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NON-TECHNICAL SUMMARY

Oxygen sensing and nutrient signalling in metabolic disease

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) Translational or applied research with one of the following aims:
  - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

obesity, diabetes, metabolism, mitochondria, hypoxia

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

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?

To study how oxygen levels and nutrients, particularly lipids, are sensed in tissues during metabolic diseases, and how this contributes to the pathology.

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?

The incidence of chronic metabolic disease continues to rise in the developed world, associated with the rise in obesity levels and associated conditions often termed lifestyle diseases. Many such conditions (e.g. type 2 diabetes, cardiovascular disease, fatty liver disease) worsen over time and whilst lifestyle changes can slow the progression, therapeutic options are very limited at present. It is vital, therefore, that we develop a greater understanding of the biological mechanisms underlying these conditions and the high mortality rates associated with them. In so doing, we can understand why mortality is so unacceptably high, as well as identifying potential targets for therapeutic intervention that could positively impact on patient health, improving survival and quality of life.

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

The direct academic benefits of this work include:

1. A better understanding of the molecular, signalling pathways at play in metabolic diseases such as obesity, diabetes and fatty liver disease. Chiefly, we are interested in developing new understanding of nutrient signalling (e.g. how fat molecules, such as those found in the diet, can regulate cell function) and oxygen sensing. In particular, we will seek to establish whether molecules involved in oxygen sensing pathways are activated in metabolic disease, and if these play a key (causative) role in the disease progression.

2. An improved understanding of the alterations in the lipid composition of tissues in metabolic disease. Lipids are a diverse class of hydrocarbon molecules that includes fat molecules and sterols such as cholesterol. Lipids have a wide range of functions and play roles in the cell including as fuel for energy metabolism in the mitochondria (the powerhouses of the cell), but also as signals that can alter cell function. Lipid composition is altered in metabolic disease, but we aim to indicate which lipids play a key (causative) role in the disease progression. In particular we wish to focus on how lipids alter the capacity of the cell (and the mitochondria within the cell) to convert chemical energy in molecules from the diet into a usable form of energy that can support cell, tissue and organ function to sustain life.

3. An indication of possible therapeutic targets (identified through the use of genetically-altered animals) and/or therapeutic strategies through the use of agents that modify lipid/oxygen sensing.

The research in this project will bring together information using a wide range of biological techniques to study the concentrations of important molecules alongside different aspects of cell, tissue and organ function, in conjunction with measurements made on living animals. For researchers working in disciplines related to ours, this comprehensive analysis will be of significant interest and provide a great resource for the analysis of associations between different sets of data, leading to the generation of new hypotheses.

The specific outputs will include new information in the form of data, resulting in publications in peer-reviewed scientific journals including primary research articles, review articles and commentary pieces. We will aim to make the supporting data underpinning these publications available whenever possible. We will share full datasets with collaborators when appropriate and more widely after we have published our own findings.

We do not anticipate generating any new products, but we will work closely with our current industry collaborators throughout the project, using existing compounds to modify metabolism and signalling pathways. As such, our work may highlight new uses for existing compounds or indicate new targets for future compounds.

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

The beneficiaries of this research, in the short to medium term (as publications arise) will include:

1) metabolic physiologists, particularly those with an interest in the factors that control lipid metabolism, in specific tissues and systemically, in response to altered conditions in the cell;

2) mitochondrial biologists, especially those with an interest in the role that mitochondria play in the hypoxia response, which includes oxygen sensing and signalling aspects, through the generation of reactive oxygen species, and the effect of hypoxia on mitochondrial respiratory function and substrate metabolism;

3) researchers interested in the molecular pathways underpinning the physiological response to hypoxia, including the hypoxia-inducible factors and their interaction with a class of free radicals, known as reactive oxygen species;

In the longer-term, the implications of these findings will become more fully understood, leading to further research into applications and here beneficiaries may include:

1) clinicians and clinical researchers with an interest in the aetiology of metabolic disease, where mitochondrial dysfunction secondary to hypoxia and oxidative stress has been implicated as playing a key role;

2) researchers in the pharmaceutical industry looking to identify new possible therapeutic targets or therapeutic approaches for the treatment of metabolic disease;

3) researchers in universities and the pharmaceutical industry with an interest in toxicology and medicines safety, since hepatic hypoxia is an underlying feature of many pathologies and is likely to alter the capacity of the liver to metabolise pharmaceutical agents, particularly those with a known effect on mitochondrial function.

The research in this project is highly integrative, including analyses of whole animal physiology alongside measures of mitochondrial respiratory function, reactive oxygen species production, morphology and tissue distribution, as well as comprehensive analysis of tissue and circulating lipid and non-lipid metabolites, free radical species and relevant transcriptional pathways. For researchers in all disciplines outlined above, the comprehensive analysis across a range of platforms will be of significant interest and provide a great resource for network analysis, leading to the generation of new hypotheses and grant proposals.

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

We will disseminate the findings of our research via open access publications, and we enthusiastically support the concept of open data, so will make all underpinning research data available upon publication. In all publications, we will target journals to ensure that our findings reach the appropriate audience and ensure that our publications are open access. Moreover, we will work with the Establishment communications team to publicise our work. We will also present the work at relevant academic conferences and through invited seminars at other universities and research institutions in the UK and overseas.

Where possible, we will aim to publish unsuccessful approaches and negative findings, which we recognise are important to prevent duplication of procedures and reduce the number of animals used.

The collaborative and interdisciplinary nature of our work, which includes academic and industry partners, will ensure that maximum benefit is derived from this research and that the findings are communicated widely and to the most likely beneficiaries in a timely manner.

Species and numbers of animals expected to be used

- Mice: 500
- Rats: 500

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.

We are using weanling and sexually-mature adult mice and rats up to 12 months of age. These are the least sentient species suitable for studies of integrative metabolic physiology. Moreover, we can capitalise on established models of metabolic disease in both species.

We carefully consider whether rats or mice are appropriate for each particular study when designing our experiments. For instance, if we need to probe the role of a particular gene or pathway, there is greater availability of genetically-modified mice than rats. Nevertheless there are some rat strains which are of interest (e.g. the Zucker fatty rat, which is predisposed to develop obesity). It is sometimes necessary to

use rats if larger quantities of tissue or blood are required than might be obtained from a mouse, and this will allow a greater number of measures to be made on a single animal, which both enhances the value of the data as a whole and reduces the total number of animals used.

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

We will be studying animal models of metabolic disease. These will typically be induced by feeding a modified diet (e.g. high-fat or high-fat/high-sugar diets). Animals may remain on these diets for up to 12 months but typically for a much shorter duration, and this would be expected to lead to altered metabolic function in tissues, obesity and possibly insulin resistance. In some cases, we will induce type 2 diabetes via the injection of a low dose of an antibiotic known to alter pancreatic insulin production. We will study some animals that have been genetically-modified in such a way that they are predisposed to develop metabolic disease or protected against the consequences of metabolic disease.

In addition, an animal may undergo one or more of the following procedures, but a typical animal will experience no more than five of these:

- Animals may be exposed to low oxygen levels (hypoxia) in a chamber. In this case, animals will be exposed to hypoxia for less than one month and at levels that they are known to tolerate well.

- Animals may receive drugs known to alter their metabolism, either through injections or orally. Not all animals will receive such drugs and the volume and number of injections used to administer drugs will be the minimum needed.

- Animals may receive injections of molecules known as tracers which allow us to understand more about the metabolic processes taking place in their cells or the oxygen levels of their tissues. Not all animals will receive such substances, and no animal would receive more than 4 injections of tracers in their lifetime, and typically a single injection of tracer would be sufficient.

- Animals may be housed alone for short periods of time (typically no more than 3-4 days, but on rare occasions up to 4 weeks). This would be necessary to measure the amount of oxygen they are consuming, their activity and food and water intake, or it may be necessary in order to match the food intake of a control animal to another animal in the study.

- Mice may be scanned using nuclear magnetic resonance (NMR) to look at their body fat percentage. Animals are briefly restrained (less than 2 minutes) in a plastic tube. This does not involve anaesthetising the mice, but is painless and harmless.

- Some animals will be anaesthetised for less than 1 hour so that we can measure the function of their hearts using an ultrasound probe. We need to anaesthetise the animals in order to obtain clear images of the heart without the animal moving around. No animal would be expected to undergo this more than 6 times in their lifetime with at least 1 week between each imaging procedure.

At the end of the experiment, each animal will be killed using the most humane method that is possible while still allowing us to collect blood and tissue samples that are not adversely affected by the method used to the detriment of the scientific analysis.

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

Animals developing features of metabolic disease will show a range of adverse effects, which might include obesity and insulin resistance. They may become lethargic. They may urinate more. They may experience some general discomfort associated with fatigue, which might resemble premature ageing, but we would not expect them to experience pain. Some genetically-modified animals might be expected to show similar adverse effects, whilst others would be protected from the impact of metabolic disease and show less adverse effects.

Animals exposed to hypoxia experience some lethargy and increased ventilation rates, particularly in the first two days. Food intake may fall for the first two days (depending on the degree of hypoxia used). All animals would be expected to recover normal food intake and activity after this.

Animals would not be expected to experience any lasting harm from injections *per se*. Tracers would not be expected to cause any adverse effects. In this project, drugs which alter metabolism would typically be expected to lessen the adverse effects associated with metabolic disease, although occasionally this may slightly worsen the effect.

Single-housing can be stressful for mice and rats, but we will minimise the time each animal is housed alone and this causes no pain or lasting harm. Where possible, animals will be re-housed in groups with their original cage-mates following a period of single-housing.

Animals anaesthetised for cardiac ultrasound imaging will feel groggy as the anaesthetic wears off, but will experience no pain or lasting harm. The ultrasound itself is pain-free and harmless,

NMR scanning for body composition analysis causes no pain, stress, or lasting or immediate harm to mice.

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)?

Most of the mice used in this project (> 75%) will experience sub-threshold or mild severities. A small proportion (< 25%) might experience a moderate severity.

Most of the rats used in this project (> 75%) will experience sub-threshold or mild severities. A small proportion (< 25%) might experience a moderate severity.

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?

The project necessitates the use of animals in a study of integrative physiology, as we seek to investigate metabolic alterations in different tissue compartments and systemically (i.e. in the blood) as well as the interactions between these compartments. Moreover, we need to use validated models of metabolic disease in carefully-controlled studies. In order to mechanistically investigate the importance of particular genes on metabolic disease, in some cases in a tissue-specific manner, we need to use genetically-modified animals.

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

We have considered cell culture models of altered metabolism, including different cell lines that reflect tissue-specific metabolic pathways. We have also considered the use of organoids e.g. liver organoids, which are miniaturised and simplified three-dimensional cultures of cells that, to some extent, reflect the function of an organ.

We have considered using clinical samples, including samples and data from large cohort studies.

Why were they not suitable?

There are currently no tissue or cell culture model alternatives to investigate the complex signalling networks associated with systemic/tissue hypoxia, mitochondrial function and lipid signalling, nor the interplay between different systems. Cultured heart muscle cells (cardiomyocytes) do not experience the same workload or blood pressures as heart cells in the body. Moreover, liver metabolism is critical for whole-body metabolic control, playing vital roles in fuel storage and synthesis of lipids, steroids and ketone bodies and in detoxification. As such, the metabolic consequences of tissue hypoxia, alongside the administration of drugs that target tissue mitochondrial function, will extend beyond specific tissues themselves altering blood lipid composition and systemic metabolism. Moreover, there are likely to be regional variations in the metabolic response across tissues such as heart and liver, which cannot be studied using cell culture methods.

We will use some cell culture models, for example to test the effects of specific lipid species on metabolic function. This will not capture the complexity of inter-organ metabolic signalling in the body, but is adequate for the mechanistic analysis of lipid signalling on metabolic function.

We will use clinical samples from patients, and data from large cohort studies to probe associations between lipid species and metabolic function. This work is important but can only identify associations between different factors. To establish cause and effect, or to reveal the step-by-step mechanism of a metabolic process, we need to use animals and to intervene in these processes through the use of drugs or genetic modifications.

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?

In order to address the scientific questions here, we estimate that we will need to run up to 20-25 separate studies. Each of these studies will typically include between 2-8 groups of animals (including appropriate control animals). Previous work in our laboratory has suggested that between 8-10 animals per group is a suitable number that is statistically-meaningful but not excessive. Some studies, however, will be smaller, pilot studies, which are not necessarily designed to optimise the statistical outputs, but to optimise e.g. drug doses, and these studies will typically use far fewer animals. Working on the basis of these numbers above, and equal numbers of rats vs. mice, we estimate that around 500 of each species will be used. These numbers are a little higher than those used on our previous licence, however, our research team has expanded and we have more questions we would like to address on this project.

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

As we design individual experiments, we carefully consider which control groups are necessary in order to address the scientific question, and we use power calculations to ensure that we are using an appropriate number of animals. We will make use of the NC3R's Experimental Design Assistant when designing such experiments.

Where relevant, factorial experimental designs will be used, rather than the one-thing-at-a-time approach. We consider carefully how to get the most out of each animal we use. For instance, many of our animal studies involve the generation of a model of metabolic disease (often reflecting a human condition such as diabetes, obesity or fatty liver disease). The nature of these diseases is such that they affect many organs of the body. As such, if we plan an animal study designed to probe the effects of diabetes on the heart, for instance, we think carefully about which other researchers/students in the laboratory can carry out work on other organs such as liver and adipose tissue from the same animals in order to address questions of relevance to their work. This reduces the numbers of animals used overall, increases the amount of data obtained from a single animal and allows us to examine links between different tissues/organs, thereby enhancing the quality of the science produced.

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

We will only set up our own breeding colonies if necessary, to avoid the wastage associated with a small colony.

We enthusiastically use pilot studies to minimise the number of groups necessary to address a scientific question. In particular, we find pilot studies to be particularly important when optimising the dose/route of administration of a new compound. For instance, a small, pilot study looking at different

doses of a mitochondrial agent allowed us to use a single, optimal dose/route in a larger study, greatly decreasing the number of animals that would otherwise have been used. We estimate that use of a pilot in this case reduced our overall animal usage by 30-40 rats.

At post-mortem we routinely collect and store all tissues relevant to our overall programme of work to ensure that the minimum number of animals are used across our studies (for example, keeping samples of different skeletal muscles to complement cardiac muscle studies). Indeed this has been a key feature and a strength of our recent work, in that we have been able to carry out multiple high-throughput analyses on an individual animal's tissues post-mortem. As an example, we recently published a paper (2020) showing remodelling of lipid composition in adipose tissue with obesity and the functional consequences. This was the fifth peer-reviewed paper to be published using mouse tissue from a study which was initially carried out in 2012. By carrying out a single, well-designed study and banking the tissue, we were able to maximise the return from 140 mice, rather than using 700 animals in separate studies.

We also collaborate very effectively with other groups and industry collaborators. During the course of our previous project, we were able to reduce the number of animals we needed to use to address some of the questions by collaborating with a long-standing industry partner based in Denmark to analyse tissue that they had already planned to collect from a rat study of diabetes. This maximised the return of data from 108 rats used in their study, and reduced the number of rats that would have been used under our previous project licence by 40.

We will continue to use similar principles in our future work and look for new opportunities to reduce the number of animals we use.

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.

We will be using mouse and rat models of commonly-occurring metabolic diseases (e.g. obesity, diabetes, fatty liver disease). These will most often be produced by feeding the animals modified diets (e.g. high-fat or high-fat/high-sugar diets). Occasionally, we will need to study animals with more advanced forms of metabolic disease, and so this will involve working with animals with genetic predisposition to develop these conditions, or alternatively, we will treat some rats with a low dose of an antibiotic drug to alter pancreatic function and induce a mildly elevated blood glucose level. In order to assess the role of some key molecular regulators of metabolic function, we will also use some genetically-modified animals. In most cases, these models will cause nothing more than mild discomfort associated with obesity and perhaps more fatigue. This will be more pronounced in some of the diabetic animals, but will not be severe.

The methods we will use to study the nature of the metabolic alterations in these animals will rely on monitoring normal physiology - e.g. blood pressure, cardiac function, O_2 consumption/CO_2 production, activity. These techniques will cause not pain, but may rely on restraining animals, briefly anaesthetising them or single-housing them (usually for short periods, although in rare cases this may be up to 2 months). In all cases, we will carefully consider which of these methods we use on each animal to balance the desires to maximise the return of data and minimise distress to each animal.

We will need to inject some substances into the animals (e.g. drugs to modify metabolism or tracers to monitor metabolic flux). In all cases we will use the least harmful route of injection that is feasible for the substance in question. We will minimise the number of injections an animal will receive in its lifetime.

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

Rats and mice are the animals of lowest neurophysiological sensitivity that are suitable for the study of mammalian metabolism. Since metabolism alters with development, and we are interested in metabolic diseases that progress over the course of a lifetime and occur in adults, we are not able to answer our scientific questions using immature animals alone. Some studies will begin in younger animals, which will be fed altered diets, but we will need to make measurements on them when they reach adult stages.

We carry out most of our analyses on organs and tissues post-mortem, however, in order to study the progression of metabolic diseases it is necessary to work with living animals and to monitor their physiology over time.

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

In all studies we monitor animals frequently and where appropriate we use a scoring sheet, which is completed alongside frequent measurement (e.g. three times weekly) of body weight, food and water intake. Moreover, in our previous project we introduced a more detailed scoring system for monitoring clinical signs when administering agents to animals. Although the agents are administered at non-toxic doses, we find this scoring system useful in understanding the interaction between such agents and e.g. hypoxic exposure, and sensitive to differences in the mild/moderate severity range. We felt this gave us a better framework to assess actual severity, whilst producing meaningful data which aided our interpretation of the biological response.

The outcomes of our project rely on the minimisation of suffering. Whilst we do not use any severe procedures, any change of environment could cause short-term distress. As such, all techniques that disturb the animal's environment or diet will involve acclimatisation periods such that data collected reflects true physiological changes due to a specific alteration, rather than generic stress. Blood pressure monitoring, for instance, will be undertaken following sufficient acclimatisation to the apparatus to minimise distress. Single-housing, for the purposes of measuring metabolic rates in a single animal, will be undertaken as infrequently as possible, and over the shortest period possible. Moreover, single-housed animals will be kept in proximity to their cage-mates and returned to group housing wherever possible.

Tissue samples will be collected from animals after they have been killed, with the exception of blood sampling from a superficial vein, which will be carried out on conscious restrained, or anaesthetised animals.

Animals will be handled during daily cage-cleaning and during studies where they will be weighed and their food/water intake assessed. This will habituate them to handling and restraint during procedures. Adequate nutrition will be provided during longer term dietary manipulation. Although there may be the occasional need to fast animals, the duration will be minimised. Animals will be housed with regular light/dark cycles (lights on for 12 hours, lights off for 12 hours) with temperature and humidity maintained in the facility and hypoxia chambers. Bedding will be regularly changed for all animals, and in particular will be carefully monitored in diabetic animals.

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

Guidance on housing/husbandry, blood sampling and handling from the NC3Rs will be followed.

To anaesthetise animals for cardiac imaging, guidance from NC3Rs will be followed, with reference made to: Flecknell (2015) Laboratory Animal Anaesthesia, 4th edition, Elsevier.

Reference will be made to PREPARE guidelines (https://norecopa.no/prepare) when planning studies.

Reference will be made to LASA (https://www.lasa.co.uk/current_publications/) guidance, e.g. on record keeping, training, and avoiding mortality.

ARRIVE Guidelines v2.0 will be used when reporting studies.

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

The PPL holder subscribes to monthly email updates from the NC3Rs and follows the NC3Rs on Twitter, and has encouraged all members of the research group to do so. We follow up by reading reports, and will join (and recommend) workshop webinars in future. We regularly include papers of relevance to the 3Rs and our own research, in our group journal club, and openly discuss how we can implement the advances in our own projects. Moreover, we attend talks and sessions at meetings of e.g. The Physiological Society, that consider advances in the 3Rs.

The advances relevant to our research are often straightforward to implement, and by feeding back to the research group we are able to ensure that improvements made by one group member become more widely adopted. For instance, refined methods of handling mice have been successfully implemented in this way, with an experienced group member demonstrating improved handling techniques to others in the group, including new starters.