Home Office

NON-TECHNICAL SUMMARY

The integrated stress response in respiratory disease

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) Translational or applied research with one of the following aims:
  - (i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

integrated stress response, pulmonary hypertension

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Cells respond to a variety of stresses by triggering a protective "integrated stress response", which "integrates" different stress signals into a single protective "stress response". We aim to identify components of this "integrated stress response" that when targeted with drugs can treat lung diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

High blood pressure in the blood vessels that supply the lungs (the pulmonary arteries) is a serious condition that affects up to 26 people per million. This "Pulmonary Arterial Hypertension" (PAH) is usually diagnosed in people between the ages of 20 and 60, affecting more women than men. It is a progressive and debilitating disease that without treatment is fatal in half of patients in 2 to 3 years. With treatment, more than half of patients can survive for 7 years, but the quality of their lives can still be impaired and this disease carries a heavy economic burden for both the patient and the health service.

A subtype of PAH called Pulmonary Veno-Occlusive Disease (PVOD) is caused by mutations of a gene called *EIF2AK4*. PVOD has an even worse prognosis than classical PAH. With no effective treatments apart from lung transplantation, death occurs in 72% of patients within the first year after diagnosis. Mutations of *EIF2AK4* affect a process in cells called the "Integrated Stress Response" (ISR). By studying how defects of the ISR cause PAH in mice, we hope to find new ways to treat this incurable lung disease.

What outputs do you think you will see at the end of this project?

In this project we will investigate the role of the "integrated stress response" in the lungs. We will study how defects in the "integrated stress response" cause some patients to develop abnormally high blood pressure in their lungs, which is also called "pulmonary hypertension". For example, we wish to discover if there is a defect in lung blood vessels in "pulmonary hypertension" or if other cells, for example cells of the immune system, cause lung blood vessels to behave abnormally. This will help us to identify which cells should be targeted by new treatments for "pulmonary hypertension".

All new information will be presented at scientific conferences and published as peer-reviewed research articles. We do not anticipate new products being produced during this project.

Who or what will benefit from these outputs, and how?

In the short term (1-4 years), the main beneficiaries will be other scientists investigating how the "integrated stress response" controls lung blood pressure.

In the medium term, (5-10 years), beneficiaries will include scientists in the pharmaceutical industry who will be provided with new targets for drug development to treat "pulmonary hypertension".

In the long term, we hope to help patients who suffer from "pulmonary hypertension" by giving them access to new treatments that will improve survival and the quality of their lives.

How will you look to maximise the outputs of this work?

All information, including positive and negative results, will be shared with the scientific community at the earliest opportunity at scientific conferences. Once data are of a sufficient quality, peer-reviewed research papers will be published. All research articles will be made freely available via Open Access in accordance with UK funder guidance. All large datasets (including gene expression and protein levels) will be made freely available in publically accessible online repositories.

Species and numbers of animals expected to be used

- Mice: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This research requires animals that have lungs, an immune system and blood vessels. There is still much we do not understand about how these cell types (lung, blood vessel and immune cells) interact and cause disease. Mice are the lowest creature in which these components can be found. Importantly, mice and humans share many very similar genes, and so we can use mice to understand how defective human genes cause disease. We need to use adult mice because our previous studies have shown that many defective genes, including one which we will study called *EIF2AK4*, cause lung problems that only show up in adult mice.

Typically, what will be done to an animal used in your project?

Mice will be bred to have genetic mutations that cause mild pulmonary hypertension. Although they are unlikely to show obvious signs of disease, we will use non-invasive tests (ultrasound or MRI scans) to determine if pulmonary hypertension is present. In experiments typically up to 6-8 weeks, mice will be given drugs (either in drinking water or by injection) that will either worsen or lessen the degree of pulmonary hypertension. In some experiments, mice will be given drugs to make them unconscious and lung blood pressure will be measured by placing a fine tube into the heart. These animals will be unconscious throughout the procedure and killed immediately after the experiment without regaining consciousness.

Some patients develop pulmonary hypertension because of mutations in certain genes. One of these genes is important in a cellular process called the "integrated stress response". This "integrated stress response" normally protects cells from a wide range of stresses including infections or poisonous chemicals. It now appears likely that a normally functioning "integrated stress response" is necessary to prevent the development of pulmonary hypertension. Therefore, in some animals we will test treatments (by giving drugs in drinking water or by injection) that target the "integrated stress response" to try to prevent pulmonary hypertension developing or to improve lung blood pressure in mice that have already developed pulmonary hypertension. In some experiments, mice will be exposed to stresses that trigger pulmonary hypertenion, such as breathing reduced levels of oxygen (by placing them into a chamber that has lower levels of oxygen than are found in normal room air) or causing inflammation (by injecting them with agents like lipopolysaccharide that trigger the immune system). These experiments may last up to 18 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected impacts from injection of substances to promote inflammation (such as lipopolysaccharide, also known as LPS) include low-grade weight loss (less than 15% of starting weight). Although we expect some inflammation in the lungs and hearts of treated mice, and that their heart chambers may increase in size slightly, we do not anticipate this to cause the animals distress or breathlessness. We base this on our previous experiments in which we gave inflammation-causing drugs (such as LPS) to healthy mice and mice with mutations causing mild pulmonary hypertension. No unexpected deaths were seen in either group. Rarely, animals treated with high doses of LPS showed evidence of distress (taking on a hunched posture with bristling of their fur); these were promptly humanely killed and in subsequent experiments we used lower doses of LPS that did not cause these signs of distress.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice

Mild	25%
Moderate	75%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice are the lowest animal in which an interaction between the "integrated stress response" (ISR), lung blood vessels and the immune system can be studied. While we have used insects to study the involvement of the ISR in vessel growth, insects do not have lungs and have only rudimentary immune systems that are very unlike those of mammals.

Clinical studies identified the gene *EIF2AK4* to be defective in many patients with pulmonary hypertension. At present, how defects of the *EIF2AK4* gene cause pulmonary hypertension remains unclear and so effective therapies cannot be developed. Mice and humans both have the *EIF2AK4* gene and our ongoing studies have shown that mice lacking this gene develop mild pulmonary hypertension (unpublished work). Using such mice with defective *EIF2AK4* genes allows us to examine the role of this gene in lung blood vessel function.

Our previous research showed that inflammation can worsen pulmonary hypertension in mice with other gene defects (such as mutations in the *BMPR2* gene). We therefore wish to test if defects of the mouse *EIF2AK4* gene can also be worsened by lung inflammation. Mice are the lowest animal in which such an interaction can be examined. Although the immune systems of mice and humans share many characteristics, there are some differences. If these difference impair our experiments, we will have access to our institution's Assessment Platform, which provides expert support with "immune-reconstituted ‘humanised’ mouse models" - mice with immune systems engineered to more faithfully model that of humans.

Which non-animal alternatives did you consider for use in this project?

We have previously used non-animal and non-mammalian models:

1. Cultured human cells.

For example, we can grow a patient's smooth muscle cells and endothelial cells from lung blood vessels removed at the time of lung transplantion and donated by the patient for research. We can produce 'blood-outgrowth endothelial cells' from blood samples donated by patients with genetic mutations that cause PAH. We have access to each of these cell models from individuals with normal *EIF2AK4* genes and from patients with defective *EIF2AK4* genes.

We can 'reprogramme' skin or smooth muscle cells from patients to behave as 'stem cells' (so-called iPSCs). These are cells that can be changed into other cell types. Blood vessel cells can therefore be made from these iPSCs. iPSCs can be genetically engineered to have the same *EIF2AK4* gene defects found in patients, allowing us to study defects even if patients are unable to donate their cells.

2. **Insects**

We have used fruit flies, an insect model, to examine the effects of ISR defects on blood vessel-like cell development

Why were they not suitable?

1. These have been used to study the effects of *EIF2AK4* defects on cell function, but such approaches cannot reliably predict the complex interactions between blood vessel cells and other cell types, for example those of the immune system.

2. While fruit fly work was useful in pointing us towards an interaction between the ISR and "BMP signalling" (a signalling system shared by insects and mammals that controls tissue development), insects lack the complexity of the human BMP signalling system, have only a very rudimentary immune system, and lack lungs.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Previous experiments performed by us and our collaborators have shown how a variety of genetic defects and chemical treatments cause pulmonary arterial hypertension. Similarly, previous studies have shown the degree of improvement in pulmonary hypertension that is possible in these animals. With this information we are able to calculate the number of mice needed in each experiment that will provide sufficiently robust results to answer our questions, without excessive numbers of animals being killed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimentals will be designed to ensure appropriate numbers of mice are used. Studies will use enough mice to generate conclusive results without being wasteful. To this end, the PREPARE guidelies will be followed to provide a framework for planning studies (see below).

Study design - each experiment will include detail of the groups of mice to be compared (gene defects and / or drug-treatments). For complex studies, a diagram is more easily interpreted. The NC3R's Experimental Design Assistant software will be used to generate plans in which experimental groups can be identified easily.

Sample size - exact numbers of mice allocated to each group will be calculated and stated for each experiment. This will be determined for each experiment depending upon: the reason for the experiment (what is the information being sought?), the size of the difference in the measured readout expected between each group (how big are differences likely to be?), and estimates of how variable any effect will be (how much "noise" will be in the information?).

I**nclusion / exclusion criteria** - these will typically be defined by animal age and any gene mutations they may have (stated in each experimental plan). Typically, genetic effects will be determined by comparison of mice without gene mutations (so called "wild-type") and genetically modified littermates.

When "homozygotes" (mice with two copies of a mutated gene) are requried (e.g. *Eif2ak4* mutant mice), heterozygote mice (mice with a single copy of the mutated gene) will be mated with other heterozygotes. When there is clear evidence that heterozygotes (mice with a single copy of the mutated gene) are no different from normal (wild-type) mice, these may be used as controls (carefully recorded) to minimise unused animals per litter. Swift and efficient genotyping (the process of determining whether a mouse has a mutated gene or not) will let us humanely kill mice of a not-needed genetic type at the earliest possible stage to minimise any suffering.

Statistical analysis - careful identification of the "experimental unit" (a mouse) in each experiment will be ensured by labelling to avoid "pseudoreplication" (accidentally remeasuring the same mouse) and "underpowered studies" (insufficient numbers of mice to make firm discoveries). This will avoid inconclusive experiments and avoid the need to excessive repetition.

Experimental animals - surplus mice will be shared between research groups, so fewer mice overall need to be bred or bought. The NC3R's breeding and colony management resources will be used to ensure best practice is followed. To locate genetically modified strains we will make use of online tools including Mouse Locator and the MRC Harwell Archive, to avoid mouse-strain duplication.

Experimental procedures - close collaboration also lets us share data and learn/use experimental techniques with minimum use of animals for the ‘learning curve’ – e.g. doses of agents that are known to activate the ISR have been based on preliminary data provided by collaborators in another institution.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our use of non-animal methods and fruit flies (Replacement) will limit the numbers of animals required for the *in vivo* (live) investigations by helping to pre-select the most effective drugs to test. Experiments will be performed in inbred strains to reduce experimental variability. Where appropriate, we will minimise surplus breeding by avoiding unnecessarily narrow specifications for animal sex, age and weight. We will freeze embryos or sperm when lines are not immediately required for studies. By sharing information with other researchers in this field, we will ensure that different groups are not unnecessarily duplicating similar experiments (except when this is necessary to ensure that research findings are robust). To this end, we will use genetically modified mice (with gene defects relevant to our research) made by other researchers, so that we are not wastefully recreating existing mice strains. In turn, we will ensure that our mice strains are available to other researchers in our field. We aim to use imaging techniques including echocardiography, CT imaging, and magnetic resonance imaging (MRI) to allow the non-invasive evaluation of heart and blood vessel function. Such ongoing evaluation of the same animal reduces the numbers of animals required in total.

Our previous expertise in using mice to study human disease and the collaborations we have established with experts in related fields will allow the lowest number of animals to be used (Reduction) and sill allow robust statistical analysis. For example, one collaborator previously required 20 animals per experimental group to allow for technical failures, but now obtains sufficient data from groups of 8 animals. Previous experience shows that 10 animals in each control and experimental group provide a 90% chance of detecting a small difference in pulmonary artery pressure or right heart chamber pressure with high statistical confidence.

When there are no appropriate prior results to allow us to design experiments with the necessary statistical strength, for example when using an entirely new drug, we will perform small pilot experiments to provide estimates of the effect size.

For each and every experiment, as part of good laboratory practice, we write an experimental protocol which includes:

- a statement of the objective(s)

- a description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animals per group), and the experimental material

- an outline of the method of analysis of the results (which may include a sketch of the analysis of variance, an indication of the tabular form in which the results will be shown, and some account of the tests of significance to be made and the treatment differences that are to be estimated)

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The simplest animal that allows us to study the development of pulmonary arterial hypertension (PAH) is the mouse. Genetic mutations that cause PAH in humans have been shown also to cause PAH in mice. Similarly, chemicals that cause human PAH, e.g. activators of inflammation, worsen PAH in mice. The degree of PAH experienced by the mice we study is typically too mild to cause obvious signs. We can detect evidence of PAH in living mice using ultrasound or MRI scans as would be performed on humans. If necessary, mice can be temporarily anaesthetised (made unconscious with medication) for scans to prevent movement that would prevent accurate measurements being made. For more accurate measurement of PAH, a fine tube is passed through the neck of the unconscious mouse and into the heart. This is only performed when the mouse is fully anaesthetised and the animal is then killed before it can regain consciousness.

Why can’t you use animals that are less sentient?

Mice are the least sentient animal in which models of pulmonary function have been developed. The mouse genome has been mapped and so can easily be "genetically engineered" (that is, mouse genes can be altered) to mimic the genetic defects causing human diseases including pulmonary hypertension. Mice also offer the practical benefits of having short breeding times and large litters. There are many laboratory tools, such as mouse-specific antibodies, that allow analysis of mouse tissues and so enable us to understand the processes occuring in disease and following treatments. Because pulmonary hypertension develops gradually in adult mice, it will not be efficient to perform

only "terminal" experiments (experiments in which animals are killed to obtain information). Instead, repeated measurements (e.g. by ultrasound or MRI scans) in the same animals provide more information and so permit the number of animals overall to be kept to a minimum.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In all instances, we will choose our models to provide robust data whilst impacting least on animal welfare. Experiments will be designed with an emphasis on animal welfare, being refined to minimise pain, suffering, distress and lasting harm as per NC3R guidance.

Bedding, nesting, enrichment - Mice will be provided with an appropriate environment, e.g. nesting material and shelter, including sufficient space and complexity to satisfy normal murine behaviours. This will be tailored to experiments as required, e.g. translucent environmental enrichment 'yellow-tubes' will be used to enable better observation of the mice while in the hypoxia chamber, removing the need to re-oxygenate them to check for altered behaviour. Additional food and water may be necessary in certain situations. For example, animals show a reduced activity when first placed into a hypoxic environment and so soft food 'mash' and or 'hydrogel' will be added to the cage.

Handling & acclimatisation - Animals will be handled by the researchers routinely performing their care and experimentation in order to minimise distress from unfamiliar experiences. When repeated or prolonged interventions are required, animals will be acclimitised to new environments (e.g. hypoxic chambers) or procedures (see non-invasive approaches below).

Observation and monitoring - Animals will be monitored at least daily and more often following interventions as appropriate. For example, injections will be carried out in the first half of the day and animals will be monitored for signs of adverse effects for a period of 1 hour after drug administration and will be monitored regularly for signs of adverse effects within 24 hours of administration, e.g. bristling of the fur, hunched posture, subdued behaviour, reduced response to stimulation, or diarrhoea. Any animal will be immediately killed humanely if it shows evidence of suffering that is greater than that specified in the relevant protocol. When animals will be placed into a hypoxic environment, they will be monitored closely at least twice-daily for adverse reaction to the intervention. Animals will be weighed three times per week during the hypoxic period and weights recorded. The respiration rates of the animals will be monitored closely and changes noted. Pilot studies will be used for unfamiliar or novel procedures to establish experimental and humane endpoints, and we will perform post-mortem examinations as a routine part of all pilot studies to investigate any unexpected deaths, seeking advice from the named veterinary surgeon as needed.

Amelioration of pain - When procedures are performed, e.g. vasectomy, painkilling medication will be provided during and after the operation, as agreed in advance with the named veterinary surgeon. Procedures will be performed using anaesthesia whenever appropriate. This will be regularly reviewed by a veterinarian to ensure that contemporary best practice is followed. Animals will be allowed to recover before further use, e.g. following vasectomy mice will not be used for mating until they are regaining weight and they are showing no adverse signs following surgery.

Administration of substances and removal of blood - We will follow the guidance of the European Federation of Pharmaceutical Industries Association and the European Centre for the Validation of

Alternative Methods. For injections and sampling, needles / catheters will be of the minimum width effective for each purpose. Adverse effects from administered substances will be limited by administered using the minimum effective volumes and frequencies to minimised discomfort, and using the lowest effective doses. Drugs will be dissolved in solutions that have been tested and deemed safe. Repeated administration may produce repeated mild distress. If the cumulative effects threaten to exceed that specified in the relevant protocol, the advice of the named veterinary surgeon will be saught and if necessary the animals will be killed humanely. For blood sampling, no more than 10% of total blood volume in 24 hours and 15% of total blood volume in any 28 day period. Animals will be weighed to ensure bleed volumes are not exceeded. Lipopolysaccharide (LPS) is a chemical that triggers the immune system and will be used in some experiments to increase pulmonary hypertension. It will be administered following existing Home Office guidance, in particulat taking into account age, sex and strain differences in LPS effects (older animals, females and outbred strains being more sensitive) with dosing being adjusted accordingly.

Non-invasive approaches - We will use "non-invasive approaches" (methods that do not involve placing tubes into the mice) where-ever possible. Lung blood pressure measurement using ultrasound scanning is possible for mice just as it is performed for human patients. Ultrasound provides measurements that similar to lung blood pressures measured directly by inserting tubes into the blood vessel, but has the advantage that it allows repeated monitoring of the same animal over several weeks, thereby reducing variability in the experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

As detailed in the **Guidelines for the Welfare and Use of Animals in Cancer Research Apply (2010)**, we will use all available knowledge to predict adverse effects and provide specialist care, especially for genetically modified animals.

Laboratory Animal Science Association (LASA) Guiding Principles for Preparing for and Undertaking Asepctive Surgery (2017) will be used to guide aseptic technique for surgical procedures (both recovery surgery and non-recovery procedures carried out under terminal anaesthesia) to ensures surgical procedure are carried out skilfully with the minimum of risk and disturbance to the mice and without infection.

Animal Research: Reporting In Vivo Experiments (ARRIVE 2.0, 2020) guidelines checklist will be used to ensure optimal reporting of data from mouse experiments can be fully evaluated and utilised. The guidelines are aimed primarily at scientists writing up their research for publication and for those who are involved in peer review

Experimental planning will following the guidance set out in the **Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE)** document (Smith et al 2018 Laboratory Animals, 52(2): 135-141).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs both by regular updated provided by our institute and from the NC3R's webpages e.g. resources page (https://www.nc3rs.org.uk/3rs-resources). We will make ongoing use of the NC3R's E-learning hub (https://nc3rs.org.uk/e-learning-resources). Refinement resources will also be accessed. Resources from the Laboratory Animal Science Association (LASA) will also be reviewed as updated, e.g. the guidance on dose selection in toxicology studies https://www.lasa.co.uk/PDF/LASA-NC3RsDoseLevelSelection.pdf will be useful to our programme of work.