EFFECTIVENESS OF CINACALCET AND DDAH2 OVEREXPRESSION ON INCREASING SYNTHESIS OF NITRIC OXIDE IN RAW 264.7 CELLS

MIREN SUMMERS

INTRODUCTION

NITRIC OXIDE AND BLOOD PRESSURE

A major risk factor to the development of cardiovascular disease (CVD) is prolonged hypertension (Hermann, Flammer and Lscher, 2006). Nitric oxide (NO) is an important regulator of blood pressure; binding to soluble guanylate cyclase (sGC) results in relaxation of vascular smooth muscle and vasodilation (Sürmeli, Müskens and Marletta, 2015) (Berg et al., 2015). Reduced bioavailability of NO coupled with increased inflammation are characteristics of a state known as endothelial dysfunction, which is often a precursor to hypertension and atherosclerosis (Hermann, Flammer and Lscher, 2006). The integrated role of inflammation in the progression of heart disease directs investigation to nitric oxide synthesis in the innate immune cells, specifically macrophages.

CONTROL OF NITRIC OXIDE SYNTHESIS

Lipopolysaccharides (LPS) are cell wall components of gram-negative bacteria that come into contact with the innate immune system via TLR4, CD14 and MD2 cell surface receptors. This begins a signalling cascade that results in the synthesis and secretion of inflammatory cytokines and nitric oxide (Knirel and Valvano, 2011).

Calcium sensing receptor (CaSR) is a G-protein-coupled receptor (GPCR) expressed on the surface membrane of macrophages. Inflammatory cytokines TNFα, IL-6 and IL-β, interact with promoters on the *CASR* gene, resulting in protein synthesis (Hendy and Canaff, 2016). Activation of nitric oxide synthase enzymes occurs downstream of pathways initiated by calcium binding to CaSR. Inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) are both synthesised by macrophages and catalyse the formation of nitric oxide (Forstermann and Sessa, 2011) (Erkens et al., 2017). Cinacalcet is a type II calcimimetic; it allosterically binds to CaSR, changing it's conformation and increasing the receptor's sensitivity to Ca^{2+}, thereby lowering the threshold for initiation of the pathways resulting in NO synthesis (Bover et al., 2015).

Dimethylarginine dimethylaminohydrolase (DDAH) regulates methylarginines which inhibit NO synthesis, the enzyme is present in 2 isoforms, DDAH1 and DDAH2; higher concentrations of the protein DDAH are associated with an increase in NO (Pérez-Hernández et al., 2014). One such methylarginine is asymmetric dimethylarginine (ADMA). At normal physiological concentrations, it binds to CaSR, sensitising it to calcium and promoting downstream nitric oxide synthesis. Pathophysiological levels, associated with CVD, competitively inhibit nitric oxide synthase enzymes, decreasing intracellular NO concentration (Dowsett et al., 2019).

This report will investigate if the use of cinacalcet on macrophages activated with LPS, results in a significantly greater concentration of nitric oxide and the proteins relevant to its synthesis and moderation. It will also aim to establish if overexpression of DDAH2 by macrophages results in significantly higher nitric oxide and relevant protein concentration when treated with cinacalcet, compared to normal levels of expression and activated cells untreated.

METHODS

CELL CULTURING

RAW 264.7 and RAW 264.7-DDAH2 overexpressing macrophages were cultured using Thermo Fisher DMEM with high glucose and GlutaMAX™ supplemented with 10% FBS (Invitrogen) and Pen Strep

(Gibco), DDAH2 overexpressing cells maintained in above media with 100µg/ml Hygromycin. $4x10^6$ cells/ml were plated into 24 well plates and cultured for 24 hours at 37°C. Untreated cells received DMEM high glucose with GlutaMAX™ with 10% FBS. Activation was achieved using 1µg/ml S. Typhosa LPS and 100ng/ml Tocris Biosciences cinacalcet was administered. Cells were treated for 6 or 24 hours at 37°C before harvesting.

GRIESS ASSAY

A Griess assay was performed to measure the concentration of nitrite secreted into the growth media from cells post-treatment. The media from each of the wells was removed, replicates were pooled in pairs producing 12 samples and solutions of 40µl/ml sample in MilliQ water were made. An Invitrogen™ Molecular Probes™ Griess Reagent Kit was used. The Griess reagent was made by combining equal volumes of N-(1-naphthyl)ethylenediamine and sulfanilic acid. Standards at µM nitrite concentrations 100, 80, 60, 40, 20, 10, 5 and 1 were made using a 1mM stock of sodium nitrite and MilliQ water. On a 96 well microplate, 75µl of standard or sample, 65µl of MilliQ water and 10µl reagent was plated out in duplicate and incubated for 30 minutes. Absorbance at 560nM was measured using a Perkin Elmer Victor X3 microplate reader.

EXTRACTION

Cell lysate was collected from half of the plated cells after removal of the media for western blotting. 500µl of PBS was used to wash each 1ml well, followed by addition of 100µl of ice cold 100mM Tris pH8.0, 1% TritonX100 containing Roche protease inhibitor and PMSF (0.2mM final). The cell lysate was triturated and pooled in an eppendorf in pairs of the same treatment and cell type. The samples were centrifuged for 5 minutes at 14000rpm at 4°C and the supernatant harvested.

RNA was collected from the remaining cells using a Qiagen RNeasy mini kit. The wells were washed with 500µl of PBS, and 175µl of Qiagen Buffer RLT was added to the wells. The contents of the wells were triturated and pooled in pairs. 300µl 70% ethanol was added to the sample before being transferred into a RNeasy mini spin column with a 2ml collection tube. The sample was spun for 15 seconds at 8000rpm and the flow through discarded. The process was repeated with 700µl of Qiagen Buffer RW1, followed by 500µl Qiagen Buffer RPE. 500µl Qiagen Buffer RPE was then added and spun for 2 minutes at 8000rpm and the flow through discarded. The column was placed in a new 2ml collection tube and spun for 1 minute at 14000rpm to dry the membrane. 30µl RNase-free water was added to the column, which was placed in a 1.5ml collection tube and spun for 1 minute at 8000rpm to elute the RNA. This was repeated once and the flow through kept.

PROTEIN ASSAY

A protein assay was performed on the cell lysate using the Bio-Rad protein assay protocols to attain protein concentrations for each sample. Standards were made up using dH_2O and bovine serum albumin at concentrations 800, 600, 500, 400, 300, 200, 100 and 50µg/ml. Samples were diluted with dH_2O to a concentration of 40µl/ml. 200µl of 200µl/ml Bio-Rad Protein Assay Dye Reagent and 20µl of sample or standard was plated out in duplicates on a 96 well microplate. Absorbance at 595nM was measured using a Perkin Elmer Victor X3 microplate reader and the data was analysed using the Prism software package.

SDS-PAGE

The proteins in each sample were separated by molecular weight using SDS-PAGE. 10% and 12.5% acrylamide gels were made. Bio-Rad Precision Plus Protein Standards were used and data from the protein assay was used to load 30µg of protein into each well. Standard protocols were followed.

WESTERN BLOT

A Western blot was used to identify the presence and quantity of the proteins DDAH2, CaSR, iNOS and eNOS. Protein was transferred onto PVDF membrane in Tris glycine 20% methanol transfer buffer. The membrane was blocked for an hour in Thermo Scientific™ SEA BLOCK blocking buffer. Blocking agent was comprised of 30% SEA BLOCK in PBS+0.1%Tween$_{20}$. 1µg/ml primary antibodies, rabbit: DDAH2, iNOS, CaSR, mouse: eNOS, β-actin, were suspended in a solution of 300µl/ml blocking agent and 700µl/ml PBS+Tween$_{20}$. The transfer was incubated in 5ml of the antibody solution overnight at 4^{o}C with shaking. The membrane was washed with PBS TWEEN 4-5 times. The transfer was placed into 100ng/ml secondary antibody, anti-rabbit or mouse respectively, in 300µl/ml blocking agent and 700µl/ml PBS+Tween$_{20}$, for 1 hour in dark at room temperature. 2-4 more washes with PBS+Tween$_{20}$, and one with PBS no Tween$_{20}$, were carried out before imaging using a LI-COR Odyssey CLX and the software package ImageStudio (LI-COR Biosciences, NE, US).

qPCR

NanoDrop™ quantitation determined RNA concentration and quality in the elutions, 260/280: 2.12(0.03), 260/230: 1.71(0.19). Applied Biosystems™ High-Capacity cDNA kit was used to prepare samples for PCR. The 2X reverse transcription master mix was prepared without RNase inhibitor according to the protocols provided. 1.8µg RNA and 10µl of master mix was added to each tube, and the volume made up to 20µl with RNase-free water. Thermal cycling conditions were 25^{o}C – 10 minutes, 37^{o}C – 120 minutes, 85^{o}C – 5 minutes, 4^{o}C – until removed. NanoDrop™ calculated a yield of 4.15(0.71)µg cDNA. 0.2µl of cDNA from each tube was diluted in 1.8µl MilliQ water and added to a well (96 well microplate) with 5µl Applied Biosystems™ Fast SYBR master mix, 0.2µl forward primer, 0.2µl reverse primer and 3.6µl MilliQ water. Forward and reverse primers for β-actin, DDAH1, DDAH2, iNOS, eNOS, CaSR, IL-6 and TNF-α were used, each treatment was plated out in duplicate. Applied Biosystems™ Quantstudio 12K Flex was used to carry out qPCR.

STATISTICS

Statistical analysis was carried out using the software packages Prism (GraphPad Inc, CA, USA) and RStudio (RStudio Inc, MA, US). Analysis of variance, Tukey testing and Welch two sample t-testing was used on data normally distributed. A significance value of $p<0.05$ was accepted.

RESULTS

NITRITE

A Griess assay was performed to measure nitrite produced as a result of NO. To ensure levels of NO were detectable, cells were incubated under treatment for 24 hours release the results can be seen in figure 1b and 1d. Analysis of variance confirmed a causal relationship between treatment and nitrite concentration in both cell lines ($n=81$, $p<0.01$). Data varied greatly at 24 hours; t-testing found the average difference in nitrite between RAW 264.7 and RAW264.7-DDAH2 to be insignificant. Both cell lines exhibited significantly greater growth media nitrite concentrations when activated with LPS. Although treatment with cinacalcet produced the highest nitrite concentrations on average, the difference compared to the LPS only treatment was not statistically significant. Data was blocked for the date the assay was completed on to reduce variance; however, the blocks were too small to perform statistical testing.

The decision was made to measure growth media nitrite at a shorter incubation period of 6 hours to investigate if DDAH2 overexpression and cinacalcet had an effect on the rate of NO release, results are presented in figure 1a and 1c. ANOVA demonstrated a statistically significant relationship between treatment and nitrite at 6 hours also. From two sample t-testing, the difference in growth media nitrite

between the cell lines, with every treatment, was found to be significant, with higher concentrations observed in cells overexpressing DDAH2. Cinacalcet treated cells showed less nitrite variation and greater average difference between cell lines than those only activated with LPS, however, Tukey post-hoc testing found the difference wasn't significant and standard error overlapped.

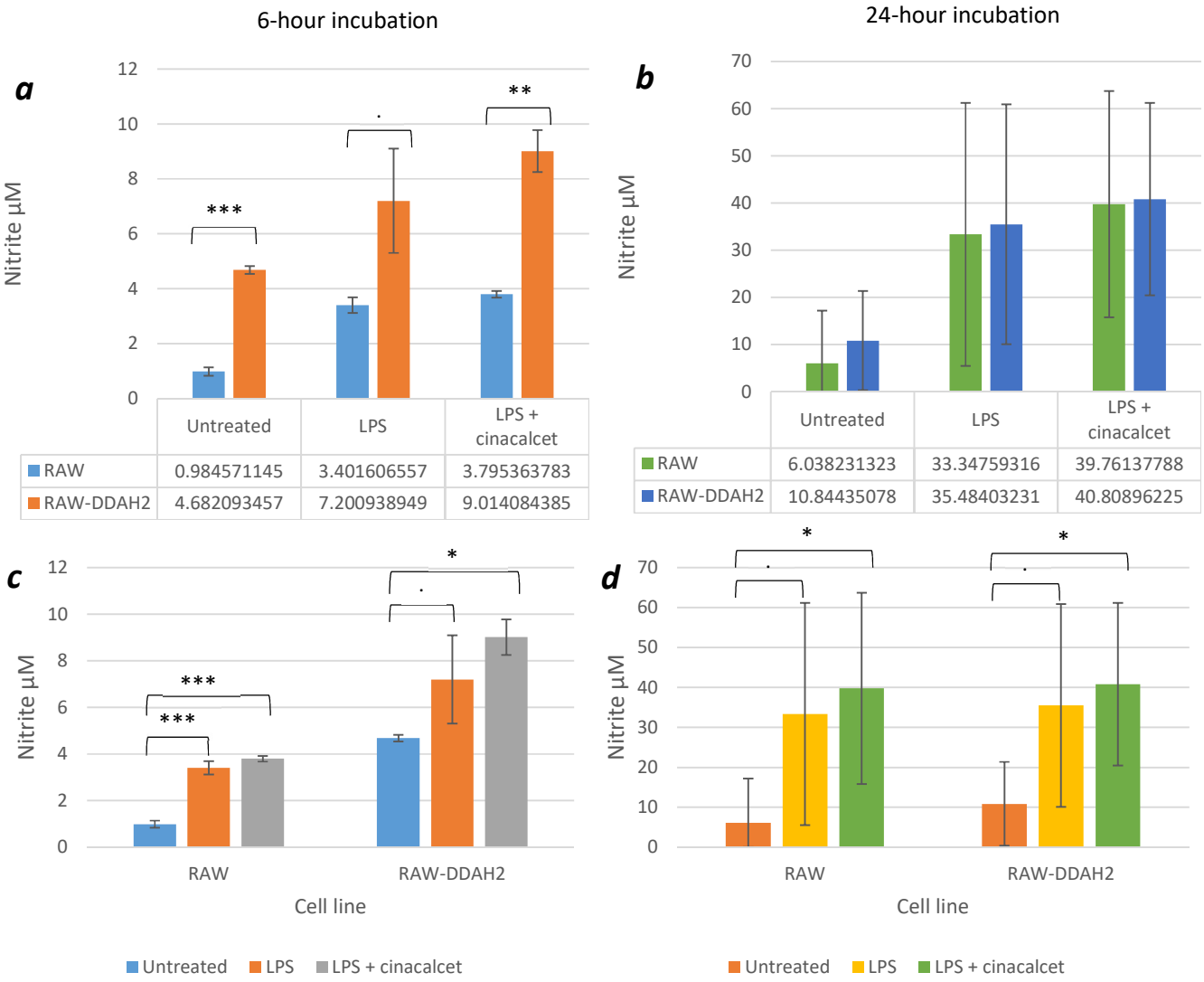


Figure 1: a/b *Comparison between cell lines, of the effect of LPS and cinacalcet on growth media nitrite concentration* ***a*** *6 hours incubation* ***b*** *24 hours incubation.* ***c/d*** *Comparison of media nitrite concentration between treatments on the same cell line* ***c*** *6 hours* ***d*** *24 hours. · - p<0.05*, * - p<0.01, ** p<0.001, *** p<0.0001. 6 hours n=24, 24 hours n=81

WESTERN BLOT

A blot was carried out on untreated RAW 264.7 cells to establish basal protein levels of RAW 264.7 cells (figure 2). DDAH2 was most prevalent, however the density varied the greatest of all the proteins. A second band at ~25kDa was observed in the lanes where eNOS was assayed for. No band was observed at 131kDa where iNOS was expected, however a faint band can be seen at ~55kDa. A dense band was observed at 60kDa and 20 kDa in the lanes CaSR was assayed in, along with a fainter band at the expected molecular weight (260kDa).

Due to insufficient protein load when carrying out the westerns, there was a lack of N numbers for statistical analysis (n=60). In the untreated cell lysate, iNOS was untraceable in both cell lines; as expected, activation resulted in expression. Analysis of variance and follow-up Tukey testing showed

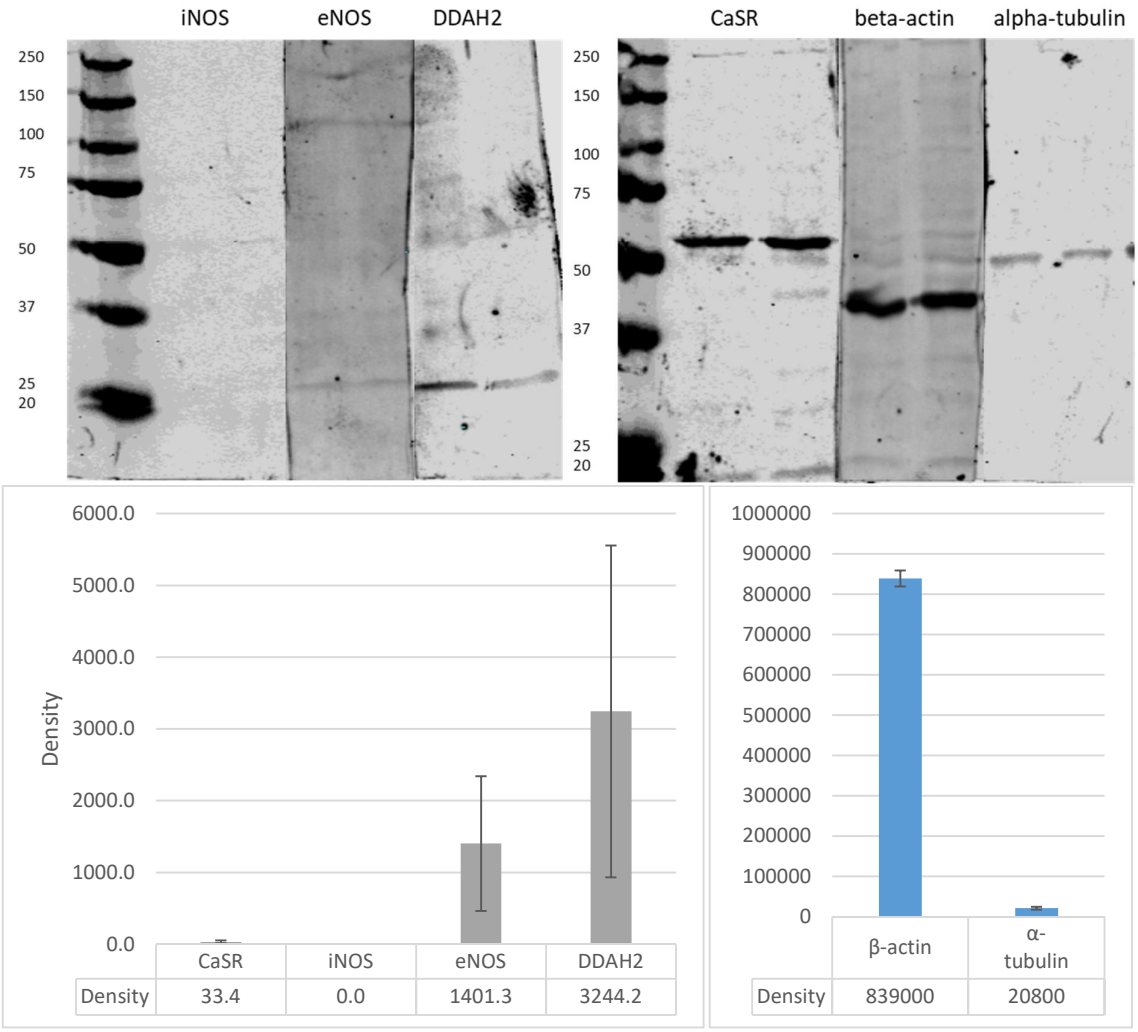


Figure 2: *Western blot of untreated RAW 264.7 cell lysate. Densitometry was normalised using housekeeping proteins β-actin and α-tubulin. Protein molecular weights CaSR dimer: 260kDa monomer: 130kDa, iNOS: 131kDa, eNOS: 133kDa, DDAH2: 30kDa.*

that cinacalcet did not statistically significantly impact iNOS concentration in either cell line (difference: 5.233, p=0.92), nor did DDAH2 overexpression (F=0.2008, p=0.68) (fig.3a) (LPS vs LPS+cinacalcet RAW 264.7: 49.18(38.48) vs 27.88(1.06), RAW 264.7-DDAH2: 48.71(15.51) vs 59.55(10.31)). CaSR expression appeared to be downregulated by LPS and more so by LPS and cinacalcet together, with highest concentration observed in untreated cells (RAW: 802.39(50.05), RAW-DDAH2: 925.85(37.02)), however, ANOVA did not find a statistically significant relationship between treatment and lysate CaSR concentration (F=5.0291, p=0.1101) (fig.3b). Average CaSR of RAW 264.7-DDAH2 cells was higher than RAW 264.7 but no causal relationship between DDAH2 overexpression and concentration was found (F=1.1384, p=0.3461). As expected, DDAH2 levels were highest in the cells that overexpressed the protein. In the RAW 264.7 cell line, levels were highest

when left untreated (48.56(653.33)); activation with LPS without the addition of cinacalcet produced the highest DDAH2 levels in the overexpressing cells (1422.09(70.31)) (fig.3c). The accuracy of data for the groups of RAW 264.7-DDAH2 incubated with LPS and LPS+cinacalcet is not accurate due to the band saturation (fig.3c). Significance testing showed no difference in observed DDAH2 between treatments (F=0.0104, p=0.9897), but the concentration significantly differed between the cell lines (F=225.35, p=0.0001147). No signal could be traced at 133kDa for eNOS, suggesting expression is unaffected by activation (fig.3d). The density of housekeeping gene β-actin was consistent (72.18(6.44)x10^4)(fig.3e).

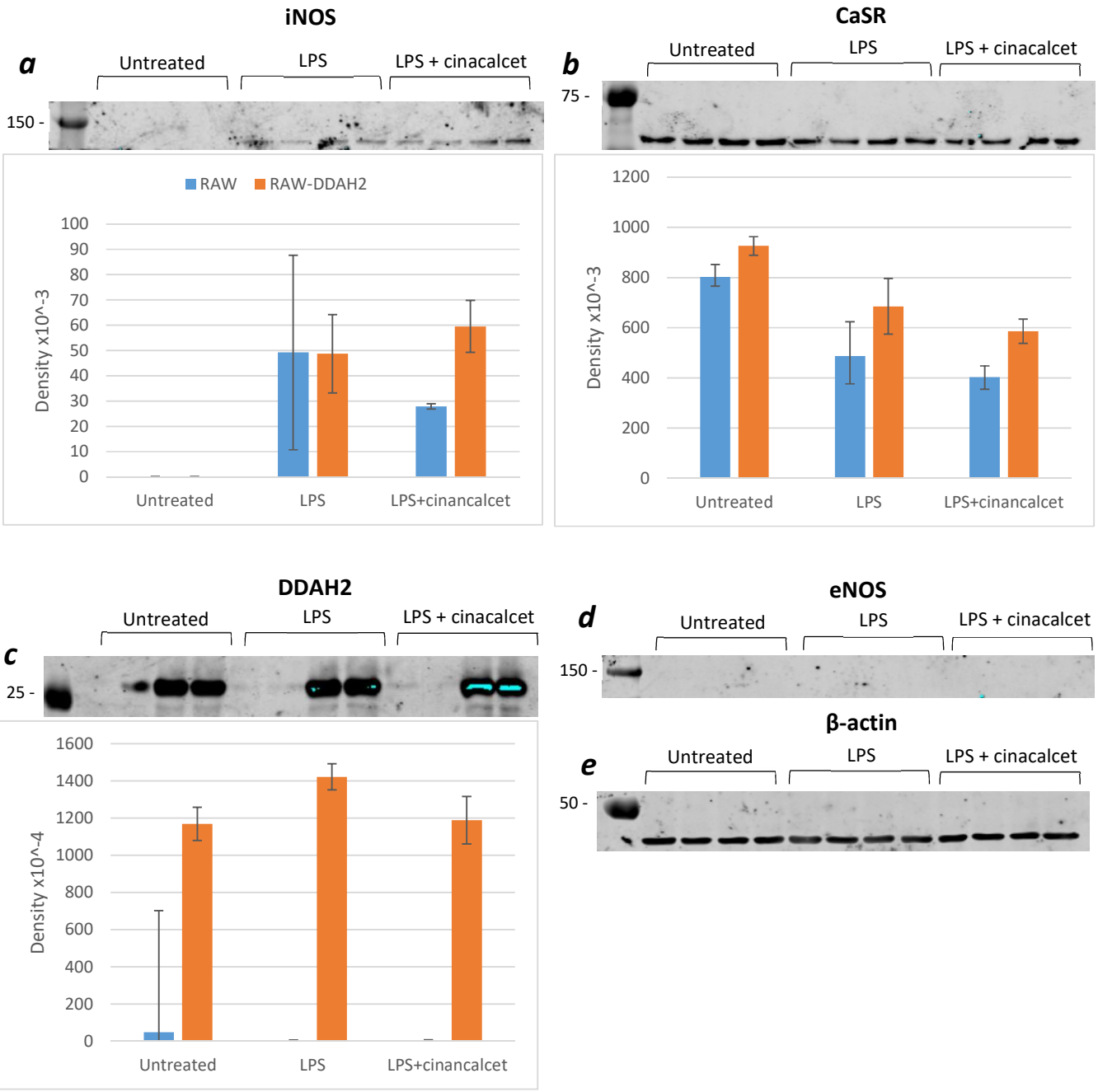
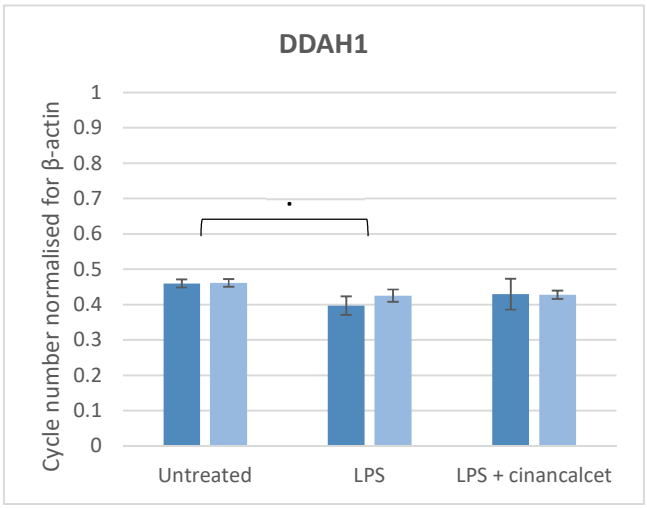
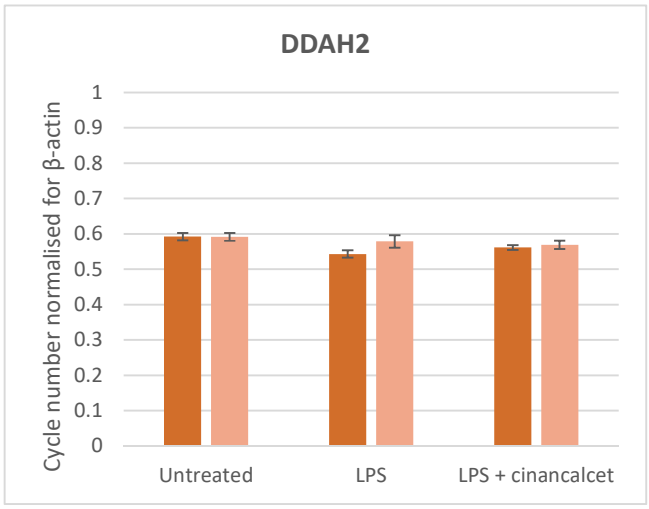


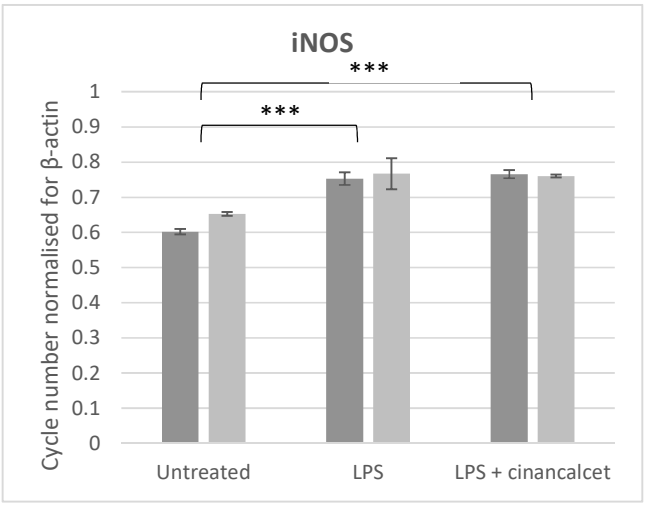
Figure 3: *Western blot and graphs showing densitometry normalised for β-actin* ***a*** *iNOS -131kDa* ***b*** *CaSR monomer – 130kDa* ***c*** *DDAH2 – 30kDa* ***d*** *eNOS – 133kDa* ***e*** *housekeeping gene β-actin*

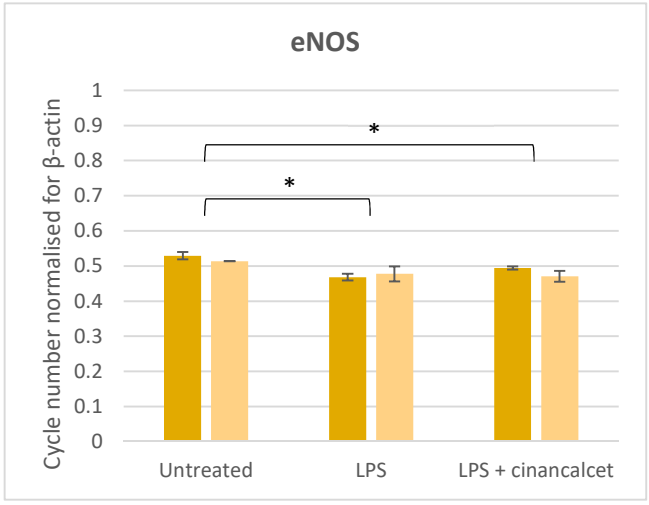
qPCR

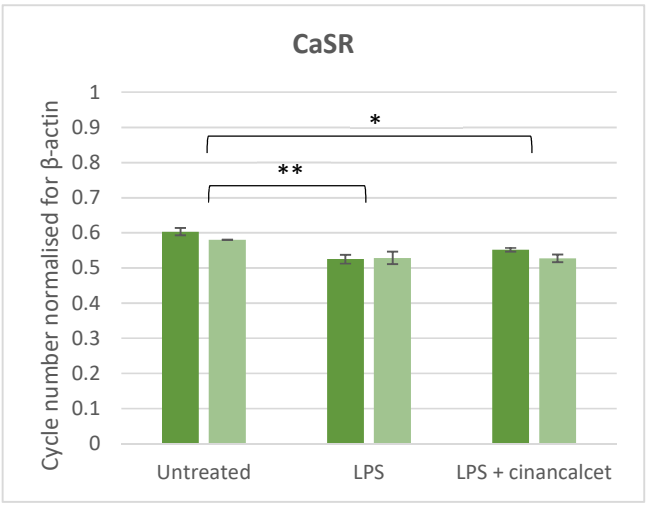
Expression of proteins after 24 hours of incubation, investigated as a proportion of β-actin is shown in figure 4. From the qPCR performed, analysis of variance and follow-up Tukey testing, treatment with cinacalcet and overexpression of DDAH2 did not statistically significantly increase expression of any of the proteins ($n=186$, $p>0.05$). Highest DDAH1 expression was observed in the untreated groups; treatment with LPS alone produced a significant decrease in expression (untreated – LPS: $p=0.0232$), LPS and cinacalcet together resulted in greater expression on average in both cell lines, however this difference was not significant ($p=0.4986$). A slight decrease in DDAH2 was observed, however testing found expression to be statistically similar in all groups ($p>0.05$). iNOS expression significantly increased when cells were activated (untreated – LPS: $p<0.0001$, untreated – LPS+cinacalcet: $p<0.0001$), however cinacalcet did not make a distinguishable difference to expression ($p=0.9816$). Activation with LPS resulted in a decrease in eNOS expression (untreated – LPS: $p=0.0024$, untreated – LPS+cinacalcet: $p=0.0091$), expression did not significantly differ with the addition of cinacalcet (LPS – LPS+cinacalcet: $p=0.6273$). A similar trend was also observed in CaSR (untreated – LPS: $p=0.0004$, untreated – LPS+cinacalcet: $p=0.0017$, LPS – LPS+cinacalcet: $p=0.4566$). As expected, IL-6 expression was significantly increased by incubation with LPS ($p<0.0001$); cinacalcet made no measurable difference to expression ($p=0.9997$). Expression of TNF-α differed between cell lines in each treatment. Both cell lines had similar levels of expression untreated. Activation with LPS alone resulted in a small increase in expression on average in the RAW 264.7 (0.8315) cells compared to the untreated (0.7877), whereas RAW 264.7-DDAH2 cells experienced a much greater increase (+0.102), however standard deviation in RAW 264.7 cells treated with LPS is much greater. RAW 264.7 expression of the protein increased when cells were incubated with cinacalcet, whereas the cells overexpressing DDAH2 saw a slight decrease on average. Significance testing found LPS alone did not statistically significantly increase TNF-α expression ($p=0.1065$), however, LPS and cinacalcet together did ($p=0.0124$). The difference induced by cinacalcet was not significant (0.298), however, due to a low N number, more testing is needed.

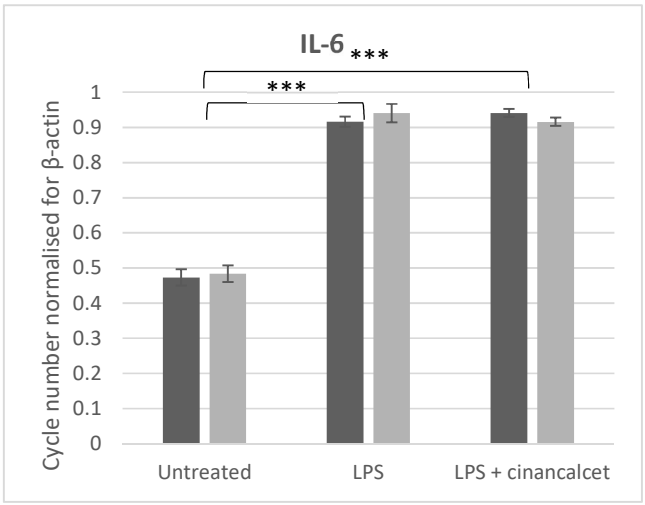

DDAH1
Cycle number normalised for β-actin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Untreated
LPS
LPS + cinancalcet


DDAH2
Cycle number normalised for β-actin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Untreated
LPS
LPS + cinancalcet


iNOS
***
***
Cycle number normalised for β-actin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Untreated
LPS
LPS + cinancalcet


eNOS
*
*
Cycle number normalised for β-actin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Untreated
LPS
LPS + cinancalcet


CaSR
*
**
Cycle number normalised for β-actin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Untreated
LPS
LPS + cinancalcet


IL-6
***
***
Cycle number normalised for β-actin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Untreated
LPS
LPS + cinancalcet

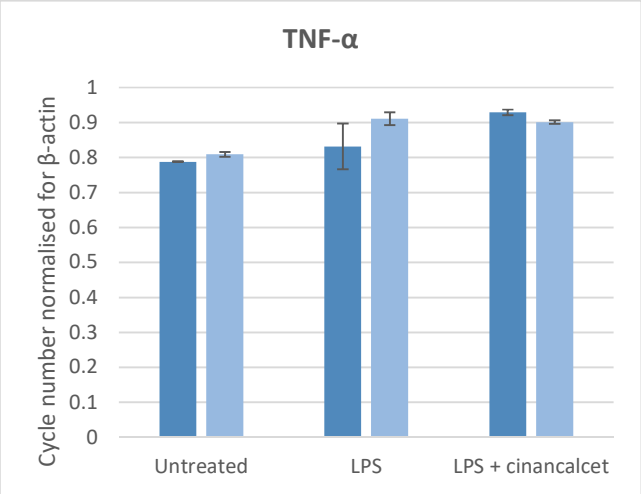


Figure 4: *Graphs of relative gene expression, qPCR cycle number at which threshold fluorescence was surpassed, normalised using β-actin. The first bar in each pair is RAW 264.7, the second is RAW 264.7-DDAH2.*

DISCUSSION

From this research, DDAH2 overexpression was found to significantly increase nitrite concentration at the 6-hour timepoint. This was not the case after a 24-hour incubation, where overexpression had no significant effect, which suggests that RAW 264.7-DDAH2 responded by producing NO faster when activated, resulting in greater levels early in the incubation period. Media nitrite in both cell lines was unaffected by cinacalcet at 6 and 24 hours, however only one concentration was assessed.

One Griess assay was performed on cells incubated for 6 hours where n=24 because no distinguishable difference was observed between cell lines and activated treatments in several assays after 24 hours. Due to lack of time, there wasn't opportunity to do replicates, making the likelihood of a false positive increased and precision decreased. With stronger evidence in favour of no significant association between DDAH2 expression and media nitrite concentration, provided by the four assays of 24-hour incubated cells, the hypothesis cannot be accepted without further research.

Future research could employ mass spectrometry for greater accuracy of measurement. Varying cinacalcet concentration and duration of exposure could impact observations at time points studied in this report. Cell media samples should be taken at more time points with replicates to achieve a comprehensive understanding of how cinacalcet and DDAH2 overexpression impacts nitrite concentration longitudinally.

Both DDAH1 and DDAH2 exhibited slight, but not significant, decreases in expression when macrophages were incubated with LPS for 24 hours compared to untreated (fig.4). A possible explanation for these observations is that the peak of expression occurred before 24 hours. Assuming concentration and transcription returned to pre-activation levels, this would also provide explanation for the statistical similarity in DDAH2 concentration between treatments (fig.3c). However, saturation in figure 3c affects the accuracy of the results; coupled with a lack of repeats, conclusions cannot be easily drawn from this data.

iNOS concentration and expression was significantly increased by LPS, however the same was not observed in treatment with cinacalcet. Griess assays demonstrated greater average difference between these two groups in nitrite concentration at 6 hours in comparison to 24 (fig.1), with higher values recorded in cell lines treated with cinacalcet, suggesting the rate of NO synthesis was impacted by cinacalcet. Nitric oxide synthase enzymes, responsible for the production of NO, are activated and synthesised in response to calcium and calcimimetics binding to CaSR. Presence of cinacalcet in the medium increases substrate availability, and subsequently iNOS and NO concentrations.

While previous research supports this theory (Forstermann and Sessa, 2011) (Erkens et al., 2017) (Bover et al., 2015), evidence collected in this report is insufficient to prove it. Difference in nitrite between LPS and LPS+cinacalcet groups wasn't found to be statistically significant. Damage and background noise in the area of the Western blot containing the iNOS bands was significant enough to make densitometry inaccurate; without repeats, this data cannot be relied upon. Measurements at more timepoints and with more repeats is required.

eNOS, despite having similar mechanisms of control to iNOS, did not exhibit the same increase in expression in either cell line when activated (fig.3e) (fig.4). The protein appeared in the western blot measuring basal concentration (fig.2) but was untraceable in the untreated lanes in figure 3d, suggesting an error in protocol or insufficient protein loading.

Cinacalcet, as predicted, did not significantly affect the expression of the cytokines IL-6 and TNF-α, however, LPS increased expression of both. Despite this cytokine response to LPS, which promotes transcription of the *CASR* gene, CaSR showed decreased expression at 24 hours (fig.4). Statistically similar or slightly decreased concentration and expression were also observed in DDAH1, DDAH2, and eNOS, when treated with LPS; contradictory to tested theories on their interaction with LPS, which predicted their levels to increase. Research by Dedrick et al. 1995, found that when LPS is administered in a single dose, as carried our in our study, expression peaked at 1-2 hours, after which plasma components neutralised the inflammatory response, and baseline levels of expression returned by 5 hours. This early peak in expression provides explanation to these observations.

The findings of this study suggest that a link between DDAH2 overexpression and increased rate of nitric oxide synthesis may exist; no such link was observed between with expression of the proteins studied. Cinacalcet did not appear to have a significant effect on nitric oxide synthesis or expression of the proteins relevant to the mechanisms of its control. However, the observations are promising and with further research, more can be discovered about the effect of cinacalcet and DDAH2 on nitric oxide release.

CITATIONS

Berg, J., Stryer, L., Tymoczko, J. and Gatto, G. (2015). *Biochemistry*.

Bobryshev, Y., Ivanova, E., Chistiakov, D., Nikiforov, N. and Orekhov, A. (2016). Macrophages and Their Role in Atherosclerosis: Pathophysiology and Transcriptome Analysis. *BioMed Research International*, 2016, pp.1-13.

Bover, J., Ureña, P., Ruiz-García, C., daSilva, I., Lescano, P., del Carpio, J., Ballarín, J. and Cozzolino, M. (2015). Clinical and Practical Use of Calcimimetics in Dialysis Patients With Secondary Hyperparathyroidism. *Clinical Journal of the American Society of Nephrology*, 11(1), pp.161-174.

Dedrick, R. and Conclon, P. (1995). Prolonged Expression of Lipopolysaccharide (LPS)-Induced Inflammatory Genes in Whole Blood Requires Continual Exposure to LPS. *Infection and Immunity*, 63(4), pp. 1362–1368

Dowsett, L., Duluc, L., Higgins, E. and Leiper, J. (2019). Asymmetric dimethylarginine is a posititve regulator of endothelial nitric oxide production by modulating calcium sensing receptor signalling. *Journal of Hypertension*, 37, p.e70.

Erkens, R., Suvorava, T., Kramer, C., Diederich, L., Kelm, M. and Cortese-Krott, M. (2017). Modulation of Local and Systemic Heterocellular Communication by Mechanical Forces: A Role of Endothelial Nitric Oxide Synthase. *Antioxidants & Redox Signaling*, 26(16), pp.917-935.

Forstermann, U. and Sessa, W. (2011). Nitric oxide synthases: regulation and function. European Heart Journal, 33(7), pp.829-837.

Hayashi, H., Hess, D., Zhang, R., Sugi, K., Gao, H., Tan, B., Bowles, D., Milano, C., Jain, M., Koch, W. and Stamler, J. (2018). S-Nitrosylation of β-Arrestins Biases Receptor Signaling and Confers Ligand Independence. *Molecular Cell*, 70(3), pp.473-487.e6.
Hendy, G. and Canaff, L. (2016). Calcium-Sensing Receptor Gene: Regulation of Expression. *Frontiers in Physiology, 7.*
Hermann, M., Flammer, A. and Lscher, T. (2006). Nitric Oxide in Hypertension. *The Journal of Clinical Hypertension*, 8, pp.17-29.
Knirel, Y. and Valvano, M. (2011). *Bacterial Lipopolysaccharides*. Vienna: Springer Vienna.
Eid, W., Dauner, K., Courtney, K., Gagnon, A., Parks, R., Sorisky, A. and Zha, X. (2017). mTORC1 activates SREBP-2 by suppressing cholesterol trafficking to lysosomes in mammalian cells. Proceedings of the National Academy of Sciences, 114(30), pp.7999-8004.
Pérez-Hernández, N., Vargas-Alarcón, G., Arellano-Zapoteco, R., Martínez-Rodríguez, N., Fragoso, J., Aptilon-Duque, G., Posadas-Sánchez, R., Posadas-Romero, C., Juárez-Cedillo, T., Domínguez-López, M. and Rodríguez-Pérez, J. (2014). Protective role of DDAH2 (rs805304) gene polymorphism in patients with myocardial infarction. Experimental and Molecular Pathology, 97(3), pp.393-398.
Sürmeli, N., Müskens, F. and Marletta, M. (2015). The Influence of Nitric Oxide on Soluble Guanylate Cyclase Regulation by Nucleotides. *Journal of Biological Chemistry*, 290(25), pp.15570-15580.

ACKNOWLEDGEMENTS

I'd like to thank James and Fiona Leiper for giving over so much time to teach and supervise me, the British Heart Foundation for their facilities, and the Scholars Scheme at the University of Glasgow for the amazing opportunity and funding.