were uniquely down regulated in FXYD1 KO mice's caveolae, which may have contributed to the disease phenotype. Within the caveolae subfractions, glutathione peroxidase 1(Gpx1), which is an antioxidant enzyme counteracting oxidative stress, and apolipoprotein A-I (Apoa1), which participates in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase, apolipoprotein C-I (ApoC I), which is an inhibitor of lipoprotein binding to LDL and catalase (Cat), which is involved in redox signalling, were significantly upregulated in FXYD1 KO mice. In addition, glutathione synthetase (GSS), a redox-signalling proteins, was upregulated in caveolae, whilst down-regulated

proteins included glutaredoxin-3 (Glrx3), a critical negative regulator of cardiac hypertrophy and thioredoxin (Txn), which has a critical role in the reversible S-nitrosylation of cysteine residues in target proteins thereby contributing to intracellular nitric oxide response.

Given the key changes to lipoprotein signalling proteins, I utilised our uniquely established mouse line of FXYD1 KO mice on the atherosclerosis-prone apolipoprotein E KO background to assess the functional impact on atherosclerosis development. Here I demonstrated that FXYD1 was involved in regulating body weight in male mice but had minimal effect on plaque development. Interestingly, FXYD1 appeared pro-inflammatory and detrimental to cholesterol metabolism, as FXYD1 KO mice had lower circulating total cholesterol and increased circulating IL-1ß. This pro-inflammatory phenotype was restricted to females, hinting at a potential unique mechanism involved in the gender differences seen in our clinics' as to cardiovascular adverse events. As inflammation is a critical driver of plaque rupture, I also examined the impact of FXYD1 absence on plaque stability using the established carotid artery tandem stenosis protocol. However, no changes were evident in either necrotic core or fibrous cap thickness in FXYD1$^{-/-}$ vs. $^{+/+}$ mice on ApoE KO background. Therefore, I conclusively showed that whilst FXYD1KO mice were prone to inflammation, they were not at increased risk of plaque development or rupture.

Conclusion: This thesis's overall findings are that FXYD1 appears to be protective against oxidative damage and proinflammatory. Overall, cardiovascular disease's effect appears to be balanced with no change in atherosclerosis plaque development or stability, whether FXYD1 was present or absent. Some of the changes in atherosclerotic mice were sex-dependent. Future studies may investigate targeting FXYD1 in specific sub-cellular regions such as the caveolae, which are redox hotspots, to lower oxidative stress without causing inflammation.

PUBLICATIONS

Lo, C. C. W., **Moosavi, S.M**., Bubb, K.J. (2018). "The Regulation of Pulmonary Vascular Tone by Neuropeptides and the Implications for Pulmonary Hypertension." Front Physiol 9: 1167.

Bubb, K.J., Tang, O., Gentile, C., **Moosavi, S.M.,** Hansen, T., Liu, C.C., Di Bartolo, B.A. & Figtree, G.A. 2021, 'FXYD1 Is Protective Against Vascular Dysfunction', *Hypertension*, p. HYPERTENSIONAHA12016884.

PRESENTATIONS

S.M.Moosavi, D.van Reyk, B.Di Bartolo, O.Tang, K.J.Bubb, G.A.Figtree, FXYD1 is associated with a female-specific pro-inflammatory and hypercholesterolemic environment: Implications for Atherosclerosis, American Heart Association's annual Scientific Sessions conference, Nov.13-17th 2020, Virtual (Poster Presentation)

Seyed Mojtaba Moosavi, Belinda Di Bartolo, Owen Tang, Kristen J Bubb and Gemma Figtree, FXYD1 may be a new signalling protein in cholesterol metabolism or handling in a mouse model of atherosclerosis, New Horizon 2019, Nov. 14-15th 2019, Sydney, Australia (Oral Presentation)

Seyed Mojtaba Moosavi, Belinda Di Bartolo, Owen Tang, Kristen J Bubb and Gemma Figtree, FXYD1 is associated with lower circulating cholesterol in a mouse model of atherosclerosis, Australian Society of Atherosclerosis Meeting, Oct. 16-19th 2019, Melbourne, Australia (Finalist) (Oral Presentation)

Table of Contents

ABBREVIATIONS

5-LO	5-lipoxygenase
ALA	Alpha-lipoic acid
AGEs	Advanced glycation end-products
Akt	Protein Kinases B
AngII	Angiotensin II
ANOVA	Analysis of variance
ApoE	apolipoprotein E
BH_4	Tetrahydrobiopterin
CAT	Catalase
Cav-1	Caveolin 1
Cav-2	Caveolin 2
Cav-3	Caveolin 3
CHD	Coronary Heart Disease
CKD	Chronic Kidney Disease
CoQ	Coenzyme Q
CoQ10	Coenzyme Q10
CVD	Cardiovascular Disease
DALYs	Disability Adjusted Life Years
DHLA	Dihydrolipoic acid
DNA	Deoxyribonucleic Acid
eNOS	endothelial Nitric Oxide Synthetase
EDRF	Endothelium-Derived Relaxing Factor
EGF	Epidermal growth factor
FDR	False Discovery Rate
FH	Familial Hypercholesterolaemia
GPX	Glutathione Peroxidase
GSH	Glutathione
GSS	Glutathione synthetase
HDL	High-Density Lipoprotein
H&E	Hematoxylin and eosin
HT	Hypertension
IP	Intraperitoneal
IS	Indoxyl sulphate
IHD	Ischemic Heart Disease
LDL	Low-Density Lipoprotein
LDL-c	Low-Density Lipoprotein cholesterol
LOX	Lipoxygenase
MAPK	Mitogen-activated protein kinase
Mn-SOD	Manganese superoxide dismutase
MPO	Myeloperoxidase
MURC	Muscle-restricted coiled-coil
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
ox-LDL	Oxidized low-density lipoprotein
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TGF-β	Transforming growth factor beta
TM	Melting Temperature
RAS	Renin-Angiotensin System

RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SDPR	Serum deprivation-response protein
SFK	Src family kinase F
STZ	Streptozotocin
SOD	Superoxide dismutase
PCR	Polymerase chain reaction
PAH	Pulmonary arterial hypertension
PKA	Protein Kinases A
PKC	Protein Kinases C
PLM	Phospholemman
PLN	Phospholamban
PTRF	Polymerase I and transcript release factor
UA	Uric acid
VSMCs	Vascular smooth muscle cells
WHO	World Health Organisation
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase

SYMBOLS

>	greater than
<	less than
α	alpha
β	beta
Δ	delta, change in
↑	increase
↓	decrease
↔	no change

UNITS OF MEASUREMENT

d	day
g	gram
hr	hour
L	litre
M	molar
m	milli
Min	minutes
mol	moles
n	nano
Pa	Pascal
Sec	second
V	volts
°C	degrees Celsius
%	percent
µ	micro

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