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Nitric oxide mediated vasodilation: Effects of inhibition of the mitochondrial electron transport chain

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Abstract

Background and purpose
Nitric oxide (NO) has been well documented as a potent, endogenous vasodilator. The effects of NO within the pulmonary and systemic vasculature have been investigated to some degree but the mechanisms behind its regulation are not fully understood. Mitochondria have been shown to regulate potassium channels that are important in the regulation of vascular tone. This study aims to observe the effects of mitochondrial inhibition of the electron transport chain at different complexes and within different vascular tissues to further elucidate this system.

Experimental approach
I examined the effects of mitochondrial transport chain inhibitors rotenone (inhibition at complex 3) and antimysin-A (inhibition at complex 1) on the relaxation of rat aortic and pulmonary vessels, induced by sodium nitroprusside (SNP).

Key results
Inhibition of mitochondrial electron transport chain at complex 1 surprisingly caused a great increase in levels of vasodilation within the pulmonary artery. Inhibition of mitochondrial electron transport chain at complex 1 also caused an increase of SNP induced vasodilation within the aorta. Inhibition of the mitochondrial electron transport chain at complex 3 resulted in a markedly decreased vasodilatory effect of SNP on the aorta.

Conclusions and implications
My results demonstrate the importance of mitochondrial activity in the regulation of NO's vasodilator action within the pulmonary and systemic vasculature. It also highlights the relevance of activity at certain complexes within the mitochondrial electron transport chain, suggesting a strong correlation between redox state and co-localised potassium channels. This promotes further research into the specificity of activity at certain complexes and the potential for further development of therapeutic NO donors in the treatment of cardiovascular disorders.

Introduction

The effects of endothelial dysfunction through processes such as aging have been well documented and for many years it has been well understood that this has major contributions to cardiovascular disorders, which are a massive burden on society, being the leading cause of morbidity and mortality world-wide, with over 17 million deaths from cardiovascular disease recorded in 2008 (Sidney and Smith 2010). Many cardiovascular diseases have been linked to some level of impairment of endothelial vasorelaxation and one of the most important signalling molecules, relating to endothelial function, is nitric oxide (NO). NO has many effects on vascular functioning through endothelial signalling, including anticoagulation, leucocyte adhesion, smooth muscle proliferation, and the antioxidative capacity (Gewaltig and Kojda 2002), however one of the most important protective effects it has is that of vasodilation. NO's vasodilatory properties are attenuated as a result of many cardiovascular pathologies.

NO achieves its vasodilatory action through a number of mechanisms. The most prominent and well understood of these is the cGMP pathway. Upon the increased production by the endothelium, NO binds to soluble guanylate cyclase (sGC) and activates it (Denninger and Marletta 1999). NO has a high affinity for sGC because of the presence of the heme protoporphyrin IX which contains ferrous form iron (Pacher *et al.* 2007). It is the single unpaired electron which give NO its high affinity to protoporphyrin IX, in comparison to other molecules. As there is only one free electron the binding of NO is a chain terminating reaction. For this reason NO has 10,000 fold greater affinity for deoxyhaemoglobin than O_2, allowing it to take effect at extremely low concentrations (Pacher *et al.* 2007). Once bound, NO activates sGC which, in turn, causes the production of cGMP. This then goes on to activate protein kinase G (PKG) which has the ability to phosphorylate a number of molecules, responsible for the cell signalling pathways which cause vasodilation (White *et al.* 2000). This includes phosphorylation of potassium channels in smooth muscle and endothelial cells. It also works by phosphorylating a serine residue on myosin light chains in smooth muscle cells to desensitise the contractile apparatus resulting in vasodilation (Mingone *et al.* 2003).

Vascular tone is regulated through a number of mechanisms. One of the most important factors to come to the forefront of recent research is through the mediation of potassium channels. In vascular smooth muscle cells these channels exist in three main groups. These are the calcium activated potassium channels, the voltage dependent potassium channels and the ATP-sensitive potassium channels (Korthuis 2011). The calcium activated potassium channels open as a result of raised intracellular calcium concentration and result in potassium flux out of the cell, resulting in cell becoming more hyperpolarised. They range from small to large conductance groupings (Gutterman *et al.* 2005) and result in the closing of calcium channels, preventing calcium influx and causing vasodilation. These channels are present on both the endothelium and vascular smooth muscle cells (Brayden 1996). The voltage gated potassium channels work to the same effect as the calcium activated channels, however their location is specific to vascular smooth muscle cells (Figueroa *et al.* 2007). The ATP-sensitive channels are mediated by the ADP-ATP ratio in the near vicinity which may link them strongly to local mitochondrial activity. They are found in both the endothelial and vascular smooth muscle cells (Nelson

and Quayle 1995). It should be noted that these channels are not only sensitive to ADP-ATP ratio but may also be activated by protein kinases as a downstream result of cGMP and cAMP activation. This highlights the importance of NO in potassium channels activity. Research has shown that, in conditions of hypoxia, levels of NO are elevated, increasing the activity of cGMP and therefore PKG, resulting in the phosphorylation and activation of these potassium channels, increasing cytosolic calcium levels in endothelial cells (Hampl *et al.* 1995).

NO is synthesised in endothelial cells as a result of intracellular calcium level perturbation (Stamler *et al.* 1994). It is synthesised from L-arginine and requires the presence of several molecules, including oxygen, heme, FAD, NADPH, tetrahydrobiopterin and calmodulin. NO is produced alongside L-Citrulline and the mediation of many of the necessary cofactors is regulated by mitochondrial activity (Toda *et al.* 2009). NO within the pulmonary and systemic vasculature is produced by eNOS (endothelias NO synthase), which is a constitutive enzyme, always present in cells and with the potential for rapid modification of its activity (Dale and Haylet 2004). In conditions of raised cytosolic calcium levels, eNOS is activated through its binding to caveolin-1 in the caveolae, causing it to migrate into the cell in its active form (Toda *et al.* 2009).

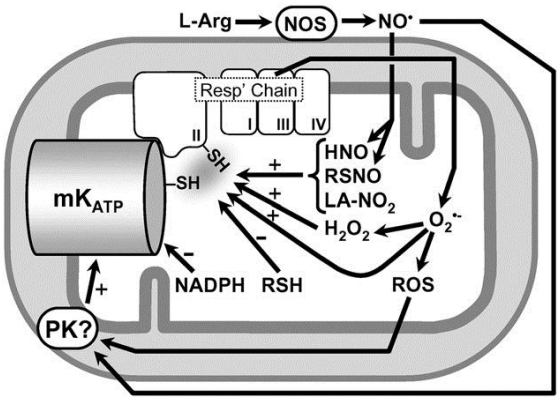

Figure 1: Taken from (Queliconi 2010). Schematic showing redox regulation of mKATP. Nitroxyl (HNO), RSNO, and LA- NO2 activate the channel, possibly via thiols on the channel itself or on complex II of the respiratory chain. The ability of low molecular weight thiols (RSH) to inhibit the channel may also be mediated via thiols on the channel or on complex II. In contrast, the effects of NADPH are likely no mediated via thiols. The ability of NO• to activate the channel may be mediated via the generation of secondary RNS (e.g. RSNO, LA-NO2, HNO), which can activate the channel via PKG-independent mechanisms, or via classical NO• protein kinase signaling. ROS (in particular H2O2) can also activate the channel, via mechanisms that may include thiol modification or protein kinase signaling. The nature of the interaction between complex II and the subunits of the mKATP channel itself remains to be elucidated.

Mitochondria have been proposed as one of the main oxygen sensing organelles within the systemic and pulmonary vasculature (Firth *et al.* 2008) and one of the main factors affecting NO's vasodilator action is mitochondrial activity. Recent research has shown the importance of mitochondrial ATP-sensitive potassium channels in the protective mechanism of ischemic preconditioning (Queliconi *et al.*

2010). These channels have been described as sensitive to reactive oxygen species (Figure 1). This means that the channels are regulated by the redox state of the localised mitochondria, determined (partially) by the presence of reactive oxygen species such as NO. As mitochondria regulate potassium channels in smooth muscle cells they have the potential to alter the effects on NO on vascular tension. It has also been noted that NO also has an independent affect upon the mitochondrial electron transport chain. It has been shown that NO displays an inhibitory effect on the electron transport chain at several complexes, including complex 1 and 3 (Brown 2007). One result of NO's inhibition on the transport chain is the production of superoxide, which has a very high affinity for NO and binds rapidly, forming $ONOO^-$. This then causes an accumulative effect on NO's inhibitory action (as $ONOO^-$ also causes inhibition).

Methods

Chemicals

Phenylephrine was purchased from Sigma Chemical Company. This was prepared into 10µM, 100µM, 1mM and 10mM stock solutions through dilation with Tyrode's buffer solution. Sodium nitroprusside was purchased from Sigma Chemical Company and was prepared into 10µM, 100µM and 1mM stock solutions with Tyrode's buffer solution. Rotenone and antimysin-A were also purchased from Sigma Chemical Company and were prepared as 5µM stock solutions with Tyrode's buffer solution.

Solutions

Tyrodes buffer solution was prepared freshly before carrying out experiments. The solution consisted of: sodium chloride at 140mM, glucose at 5.55mM, sodium bicarbonate at11.9 mM, potassium chloride at 2.68 mM, sodium dihydrogen orthophosphate 0.4 mM, calcium chloride at 1.84mM and magnesium chloride at 1.4mM. All of these components were provided by the University of Plymouth.

Preparation of tissue

Male Wister rats (250-450g) were killed by cervical dislocation and decapitation in accordance with Schedule 1 (Appropriate methods of humane killing) to the Animals (Scientific Procedures) Act 1986. An incision was then made into the peritoneum and then up through the centre of the rib cage to the neck in order to access the heart. Connective tissue was cut away with precision scissors whilst using forceps to gently pull away the heart by holding the lungs away from the body. The descending aorta was then cut away from the spine (through connective tissue) and cut off as far down as possible. Any tissue not immediately in use was kept in Tyrode's buffer solution and on ice to keep the tissue from dying. As much descending aorta as possible was removed from the heart and cleaned in a petri dish with fresh buffer solution. This involved cutting away all remaining connective tissue and fat with small scissors whilst using fine forceps to hold the vessel at the edges, gently so as not to damage the endothelial lining or affect vasodilatory action. The rest of the heart and lungs were then taken from the ice and placed into a petri dish lined with a base of wax and filled with buffer solution. Both of the lungs and the heart were held apart in the wax with pins. All remaining connective tissue, fat and unwanted vessels were carefully removed, leaving the heart and two lungs attached by the pulmonary artery. Each end of the vessel was clamped with forceps and then cut, as close to the

lungs/heart as possible to provide the most artery for experimentation. Both the aorta and pulmonary artery were then cut into appropriately sized sections: 5mm for aorta and 2mm for pulmonary artery. These segments of blood vessel were then kept in buffer, on ice until needed.

Organ bath setup

The volume of both organ baths 1 and 2 was measured at: 74ml for organ bath 1, and 68ml for organ bath 2. These were filled to their levels with buffer solution and kept at 37.5ºC in a water bath at all times. Hooks were prepared from stainless steel wire at a length of 7mm each. Two hooks were carefully slid through the lumen of the blood vessel, with one fixed to the base of the organ bath and the other attached to an isometric force transducer above (AD Instruments Isometric Force Transducer) as described previously (Kato *et al.* 1990), in order to suspend the vessel segments (As shown in *Figure 1*). The isometric force transducer was then calibrated to display force in grams with the LabChart software. This involved recording the addition of a 1g weight to the transducer and setting this change in weight as 0g-1g on the software. Blood vessel segments were suspended in the organ bath, in buffer, at 2g tension. Each vessel was allowed 30 minutes to equilibrate. Carbogen, a mix of 95% oxygen and 5% carbon dioxide, was bubbled through the organ bath to provide oxygen for the tissue to survive. Forces were achieved through inducing constriction and dilation of the blood vessels through the use of phenylephrine and sodium nitroprusside respectively. Isometric tension forces were recorded using a force transducer coupled to an amplifier. These signals were converted to digital signalling through LabChart software. The information was constantly recorded by the LabChart software in a visual (graph) format.

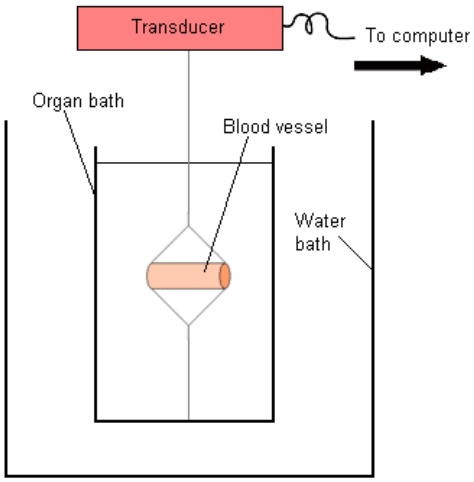

Figure 2: Organ bath setup. Shows mounting of vessel strip attached to isometric force Transducer, which is linked to the computer and LabChart software.

Confirmation of tissue integrity

Before carrying out an experimental procedure on any piece of tissue it was first established whether the vessel was undamaged (within reason) through the dissection and mounting process. This involved adding phenylephrine at 1µM and

3µM concentrations and observing the level of contraction induced. With any significant contraction induced it was considered that the tissue was undamaged in terms of vasodilatory properties as it was still functioning perfectly with respect to the experiment.

Data analysis and stats

The data was analysed with the LabChart software by extrapolating the data into an excel spread-sheet. Average points of tension were taken at the equilibrated resting point and after the addition of any drugs to produce a table displaying the changes in tension at any point through each experiment. These figures were then converted into values to display the actual change in tension by taking their values from the maximal contraction. These new figures were then converted again to give percentages of contraction which were plotted against drug concentrations.

Results

Contraction of vessels with phenylephrine

Addition of phenylephrine achieved 100% contraction at a concentration of 100µM in strips of rat aorta, suspended in Tyrode's buffer solution. At a concentration of 1µM however, the average contraction was over 50% (Fig.3) and upon administration of phenylephrine at 3µM contractions of 60%+ were consistently achieved. To avoid disruption of the mechanisms under investigation, a concentration of 3µM phenylephrine was chosen to induce contraction of aortic strips in each experimental procedure.

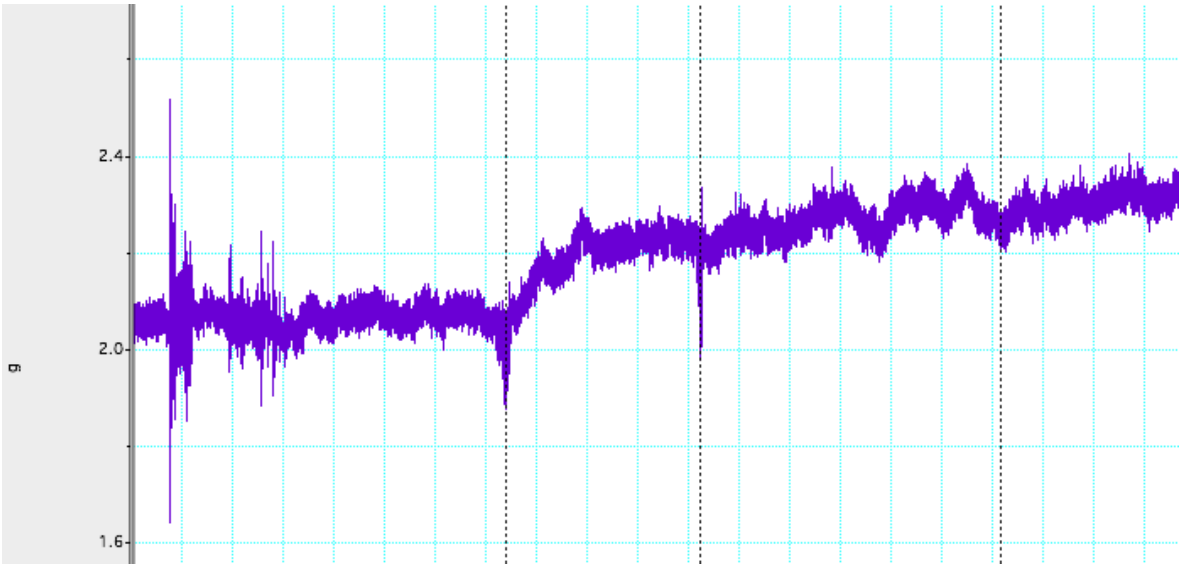

Figure 3: Contraction of pulmonary artery with phenylephrine (Screen shot from LabChart Shows contraction of pulmonary artery with addition of 300nM, 1µM and 3µM phenylephrine; indicated by the first second and third dotted lines respectively).

Relaxing of vessels with sodium nitroprusside

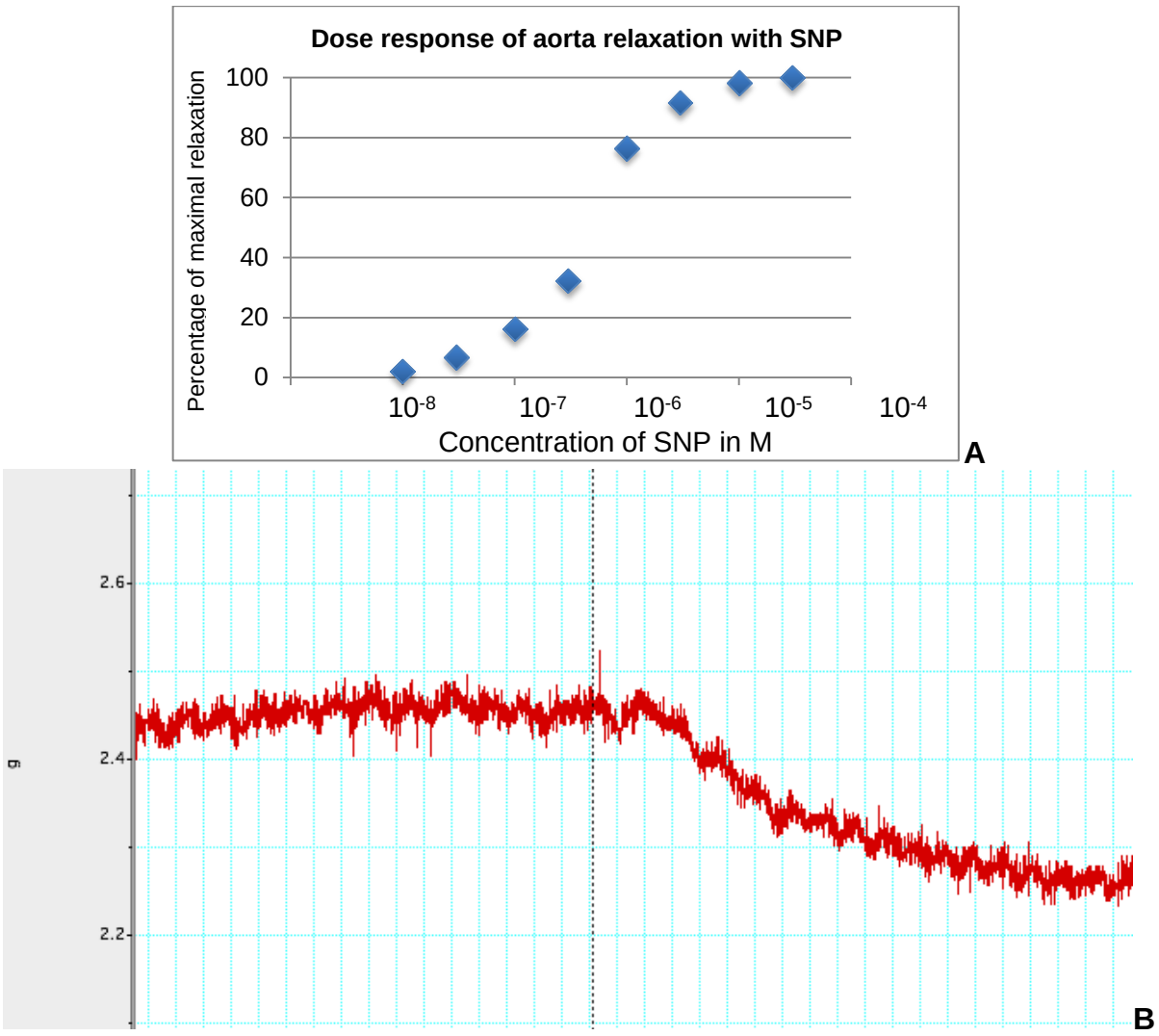

Figure 4: A: Dose response of aorta to SNP. Points on graph indicate the percentage of maximal relaxation of the aorta at a certain concentration of SNP, ranging from 10nM to 30µM SNP. **B**: Screen shot from LabChart. Shows relaxation of aorta with the addition of 1µM SNP.

Sodium nitroprusside (SNP) was used as the NO donor used to create a model of endothelial and pharmacological functioning in both pulmonary artery and aortic vessels. In sections of pulmonary artery the maximum relaxation was induced at a concentration of 300µM SNP, but high levels of relaxation were also achieved at much lower concentrations (Fig.5). On average, 3µM SNP resulted in a relaxation in excess of 80% (Fig.5) and a concentration of 300nM SNP caused an average relaxation of 69.36%. This shows the rapid activity of SNP at relatively low concentrations, with regards to vessel dilation. The findings show that the pulmonary vascular tissue has a high sensitivity to SNP at low concentrations as an average of 40% of maximal relaxation was achieved with just 30nM SNP. The effect of SNP on the dilation of aortic tissue yielded less drastic effects, with a concentration of 300nM

causing only 32% of maximal relaxation (Fig.4 **A**), however maximal relaxation was reached at a lower final concentration of 30µM SNP. It is interesting to note that, at a concentration of 1µM SNP, both the pulmonary artery and aortic tissue average percentages of relaxation were almost identical; 76.5% with pulmonary and 76.3% with aorta. This concentration appears to be the point at which aortic tissue becomes sensitive to SNP as the difference in average relaxation rises by 44% with the increase from 300nM to 1µM SNP.

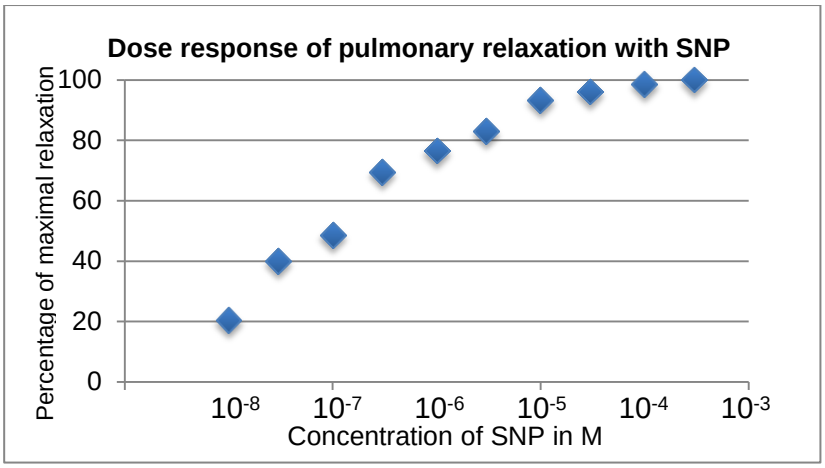

Figure 5: Dose response of pulmonary to SNP. Points on the graph show the percentage of maximal relaxation of the pulmonary artery at certain concentrations of SNP, ranging from 10nM to 300µM SNP.

Relaxation of pulmonary artery in the presence of rotenone

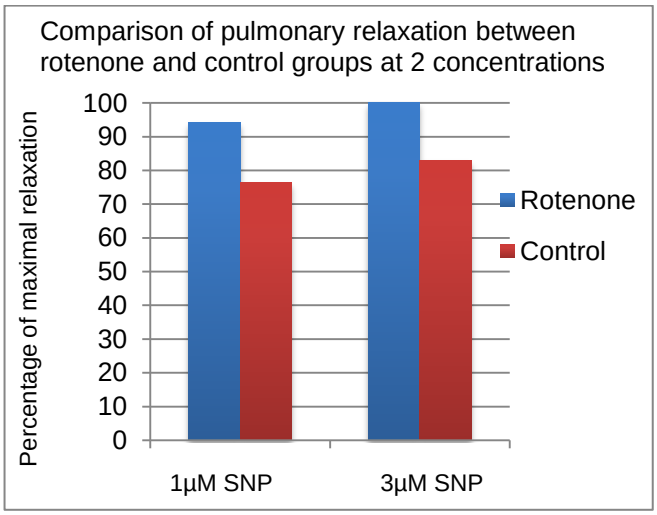

Figure 6: Effects of rotenone on pulmonary dilation with SNP. Blue bars show the percentage of maximal relaxation of pulmonary artery at 1µM and 3µM SNP, in the presence of rotenone. Blue bars show percentage of maximal relaxation of pulmonary artery at 1µM and 3µM SNP, with no rotenone present (control group).

In the presence of rotenone (a mitochondrial electron transfer chain inhibitor at complex-1) the effects of SNP on the dilation of pulmonary artery sections were significantly increased in comparison to the control experiments (Fig.6). In the control studies a concentration of 1µM SNP resulted in 76.5% of maximal relaxation, but in the presence of rotenone this value was increased to 94%. Furthermore at 3µM SNP, whilst rotenone was present, maximal relaxation was induced, whilst the control model had only reached 83% at this concentration. Any concentration of SNP above 3µM, in the presence of rotenone, always induced 100% of maximal relaxation in pulmonary artery strips. This shows the effect of rotenone on the sensitivity of pulmonary artery tissue to SNP, which is already highly sensitive to the drug.

Relaxation of aorta in the presence of rotenone

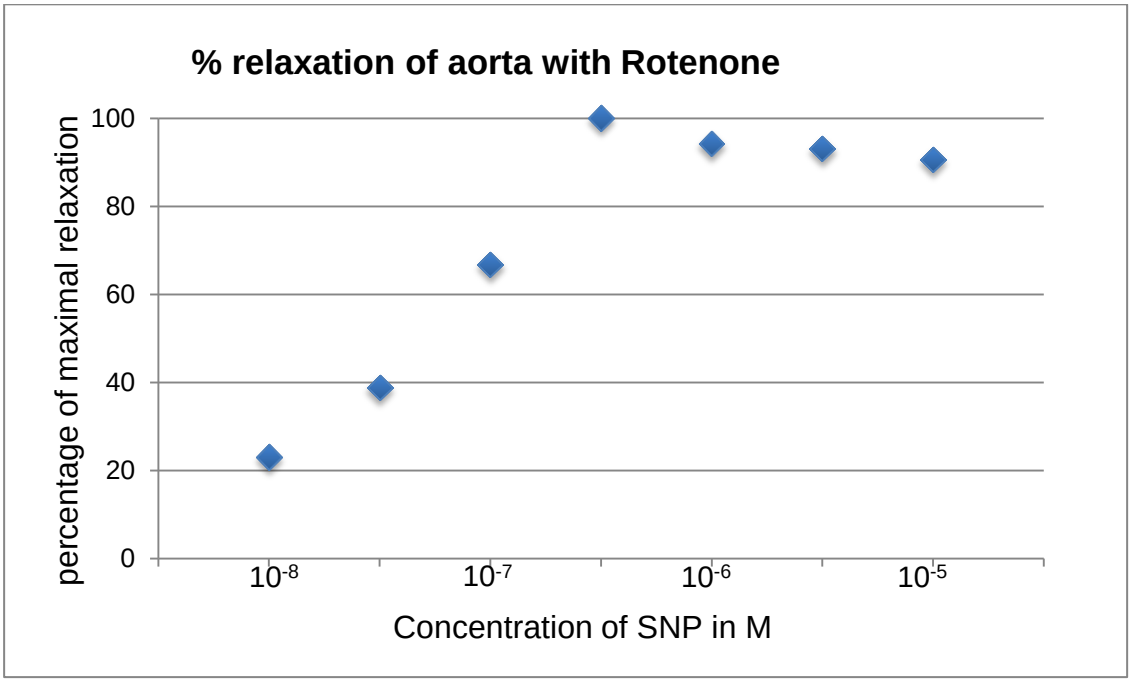

Figure 7: Example of dose response of aorta to SNP, in the presence of rotenone. Graph shows percentage of maximal relaxation of aorta at certain concentrations of SNP whilst rotenone was present. Graph shows 100% relaxation at low concentration of 300nM SNP.

The addition of rotenone to strips of aorta resulted in drastic effects on the vasodilatory action of SNP, with a variety of different results extracted from various experiments. The most prominent contrast to control experiments was the dramatic reduction in the concentration of SNP needed to achieve maximal relaxation of the tissue. Due to the large variation in the figures obtained from each experiment, it was not possible to conclude any specific concentration at which maximal relaxation was achieved however in some cases this was as low as 30nM SNP. It should be noted that in one particular experiment (Fig.7) this concentration (30nM SNP) only caused 39% of maximal relaxation, with 100% relaxation being reached at a concentration of 300nM SNP. Any values for relaxation above 300nM SNP were considered arbitrary as, by this point, maximal relaxation had already been achieved and values tended

to fall lower than 100%. This is assumedly due to other factors, such as disturbance from gas bubbles or natural constriction, as opposed to the effects of the rotenone and SNP. In nearly every experiment of aortic dilation in the presence of rotenone it was only the first or second addition of SNP which resulted in relaxation. Any concentrations of SNP over 100nM which were added had no vasorelaxant action on aortic strips at all. Another interesting result from the addition of rotenone was not in the effect it had upon the vasorelaxant effects of SNP but rather a direct action on the aortic tissue itself. In one experiment the addition of rotenone alone was enough to induce vasodilation. This caused difficulty in acquisition of a dose response to SNP, as the tissue was already relaxing significantly. This result led the decision to change the protocol by adding the rotenone before contraction of the tissue, with phenylephrine, in further experiments.

Relaxation of aorta in the presence of antimysin-A

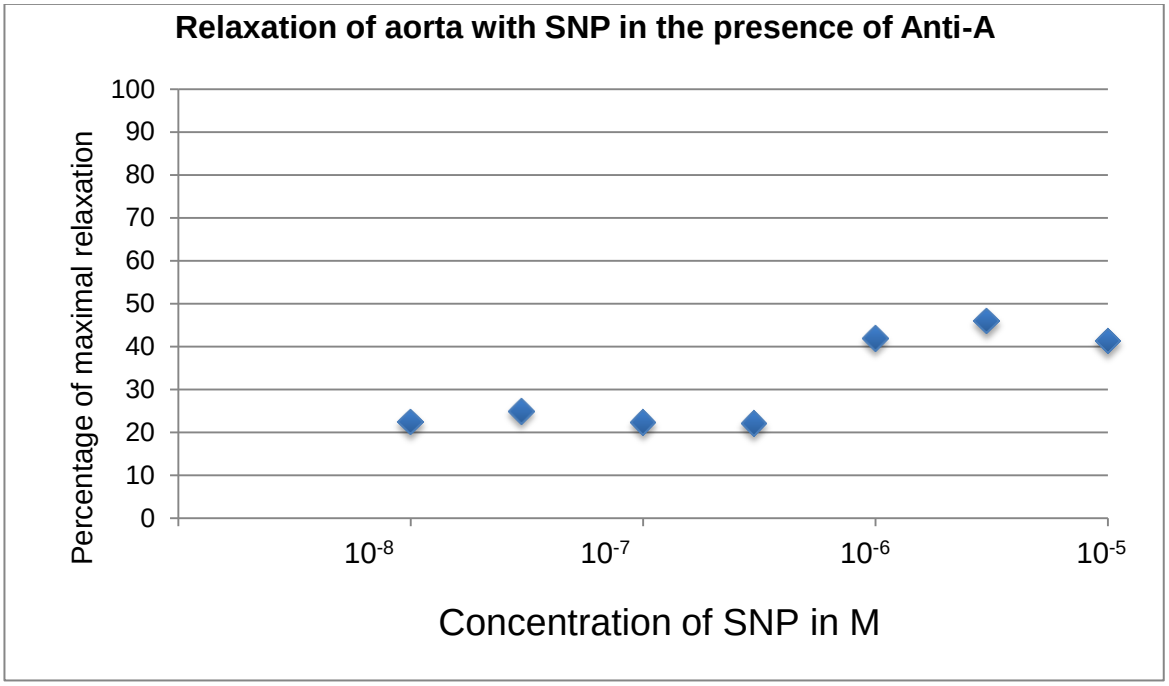

Figure 8: Effect of Antimysin-A on SNP induced dilation**.** Graph shows percentage of relaxation of aorta at different concentrations of SNP, in the presence of antimysin-A. Markedly low levels of relaxation at high concentrations of SNP (with comparison to controls).

In none of the experiments with the inclusion of the mitochondrial electron transport chain inhibitor (at complex-3) antimysin-A was maximal relaxation from SNP achieved. In one of these experiments a value of 65% of maximal relaxation was reached with 3µM SNP; however the average value at this concentration was 46%. Though 65% relaxation was achieved at this concentration, values from lower concentrations of the same experiment showed no trend whatsoever. When the concentration of SNP was raised from 30nM to 100nM the level of relaxation actually dropped by 5%. On the whole values retrieved from the lower concentrations of SNP did not differ greatly from those of the control groups (Fig.8), but the aortic tissue samples were not very sensitive to SNP at low concentrations (with comparison to

the pulmonary tissue). It was at concentrations of 300nM and above that the effects of the antimysin-A were most notable in aortic tissue, with the average relaxation at this concentration being only 22% compared with 32% relaxation in control groups. As the concentration increases the effects of antimysin-A become more prominent, with the tissue failing to relax in response to the SNP. The direct inhibitory effects of antimysin-A on SNP's action is highlighted by the consistency of the concentration at which SNP makes a significant change to vessel relaxation. In both control and antimysin-A treated experiments it was at 1μM SNP that the percentage of relaxation was significantly increased. It was halved however in the antimysin-A group, with the average increase in relaxation dropping from 44% to 22%.

Discussion

Vasodilation through NO

This study highlights the importance of mitochondrial activity in the mediation of NO induced vasodilation in both aortic and pulmonary artery tissue. It has also shown the variation present between these two tissues with respect to their sensitivity to NO. Typically, NO is released by the endothelium in response to increased intracellular calcium concentration, in the endothelium. SNP was used as an NO donor drug to create a model of endogenous NO within vascular smooth muscle and endothelium. The study's results showed the marked variation in NO sensitivity between the pulmonary artery and aorta, with concentrations of 300nM inducing over double the level of relaxation in pulmonary artery tissue than in the aortic samples. This supports previous findings that pulmonary vascular regulation is strongly influenced by NO (Merkus *et al.* 2004).

Mediation of vasodilation by mitochondria

Mitochondria play a crucial role in the regulation of vascular tone in both pulmonary and systemic vascular tissue. Specifically within the pulmonary vasculature, mitochondria have been proposed as the main organelle responsible for detection of oxygen concentration (Firth *et al.* 2008). Mitochondria have been shown to produce oxygen radicals in proportion to oxygen tension and play a role in the regulation of second messenger metabolism (Poburko *et al.* 2004). This study aimed to investigate the effects of inhibition of the mitochondrial electron transport chain at different complexes to help elucidate the mechanisms through which the mitochondria mediate vasorelaxation. It has been shown previously that, specifically within the pulmonary vasculature, mitochondria are co-localised to voltage gated potassium channels, responsible for the control of vascular tone (Bonnet and Archer 2007). Mitochondria have been shown to mediate voltage gated potassium channels through magnesium ion and ATP-dependent mechanisms within pulmonary artery smooth muscle cells (Firth *et al.* 2008). It is known that in times of hypoxia the decreased oxygen concentration is detected by mitochondria which then inhibit potassium channels within the pulmonary artery; resulting in cell depolarisation and calcium influx. The pulmonary vasculature is unique in response to low levels of oxygen in that the blood vessel will contract, as opposed to relaxation induced within the systemic vasculature. By inhibiting the mitochondrial electron transport chain at complex 1 it was hypothesised to disrupt mitochondrial regulation of potassium channels, imitating conditions of hypoxia. The results obtained however gave opposing data, as rotenone would assumedly cause a reduction in SNP's ability to induce vasodilation. It was instead found that the addition of rotenone increased the

vasodilation induced by SNP. As rotenone disrupts the mitochondrial electron transport chain it causes a change in the redox state surrounding the mitochondria. By adding rotenone at the beginning of the experiment the redox state of the mitochondria was altered before any addition of phenylephrine of SNP, which could cause changes in protein modification. This could be a potential cause of the unexpected results obtained. It is also possible that too high a concentration of rotenone was used leading to unwanted effects, as previous research shows use of lower concentrations (Navarro *et al.* 2010). Previous experiments into the effect of mitochondrial inhibition on pulmonary vasodilation were focused on the smooth muscle cells. As the endothelium was most likely still intact in this experiment, and NO has been shown to have opposing effects in endothelium and smooth muscle cells, this is a possible reason for the unexpected values recorded. Though mitochondria have been linked with potassium channel regulation in pulmonary smooth muscle, this relation has not been observed in aortic tissue. For this reason the same effects of inhibition of the mitochondrial transport chain were not expected in aortic strips. The results however again showed an increase in vasodilation from SNP with the presence of rotenone. It is possible that the addition of rotenone alone caused a change in redox state of the mitochondria present and resulted in changes in potassium channel activity. This idea is supported by the fact that in some cases the addition of rotenone alone actually resulted in vasodilation within the aorta. This suggests some possible unexplored linkage between mitochondria and potassium channels outside the pulmonary vasculature.

Unlike the results obtained from experiments with rotenone, the use of antimysin-A to inhibit the mitochondrial electron transport chain at complex 1 caused an inhibition of vasodilator functioning of NO. Whilst the general trend of dilation was similar to that of the controls, with the same sensitivity to concentration, the effects of SNP were stunted, with much lower average values obtained at each concentration. The stark contrast between results from experiments with rotenone and antimysin-A highlight the specificity of certain complexes within the mitochondria and shows the importance of mitochondrial function in the regulation of vascular tension within the systemic vasculature.

Limitations of experiment

Many issues occurred during the conduct of these experiments which would call for changes if they were to be repeated. The first problems to identify themselves were with the dissection of the rats. Whilst the entire aorta is a fairly large and identifiable structure, with easy dissection, the pulmonary artery of a rat is a very small vessel. On top of this, it is partly concealed by the trunk of the aorta, connective tissue and surrounding fat. Removal of surrounding fat and connective tissue warrants extreme care and precision, and even when taking care it is very easy to accidentally cut into the pulmonary artery and cause problematic damage to the tissue. Another problem is with the removal of surrounding vessels; even when the pulmonary artery is left undamaged, the removal of other vessels (in order to open access to the pulmonary) results in the outlet of a large level of blood and clots, which mask the pulmonary artery even further. If this experiment were to be repeated there should be the proposal of an alternative dissection setup. Firstly, a slightly angled setup, with a slow and steady flow of buffer over the tissue would allow consistent washing of the heart and lungs whilst dissecting. This would prevent the build up of blood and clots, which hinder vision of the tissue. It would also be appropriate to include a dissection

microscope for the pulmonary dissection, as this involves a high level of precision and deals with a very small vessel.

Time was another factor with serious implications on the control and reliability/reproducibility of the experiments. As time was limited to around 2 months, along with laboratory availability, the number of experiments which could be carried out was limited. Ideally there would have been further repeats of the effects of rotenone on pulmonary dilation to acquire realistic average values at each concentration. This means that if the experiments were repeated then they may produce significantly different results. There was also no time available to investigate the effects of antimysin-A on the dilation of the pulmonary artery, which would have provided further information to elucidate the difference in mechanisms of vasorelaxation between the two tissue types. Further more, repeats of the experiments using Angeli's salts, in replacement of SNP, would have provided interesting information on the use of the novel nitroxyl (NHO) ion, as opposed to endogenous NO for pharmaceutical use in vascular disorder therapy. As the experiments were carried out over a 2 month period, the size of the rats involved varied greatly due to growth. Ideally the weight of the rats used would not vary more than 100g, but by the end of the experimentation period certain rats had grown much larger than anticipated, increasing the range in weights to 200g. As the control experiments were generally carried out earlier on in the 2 month period the average weight was most likely much lower for the control rats. This could cause unwanted variation and lower the reliability of the results obtained. There is also the time between death of the rats and removal of the desired tissues. As all rats for use in a single day's experimentation were killed at the start of the day, there were issues concerning the quality of the tissue and actual removal of the tissue with later experiments. The longer time between death and dissection means an increase in the level of clotting of the blood, adding difficulty to the dissection. It also means that the tissue is much more likely to have died, and results from older tissue samples may have produced lower values, skewing the averages used in the results.

Finally the apparatus used were not all ideal for the acquisition of perfect data. One issue was the setup of the gas bubbles through the organ bath. The tube for gas delivery was only able to pump gas fairly close to the suspended tissue. This often caused a great deal of disturbance and 'noise' on the LabChart software. The control of the gas outlet was also fairly unresponsive, resulting in many cases of overly vigorous oxygen bubbling. This could disturb the tissue and affect results. There was also a common occurrence of large gas bubbles becoming trapped within the fluid outlet pipe. These could be difficult to remove and would have altered the total volume of the organ bath, affecting the precision of the concentrations of drugs added to the experiment. At one point during experimentation the carbogen gas tank ran out and supplies were depleted for some time after this. This put a hold on any further experimentation and added a significant burden on the time restrictions already in place. The clamps that held the base of the organ bath in place were also inadequate. On many occasions, once equilibrating, the organ bath would slip within the clamp and caused the need to reset the equipment. This wasted time but also raises the possibility that slightly less drastic slips (during experiments) may have been missed, or interpreted as data relating to vasorelaxation. Were this study to be repeated, a much more reliable clamp setup would be needed to ensure consistency of results. The last consideration of lab apparatus hindrances is in the actual

laboratory itself. As this was often in use by others, space was limited, meaning that certain aspects (such as dissection) had to be carried out in slightly inappropriate areas, such as spaces with low levels of lighting. When carrying out a difficult dissection a well-lit workplace is essential and this was often not available.

Conclusions and considerations for further research

In conclusion the relationship between mitochondria and NO induced vasodilation is still yet to be fully understood. The importance of voltage gated potassium channels has become clear and the regulation of these channels through mitochondrial activity shows great importance in both the pulmonary and systemic vasculature. Future research should aim to investigate the effects of inhibition at different mitochondrial complexes whilst under conditions of low oxygen concentration within the pulmonary artery. It should also be recognised that reactive oxygen species produced by mitochondria themselves also contribute to vascular tone regulation and therefore careful examination of the relationship between mitochondrial and endothelial reactive oxygen species may provide vital information on how this system is regulated. Furthermore, a better understanding of how mitochondria regulate vasodilation could provide useful insight into the morphological changes in mitochondria, presumed to be an underlying cause of many cardiovascular diseases, including diabetes mellitus.

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References

Bonnet S and Archer SL (2007) Potassium channel diversity in the pulmonary arteries and pulmonary veins: Implications for regulation of the pulmonary vasculature in health and during pulmonary hypertension. *Pharmacology and Therapeutics.* **15**(1):56-69.

Brayden JE (1996) Potassium channels in vascular smooth muscle. *Clinical and Experimental Pharmacology and Physiology* **23:** 1069-76.

Brown GC (2007) Nitric oxide and mitochondria. *Frontiers in Bioscience* **12:** 1024-33.

Dale MM and Haylet DG (2004) *Pharmacology Condensed.* London: Elsevier Limited. 39.

Denninger JW and Marletta MA (1999) Guanylate cyclase and the NO/cGMP signaling pathway. *Biochimica et Biophysica Acta* **1411** (2-3): 334-350.

Figueroa XF, Chen C-C, Campbell KP, Damon DN, Day KH, Ramos S and Duling BR (2007) Are voltage dependent ion channels involved in the endothelial cell control of vasomotor tone? *American Journal of Physiology and Heart Circulation Physiology* **293:** 1371-1383.

Firth AL, Yuill KH and Smirnov SV (2008) Mitochondria dependent regulation of Kv currents in rat pulmonary artery smooth muscle cells. *American Journal of Physiology. Lung Cellular and Molecular Physiology* **295** (1):L61-70.

Gewaltig MT and Kojda G (2002) Vasoprotection by nitric oxide: mechanisms and therapeutic potential. *Cardiovascular Research* **55:** 250-260.

Gutterman DD, Miura H and Liu Y (2005) Redox modulation of vascular tone: focus of potassium channel mechanisms of dilation. *Arteriosclerosis Thrombosis and Vascular Biology* **25:** 671-678.

Hampl V, Cornfield DM, Cowan NJ and Archer SA (1995) Hypoxia potentiates nitric oxide synthesis and transiently increases cytosolic calcium levels in pulmonary artery endothelial cells. *European Respiratory Journal* **8:** 515-522.

Kato T, Iwama Y, Okumura K, Hashimoto H, Ito T and Satake T (1990) Prostaglandin H2 may be the endothelium-derived contracting factor released by acetylcholine in the aorta of the rat. *Hypertension* **15:** 475-481.

Korthuis RJ (2011) Control of blood flow to cardiac and skeletal muscle during exercise. *Skeletal Muscle Circulation.* California: Morgan and Claypool Life Sciences.

Mingone CJ, Gupte SA, Lesaki T and Wolin MS (2003) Hypoxia enhances a cGMP-independent nitric oxide relaxing mechanism in pulmonary arteries. *American Journal of Physiology. Lung, Cellular and Molecular Physiology* **285** (2): L296-304.

Navarro A, Bandez MJ, Gomez C, Repetto MG and Boveris A (2010) Effects of rotenone and pyridaben on complex I electron transfer and on mitochondrial nitric oxide synthase functional activity. *Journal of Bioenergetics and Biomembranes* **42** (5):405-412.

Nelson MT and Quayle JM (1995) Physiological roles and properties of potassium channels in arterial smooth muscle. *American Journal of Physiology* **268:** 799-822.

Pacher P, Beckman JS and Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. *Physiological Reviews* **87** (1): 315-424.

Poburko D, Lee CH and Breemen C (2004) Vascular smooth muscle mitochondria at the cross roads of Ca(2^{+}) regulation. *Cell Calcium* **35** (6):509-21.

Queliconi BB, Wojtovich AP, Nadtochiy SM, Kowaltowski AJ and Brookes PS (2010) Redox regulation of the mitochondrial K_{ATP} channel in cardioprotection. *Biochimica et Biophysica Acta.* **1813:** 1309-1315.

Sidney C and Smith Jr (2010) Screening for High-Risk Cardiovascular Disease: A Challenge for the Guidelines. *Archives of Internal Medicine*. **170** (1): 40-42.

Stamler JS, Loh E, Roddy M, Currie KE and Creager MA (1994) Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy

humans. *Journal of the American Heart Association. Circulation* **89** (5): 2035-2040. http://circ.ahajournals.org/content/89/5/2035.full.pdf+html

Toda N, Ayajiki K and Okamura T (2009) Cerebral blood flow regulation by nitric oxide: recent advances. *Pharmacological Reviews* **61** (1): 62-97.

White RE, Kryman JP, El-Mowafy AM, Han G and Carrier GO (2000) cAMP-dependent vasodilators cross-activate the cGMP-dependent protein kinase to stimulate BK_{Ca} channel activity in coronary artery smooth muscle cells. *Circulation Research* **86:** 897-905.