University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. **Student**

   Surname: Gamwell Forename: Jonathan

   E-mail address: 2148458g@student.gla.ac.uk

2. **Supervisor:**

   Surname: Salt Forename: Ian

   E-mail address: ian.salt@glasgow.ac.uk

3. **Research Project Report**

   3.1 Project Title:

   The effect of experimental hyperglycaemia on eNOS interactions with protein kinases

   3.2 Project Lay Summary:

   Blood vessel dysfunction is often responsible for heart attacks and strokes in diabetic patients. The dysfunction relates to an inability of cells lining the blood vessels to produce a chemical, NO, which causes relaxation and hence opening up of the vessels. This happens during conditions of high blood glucose and also high insulin levels, as found in Type II diabetes but quite how it happens is unknown. The aim of this project is to study two targets in the cells which might be involved in the process and which might be future drug targets to counteract cardiovascular problems in diabetics.

   3.3 Start Date: 26/6/17 **Finish Date:** 4/8/17

3.4 Original project aims and objectives:

Taken together, the published and unpublished data from the Salt laboratory indicate that prolonged culture of human endothelial cells in insulin or glucose mimics the impaired NO synthesis observed in hyperinsulinaemia and diabetes respectively. The mechanism underlying these detrimental effects remains elusive, however. We hypothesise that impaired NO synthesis due to culture in high glucose is associated with altered eNOS interaction with regulatory protein kinases. The studentship will address the following research questions:

i. Does high glucose alter eNOS association of with the protein kinases Akt or AMPK?

ii. Does the activity of Akt or AMPK influence their association with eNOS?

3.5 Methodology:

Human Umbilical Endothelial Vein Cells (HUVECs) were cultured using standard sterile techniques. When ready, the cultures were treated with either AMPK or Akt inhibitors and activators and controls (see Figures 1a and 1b). Cells were lysed, collected and frozen. N=3 replicates of each treatment were prepared in this manner.

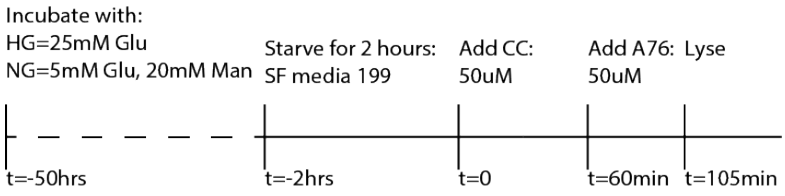

Figure 1a- *Timeline of preparation for AMPK experiments*

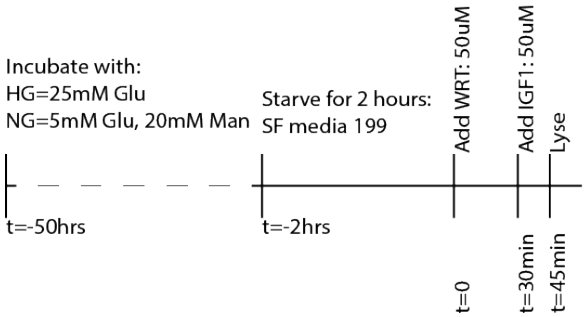

Figure 1b- *Timeline of preparation for Akt experiments*

Samples of cell lysates were prepared for co-immunoprecipitation of eNOS and any bound proteins by the following method. A Bradford assay was carried out to determine the volume of cell lysate required to yield 10µg of protein. Agarose beads conjugated to an eNOS antibody were incubated with those lysates overnight in order to bind the eNOS. Samples and controls were loaded onto SDS gels for Western blotting. The resultant membranes were probed and reprobed for eNOS, p-eNOS S1177, AMPK, AKT and Cav1 in both the Akt and AMPK sets. The original lysates were also analysed by Western blot, probing and reprobing for the following:

AMPK sets- AMPK, p-AMPK T172, ACC, p-ACC S79, AKT, p-AKT S473, Cav1, β-tubulin

AKT sets- AMPK, AKT, p-AKT S473, Cav1, β-tubulin

Membranes were scanned (LICOR) and the images assessed for relative protein density (Photoshop).

N=1 set of cells were prepared for confocal imaging to determine the degree of colocalisation of Cav1 and eNOS. The set was treated with controls and with AMPK activators and inhibitors. A further 3 sets were then examined for vehicle versus Compound C only. Slides were imaged using a confocal microscope and the images were assessed using the JACOP plug-in for Image J and analysed using the Pearson Coefficient of Correlation technique.

N=3 sets of human arterial endothelial cells (HAECs) were treated with AMPK activators and inhibitors along with insulin to determine the influence of these compounds and of hyperglycaemia on insulin-stimulated NO production by a direct NO assay using an NO measurement device.

The following techniques were all new to the student:

- All sterile culture and cell treatment techniques
- The preparation and transferring of gels and the incubation, washing and analysis of membranes.

- Use of confocal microscopy
- Use of NO assay equipment

3.6 Results:

Analysis of the Western blot images reveals that the amount of Cav1 bound to eNOS in untreated cells in high glucose is significantly ($p<0.001$) greater than in normal glucose conditions. Furthermore, the increase is significantly ($p<0.001$) more marked in the cells incubated (longer, see Figure 1) for the AMPK experiments than the Akt experiments.

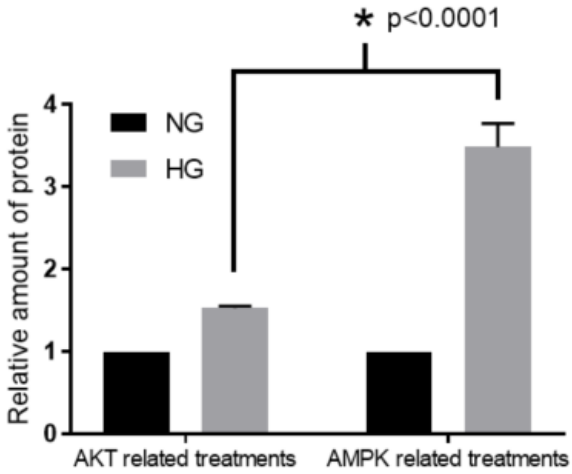

Figure 2- *Levels of Cav1 bound to eNOS in 5 sets of HUVECS post-incubation (normalised to normal glucose). Data presented as mean+/-SEM.*

When considering the treatments by 2-way ANOVA, Akt stimulation/ inhibition led to no significant difference between high and normal glucose in Cav1 binding to eNOS. AMPK stimulation/ inhibition tended towards a significant ($p=0.051$) increase in the high glucose sets but, contrary to visual inspection of the results, no significant statistical differences between the inhibitory Compound C and stimulating A76.

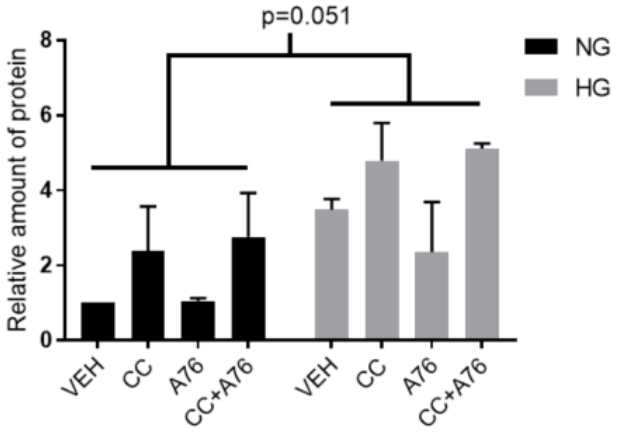

Figure 3- *Levels of Cav1 bound to eNOS in 3 (NG) and 2 (HG) sets of HUVECS post-treatment (normalised to normal glucose). Data presented as mean+/-SEM.*

No significant differences between high and normal glucose conditions were found for any of the other proteins which were probed for. These included pAMPK T172 and pACC S79, which might have indicated that AMPK activity was responsible for the variations in Cav1 binding in the AMPK sets.

The confocal imaging (Figure 4) showed no significant difference (p-0.5) between treatment with Compound C and the control, hence there is no evidence that Cav1 to eNOS binding is altered by the treatment.

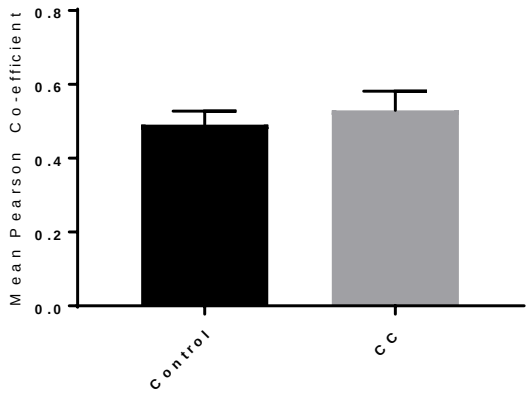

Figure 4- *Colocation of Cav1 and eNOS post treatment. Data presented as mean+/-SEM.*

The NO assay revealed that all treatments significantly ($p<0.001$) inhibit NO production, including high glucose incubation alone.

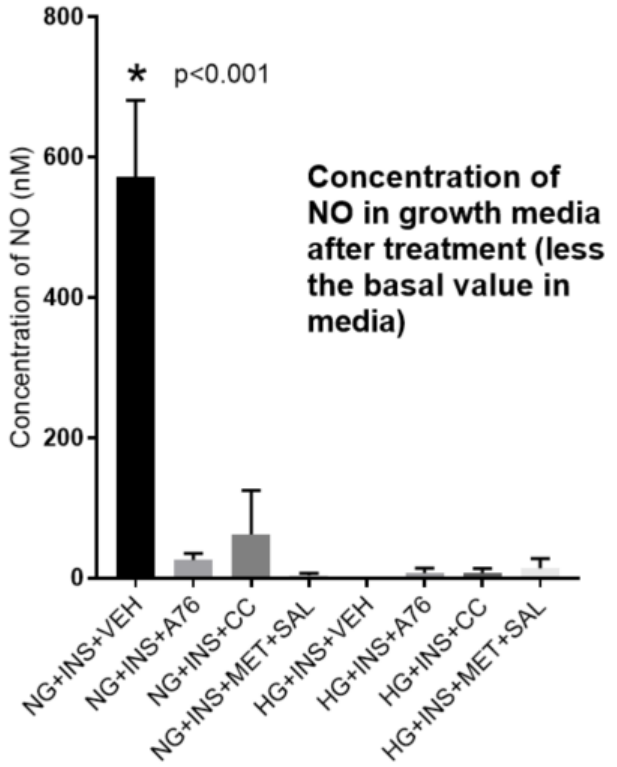

Figure 5- *NO production of HAECs post treatment. Data presented as mean+/-SEM.*

3.7 Discussion:

The results indicate that incubation of HUVECs for 48 hours in high glucose (25mM) results in significantly more binding of Cav1 to eNOS than incubation for 48 hours in normal glucose (5mM glucose and 20mM mannitol). Note that this concentration of glucose is not uncommon in pre-diabetic patients or patients with poorly-controlled diabetes. This primary result confirms those made by the Salt lab in the past and is significant because Cav1 has an inhibitory effect on eNOS and hence an inhibitory effect on vasodilation. The question, then, is whether this is mediated by the kinases Akt or AMPK or whether there is another modulating pathway(s).

The pattern of binding indicated in Figure 3, where AMPK inhibition by Compound C seems to increase binding and activation by A76 seems to hold or decrease binding is not supported by the ANOVA analysis. An initial suggestion of an increase in colocation with Compound C treatment is refuted by the data presented in in Figure 4. The data shows that the levels of p-AMPK T172 and p-ACC S79 (ACC is phosphorylated by AMPK)

are unchanged by A76 stimulation, suggesting that increases in AMPK activity have no effect. All together, these results suggest that the change in Cav1 binding which is triggered by high glucose is not mediated by AMPK but by some other pathway.

With regards to Akt, there is no evidence that changes in Akt have any impact on eNOS phosphorylation or Cav1 binding.

4. **Reflection by the student on the experience and value of the studentship:**

The studentship was a very enjoyable experience, working with some talented and enthusiastic people and learning from their combined experiences.

On a practical level, I learned several techniques, their applications and limitations, namely sterile cell culture, preparation and running of gels, immunoprecipitation, the NO assay and confocal microscopy and data analysis. Outside of the lab, I may not use these on a day-to-day basis but familiarity with them will stand me in good stead while reviewing (and criticising) published research.

From the point of view of results, it was pleasing to be able to repeat the group's previous observations relating to the Cav1 protein, even if it is not something new, because the trend in published science which cannot be repeated is a worrying one. It is reassuring to know that this piece of science can be reproduced, even by someone inexperienced. Experiencing "negative" results was also beneficial as a reminder that much of science is about ruling out possibilities rather than discovering the key to a problem.

Overall, this has been an interesting and rewarding studentship and a good chance to experience a research environment and to investigate an area outside of but not far removed from the core of my degree subject. At the start of my degree, I would not have imagined continuing on to a PhD but as the course has progressed, the idea has become more interesting and this funded studentship has provided an invaluable opportunity to see what that might entail in a lab environment.

5. **Dissemination:**

   Amongst the immediate research group only.

6. **Signatures:**

Supervisor	Date	Student
	Date	
27/8/17		27/8/17