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Submission Form animal DEC- UM *Versie 2006-English* **Herziene versie**	**DEC-NR: 2011-028 Received: 01-03-2011**

DEC Date of approval #	Request Type [2]	VROM/GGONR[3]	LNV**/CBDNR[4]
17-03-2011	New / ~~Revised / Pilot~~	95-081	

Main Project	CARIM	~~NUTRIM~~	~~Psychology~~	~~GROW~~	~~biomaterials~~	~~other UM~~	~~None UM~~
Sub Project	~~1.~~ 2. ~~3.~~	~~1. 2. 3. 4.~~	~~1. 2. 3.~~	~~1. 2. 3.~~			

Financial Manager		Budget number	to be determined

Title of the research:

Regulated sugar for my sweetheart: Glycogen-targeting of AMP-activated protein kinase (VIDI project)

Start date 01.04.2011 End date [9] 01.04.2015 *Duration of test [10]: 20 weeks*

	Name	Tel (+ Tel private only PI, Replacement PI, and Safety Deputy	E-mail:	Type of Permit	Cap. group /Department
1. Principal (Responsible) Investigator (PI)				Art.9	
2. replacement of PI				Art.9	
3. Deputy for Biological Safety (VM) GGO[7]				Art.9	
4. Other personnel					

ctrl/exp/sham	ctrl	exp	exp		
Species	01	01	01		
Strain	C57BL/6J	C57BL/6J	C57BL/6J		
Genetic Construct	-	AMPKβ2-T148A	AMPKβ2-T148D		
Origin (supplier)*	01	01	01		
Number	216	216	216		
Gender	m	m	m		
Animal immune competent?	Yes	Yes	Yes		
Age/Weight	12 weeks	12 weeks	12 weeks		
The purpose of the test *	31	31	31		
Relevance of the test *	01	01	01		
Toxicological studies *	01	01	01		
Special techniques *	01	01	01		
Anesthesia *	04	04	04		
Pain treatment *	01	01	01		
Level of discomfort *	02	02	02		
Final status of animal *	01	01	01		

Title: *Regulated sugar for my sweetheart: Glycogen-targeting of AMP-activated protein kinase (VIDI project)*

1. The purpose of the test.

The test shall be performed in the context of a VIDI project awarded to in the grant round year 2010. The VIDI project is entitled: **Regulated sugar for my sweetheart: Glycogen-targeting of AMP-activated protein kinase.**

The shuttling of AMP-activated protein kinase (AMPK) to and from glycogen is not understood. Based on the evidence that AMPK looses its affinity for carbohydrates resulting from its autophosphorylation, the proposed research addresses the physiological consequences of the AMPK glycogen binding dynamics.

The AMP-activated protein kinase (AMPK) is a crucial sensor and regulator of energy metabolism on the cellular and whole body level (see recent reviews [1,2]). AMPK is a heterotrimer composed of the catalytic α, and regulatory β and γ-subunits. Each of the subunits occur in different isoforms (α1,α2,β1,β2,γ1,γ2,γ3) giving rise to 12 possible AMPK complexes. The α-subunits encode a conventional serine/threonine kinase domain, the β-subunits carry a carbohydrate-binding module (CBM) allowing AMPK to attach to glycogen particles and the γ-subunits bind adenosine nucleotides via an arrangement of four consecutive cystathionine β-synthase domains. Once activated through phosphorylation by upstream kinases, AMPK phosphorylates itself (autophosphorylation) and its target proteins, thereby stimulating ATP-producing catabolic pathways and inhibiting energy consuming anabolic processes. Therefore, AMPK activation affects the metabolism of fatty acids, proteins and glucose. Focusing on the latter fuel source, AMPK activation leads to elevation of cellular glucose import, is facilitating glycolysis and inhibiting glycogen synthesis. Altogether, AMPK action delivers extracellular glucose to the cytoplasm thus fuelling glycolysis to match the immediate energy need, which is facilitated by shunting glucose away from glycogen to prevent built-up of stores.

Muscles are the major glucose sinks of the body and express high levels of the AMPK β2 isoform. The probable site of autophosphorylation mediating the loss of AMPK glycogen affinity has been identified as Thr-148 in the AMPK β2-subunit. Thus, after confirmation of the biological relevance of this regulatory site in cell culture, the mutant mice that need to be generated are β2-T148A and β2-T148D transgenics, which are predicted to constitutively bind or being unable to bind glycogen, respectively. The aim of the experiments described herein is to analyse the physiological consequences of the transgene on relevant metabolic parameters.

Feeding with high-fat diet has been shown to induce insulin resistance (a hallmark of diabetes development) in mice and thus has been widely used as a pre-diabetic mouse model. Hence, the three mouse strains (Wild-type controls, T148A and T148D) will be divided to subgroups either fed on normal diet or fed on a high fat diet. These data will give an indication whether changing the subcellular localization of AMPK (i.e by drugs) can be used to prevent the onset of T2D.

2. Social relevance and / or scientific interest

As stated above, the commuting of AMP-activated protein kinase (AMPK) to and from glycogen is not understood. The studies will address the signals preventing AMPK from associating with glycogen. Following from detailed investigations of proteins, cells and organisms, including rodent disease models and transgenics, we aim at delineating the molecular mechanics, cellular consequences and physiological impact of this new mechanism of control.

The prevalence of type 2 diabetes (T2D) is rising rapidly and there is evidence that this is due to the population taking less exercise and becoming more sedentary, coupled with the increased consumption of high-fat food. Hence, the planned feeding experiments with low fat (control) vs. high fat diet are used to simulate a problem of societal dimension. These feeding experiments will be carried out with the transgenic mice also to investigate whether changing the subcellular localization of AMPK (i.e by drugs) can be used to prevent the onset of T2D. Thus, the research furthermore aims at exploring the potential to interfere with this process by chemical compounds for the benefit of mankind, i.e. type-2 diabetics and Wolf-Parkinson-White (WPW) patients.

With regard to the potential clinical use of the generated knowledge interfering with glycogen localization of muscle-specific AMPK β2 may favor blood glucose clearance into muscular glycogen and thus could prove beneficial for treatment of T2D. Conversely, enhancing glycogen-binding of AMPK-β2 may alleviate WPW.

3. Alternatives

Because it is not possible to use patient material for this research in view of practical and ethical reasons, the experiments will be carried out with transgenic mice. Transgenesis is a widely used and powerful technology in metabolic research. But first, the studies will use appropriate cell culture models to verify that the hypothesis that is addressed with the animal experiments stands on a solid ground. However, only the generation of transgenics can ultimately show the physiological consequences of the mutations, which provides important information about the therapeutic potential of interfering with AMPK localization.

Furthermore, we will study contraction-induced cardiac AMPK signaling. In this respect, heart-derived immortalized cell lines are no alternative because these cells are not capable of exhibiting contractile performance in contrast to cardiomyocytes freshly isolated from the mouse heart. These freshly isolated cardiomyocytes are the only cell model that can be electrically stimulated to undergo controlled contractions.

4. Ethical consideration

In the ethical consideration, the intrinsic value of the experimental animals is taken into account. The degree of discomfort and the number of experimental animals will be limited to the minimum. Experience for previous experiments plays an important role in this respect. Through proper treatment of the mice and use of anesthesia the degree of discomfort will be limited. Usage of duo/triple-detection techniques (simultaneous measurement of [^{3}H]glucose and [^{14}C]palmitate, and Western detection of more than one proteins within one gel) and usage of the

most suitable statistical analysis will decrease the number of experimental animals.
When taking into consideration the instrumental value, the experiments will be performed with a well-defined aim. The major complication and most important cause of death in diabetic patients is heart failure. Research aimed at studying the role and mechanism of binding of AMPK to glycogen will reveal whether this process can be used as target for treatment of heart failure in diabetic patients. This research is aimed at treatment as well as prevention of diabetic cardiomyopathy.
Taken together, we expect that the importance of the anticipated results outweigh the discomfort of the experimental animals.

5. Scientific Background

The storage of glucose into muscular glycogen is diminished in type-2 diabetes (T2D). AMP-activated protein kinase (AMPK) is constantly monitoring energy levels by sensing the cellular adenine nucleotide content and, in response to various stresses, adjusts the flux of key metabolic pathways to maintain the balance of cellular fuel intake and expense (see recent reviews [1,2]). These functions are especially important for adaptation to workload in muscle, where the AMPK-$\alpha\beta\gamma$ heterotrimer is capable of localizing to glycogen via its high-affinity β2-subunit carbohydrate-binding module (CBM). Upon activation, AMPK increases insulin-independent glucose import and concomitantly inhibits glycogen synthase located at glycogen particles thus shifting glucose metabolism away from storage towards glycolysis. Preliminary evidence shows disappearance of binding-affinity for carbohydrates in active AMPK, implicating AMPK to cycle between glycogen-bound and free situations thus affecting glycogen dynamics. In keeping with this notion, mutations of heart abundant AMPK-γ2 cause Wolff-Parkinson-White syndrome (WPW) [3] characterized by cardiac glycogen accumulation and metabolic hypertrophy.

Apart from studying the AMPK glycogen signaling in cellular systems, the research also addresses the muscular AMPK fuel signalling in transgenic mice over-expressing two different AMPK β2 mutants, which (a) turn AMPK incapable of binding to carbohydrates or (b) cause it to localize at glycogen particles constitutively. Autophosphorylation at Thr-148 of the AMPK β2 subunit has been shown to inhibit its glycogen binding. Further, the β2 T148D mutant AMPK refrains from binding to carbohydrates, whereas the T148A mutant kinase retains binding affinity despite being autophosphorylated. Hence, the evidence suggests that the planned AMPK β2 T148A and T148D trangenics will prove adequate for our studies.

In our proposal we aim at studying glycogen signaling and the metabolic consequences of altered AMPK subcellular localization. We include the analysis of fatty acid handling because AMPK was also shown to regulate the CD36 fatty acid transporter [4]. Furthermore, it is well established that the imbalance between food intake and consumption greatly contributes to the development of T2D in humans and mice. Therefore the feeding of high-fat diet to mice is a very relevant model for our studies (see also point 1).

The most important determinants of the metabolic flux in the heart are sarcolemmal contents of the fatty acid transporter CD36 and the glucose transporter GLUT4 [5]. Short-term regulation of the sarcolemmal contents of CD36 and GLUT4 occurs by reversible translocation from intracellular storage compartments. Therefore, to predict any effect of the AMPKβ2 mutation on the cardiac metabolic flux it is imperative to measure cellular LCFA (palmitate) uptake and glucose (deoxyglucose) uptake, as well as subcellular localization of CD36 and GLUT4. Furthermore, one of the most established metabolic actions of AMPK activation is stimulation of LCFA oxidation [5]. Therefore, we will measure palmitate oxidation as a metabolic readout of AMPK activation.

Taken together, the determination of metabolic parameters will be necessary (i) to profile the transgenic mouse models (i.e. AMPK β2 T148A/D) and (ii) to monitor the effects of high-fat diet in conjunction with the same transgenics.

6. Scientific Review

This DEC protocol is scientifically judged and approved by
).

This DEC protocol encompasses the animal experiments described in NWO-ALW VIDI project
, awarded to , and to be executed in the period April 2011 – April 2016.

7. Choice experimental animal
The AMPKβ2-T148A- and AMPKβ2-T148D mutant mice, displaying decreased and enhanced glycogen binding, respectively, are suitable models to study the role of glycogen binding in AMPK activation and AMPK-mediated substrate utilization.

7a. Species, strain / origin / final destination
The AMPKβ2-T148A- and AMPKβ2-T148D mutant mice will be generated on a C57Bl/6J background. The construction of these mutant mouse strains will be performed by a company specialized in the generation of transgenic mouse models. The mice will be transported to Maastricht according to European guidelines. The breeding, caretaking and harvesting of these mice will be done in the CPV.

7b. Gender
Male mice are preferred for the planned experimental approach. Females are disadvantageous in this respect because the menstruation cycle has a large impact on muscle glycogen content [6].

7.c. Number
We wish to initiate a breeding program with heterozygous mice from the two AMPKβ2-mutant strains obtained from Upon breeding of a sufficient number of mice (also including mice to maintain the breeding of the strains), all mouse groups will be subjected to a low fat (control) diet and a high fat diet. Then the mice will be used for isolation of cardiomyocytes for measurement of parameters of cardiac metabolism in 6 distinct experiments as mentioned below.

Cardiomyocytes will be used for the following experiments:
Experiment 1: Rate of substrate uptake ([^{3}H]deoxyglucose/[^{14}C]palmitate)
Experiment 2: Content of glycogen
Experiment 3: Phosphorylation of proteins within the AMPK signaling pathway
Experiment 4: Translocation of CD36 en GLUT4
Experiment 5: Rate of [1-^{14}C]palmitate oxidation
Experiment 6: Content of high-energy phosphates (AMP, ADP, ATP, creatine-phosphate)

Based on our experience with similar experiments with cardiomyocytes in other transgenic mice (a.o., AMPKα2-knockout mice, see [7]) we estimate that the percentage of drop-out of the mice is 25%. This percentage of drop-out is due to the difficulty to cannulate the mouse aorta (the critical step in SOP1). **Namely, the mouse aorta is very small and fragile, and opening and cannulating it requires microsurgery and a very steady hand.Moreover, this step has to be carried out very rapidly in order to minimize the exposure of the heart to oxygen-poor conditions.** In 25% of the cases the aorta ruptures during the cannulation, so that the heart cannot be perfused with collagenase for the isolation of cardiomyocytes. **Because we use mouse cardiomyocyte isolation already for several years, in which we modified and further optimized the procedure, we expect that there is no further experimental optimalization (and hence, decrease in drop-out percentage) possible. Note that our group is one of the very few groups in the world able to perform metabolic measurements with mouse cardiomyocytes. This is mainly due to the difficulty in cannulating the mouse aorta.**

Formule of Sachs: Based on our earlier experience, the magnitude of the effects of the radioactive metabolic experiments (1 & 5) amounts to 40% with a variation of 25%, the magnitude of the effects of the metabolite content experiments (2 & 6) amounts to 30% with a variation of 20%, and the magnitude of the phosphorylation/ translocation experiments (3 & 4) amounts to 50% with a

variation of 30%. Hence, for experiments 1 & 5, the formule of Sachs predicts $15.7 * (25/40)^2 = 6.13$ animals per experiment, and after correction for drop-out, the number of animals per group will increase to $6.13 * 1/(1-0.25) = 8.17$ (i.e., n = 9). For experiments 2 & 6, group size will amount to $15.7 * (20/30)^2 * 1/(1-0.25) = 9.30$ (i.e., n = 10). For experiments 3 & 4, group size will amount to $15.7 * (30/50)^2 * 1/(1-0.25) = 7.54$ (i.e., n = 8).

We wish to subject each mouse strain to 2 diet groups (low fat and high fat diet), and then perform these 6 cardiomyocyte experiments. We also wish to use 2 different AMPK-activating stimuli (oligomycin and electrostimulation, see section 8). The total amount of mice per strain will amount to (2 diets * 2 experiments * 2 AMPK-stimuli * 8 animals/group) + (2 diets * 2 experiments * 2 AMPK-stimuli * 9 animals/group) + (2 diets * 2 experiments * 2 AMPK-stimuli * 7 animals/group) = 192.

8. Experiment

When the mice will be 12 weeks of age, the AMPKβ2-mutant groups and the WT group will be divided into groups receiving a 20 weeks' low fat diet, and groups receiving a 20 weeks' high fat diet. Upon termination of the diet, the mice will be anesthesized, and used for the isolation of cardiomyocytes. The cardiomyocytes will be suspended and divided over 6 incubations (see Fig. 1-3). All incubations will be carried out in duplo. The first duplo serves as zero-time control (or blanco; Bl), the second duplo serves as basal condition (Control; Ctrl) and the third duplo involves the AMPK-stimulated condition (treatment with either electrostimulation (ES) or oligomycin (Oli)). Oligomycin is the most potent and specific pharmacological AMPK activator, and electrostimulation the most physiological AMPK activator [8]. Then, the cardiomyocyte incubations will be subjected to each of the 6 experiments. The effect of AMPK stimulation on parameters of cardiac metabolism will always be assessed relative to the basal condition (Ctrl). **But prior to dividing the value of the AMPK-stimulated condition (ES or Oli) over the value of the basal condition (Ctrl), both values will be corrected for background via subtraction of the blanco (Bl). Hence, all these 6 incubations serve to measure the effect of one AMPK activator in relation to the basal situation. Note that for each of the 6 experiments the cardiomyocyte reactions will be terminated in a different manner, and the lysates treated in a different manner.**

1. Rate of substrate uptake

After 20 min pre-incubation in the absence or presence of oligomycin/electrostimulation, a mixture of [^{3}H]deoxyglucose and [^{14}C]palmitate will be added to each of the cardiomyocyte incubations. After 3 min, the cardiomyocytes will be centrifuged and washed 3x. The final cell pellet will be dissolved in scintillation fluid for the detection of ^{3}H and ^{14}C.

2. Content of glycogen

After 20 min pre-incubation in the absence or presence of oligomycin/electrostimulation, cardiomyocytes will be subjected to ethanol precipitation, after which the glycogen pellet is incubated with amyloglucosidase. Subsequently the glucose concentration will be measured with the glucose oxidase method.

3. Phosphorylation of proteins within the AMPK signaling pathway

After 20 min pre-incubation in the absence or presence of oligomycin/electrostimulation, cardiomyocytes will be pelleted by centrifugation, dissolved in electrophoresis-sample buffer, and used for Western detection of phosphorylation of AMPK and phosphorylation of the AMPK substrates acetyl-CoA-carboxylase (ACC) and AS160.

4. Translocation of CD36 and GLUT4

After 20 min pre-incubation in the absence or presence of oligomycin/electrostimulation, cardiomyocytes will be homogenized by douncing, after which the lysates will be subjected to gradient density centrifugation for the collection of distinct subcellular membrane fractions. The fractions will be used for Western detection of CD36 and GLUT4.

5. Rate of [1-^{14}C]palmitate oxidation

After 20 min pre-incubation in the absence or presence of oligomycin/electrostimulation, [^{14}C]palmitate will be added to each of the cardiomyocyte incubations. After 20 min, cardiomyocyte metabolism will be terminated by addition of perchloric acid, and produced $^{14}CO_2$ will be measured by base trapping [9].

6. Content of high-energy phosphates

After 20 min pre-incubation in the absence or presence of oligomycin/electrostimulation, cardiomyocytes will be pelleted by centrifugation and freeze-dried. Then, concentrations of phosphocreatine, ATP, ADP en AMP will be determined via HPLC.

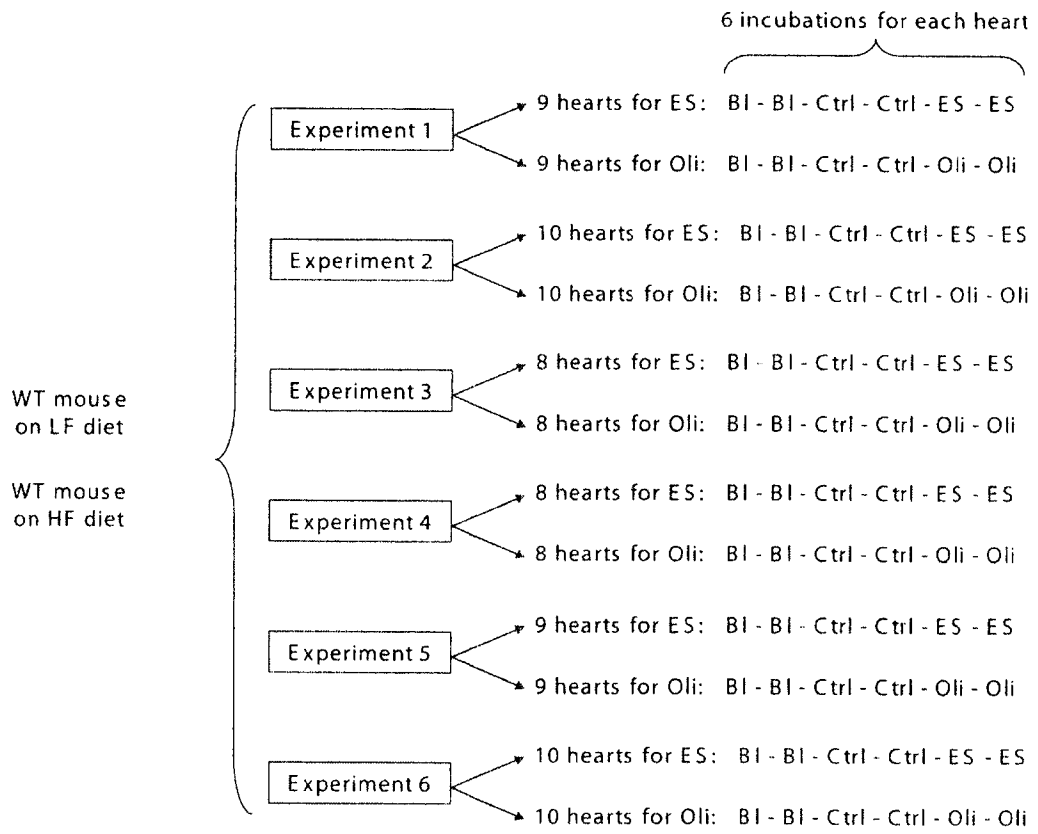

Fig. 1: Planned experiments with WT mice (ES, electrostimulation; Oli, oligomycin; Bl, blanco, Ctrl control). Total: 108 * 2 = 216.

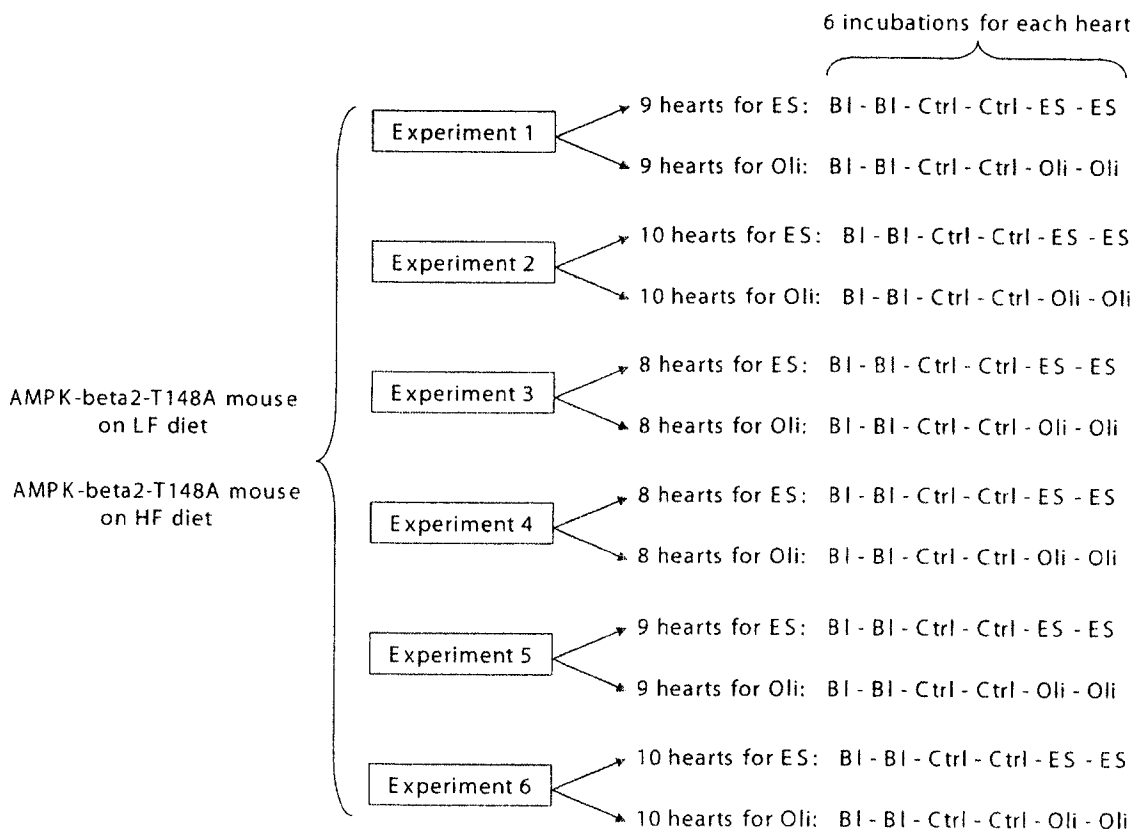

Fig. 2: Planned experiments with AMPKβ2-T148A mice (ES, electrostimulation; Oli, oligomycin; Bl, blanco, Ctrl control). Total: 108 * 2 = 216.

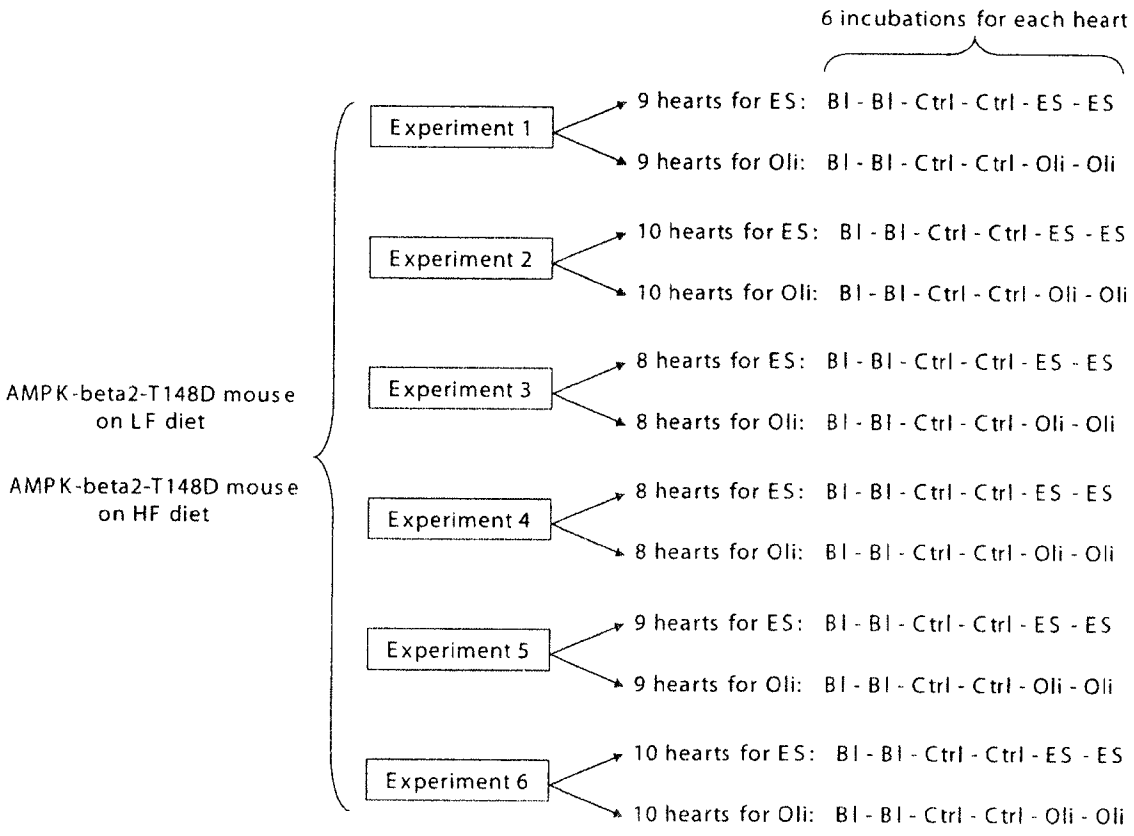

Fig. 3: Planned experiments with AMPKβ2-T148A mice (ES, electrostimulation; Oli, oligomycin; Bl, blanco, Ctrl control). Total: 108 * 2 = 216.

9. Experimental conditions

9a. Anesthesia
Under complete anesthesia via an intraperitoneal injection with pentobarbital (120 mg/kg) diluted in physiological salt solution (1:10), the heart will be removed. Other means of anestesia, unfortunately, are not possible. Analgesie (like NSAID's, lidocaine or buprenorphine) worsens the viability of the cardiomyocytes. Upon opening of the thorax, the heart will be excised. The mouse dies in the experiment.

9b. Pain
Not applicable.

9c. Euthanasia en Humane endpoints
In case the personnel of the CPV or the experimentators would notice a high degree of discomfort for certain animals during the caretaking or during the experiments, these animals will be sacrificed upon consulting local certified personnel. Features of discomfort include: weight loss (10-15%), lack of explorative behaviour, loss of appetite for food or drinking water, skin problems, or lack of feaces production. Euthanasia will be carried out by means of exposure to a low concentration of CO_2, followed by exposure to 100% CO_2.

Care

10a. Discomfort
Intraperitoneal injection and removal of the mouse heart after anesthesia: slight/modest
During the breeding and during the low/high fat diet regimes, the mice will experience no inconvenience.

10b. Wellness Evaluation
Not applicable

11. Care and housing
Housing:
All animals will be housed in the quarantaine room of the CPV **in group** according to the rules and guidelines of the CPV. The diets and drinking water will be supplied to the mice *ad libitum.* The cages will be enriched with rodent toys (e.g., piece of wood, the inside of a toilet roll).

Diets:
D12450B low fat (control) diet (10% fat, 23% protein, 35.5% carbohydrates) and D12492 (is now replaced by D12451) high fat diet (60% fat, 16.4% protein, 25.5% carbohydrates) are from the company 'Research Diets'

12. Expertise
The responsible researchers and collaborators are licenced to design and execute animal experiments according to art.9. Expertise is evident from many years of experience with similar animal experiments.

13. Standard Operation Procedures (SOP)
SOP1: Anesthesia of the mouse and removal of the heart

- Intraperitoneal injection: pentobarbital 120mg/kg body mass diluted in physiological salt solution (1:10). **Other anesthetics will be considered if available.**
- Laying the mouse on its back, followed by fixation
- Desinfecting the skin of the thorax and making an incision in the skin
- Opening the thorax with a scissor
- Cutting out the heart with a scissor and removing adhering lung tissue, fat tissue and thymus from the heart
- Removing of lung or fat tissue from the aorta with small forceps and opening it with a scissor.
- Canulating the aorta
- Isolation of cardiomyocytes via Langendorf perfusion with collagenase type-2-containing medium

Relevant literature

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4. Luiken JJ, Coort SL, Willems J, Coumans WA, Bonen A, van der Vusse GJ, Glatz JF. Contraction-induced fatty acid translocase/CD36 translocation in rat cardiac myocytes is mediated through AMP-activated protein kinase signaling. Diabetes. 2003;52(7):1627-1634.
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6. Hackney AC. Effects of the menstrual cycle on resting muscle glycogen content. Horm Metab Res. 1990;22(12):647.
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8. Luiken JJ, Coort SL, Willems J, Coumans WA, Bonen A, van der Vusse GJ, Glatz JF. Contraction-induced fatty acid translocase/CD36 translocation in rat cardiac myocytes is mediated through AMP-activated protein kinase signaling. Diabetes. 2003; 52(7):1627-34.
9. Glatz JF, Veerkamp JH. A radiochemical procedure for the assay of fatty acid binding by proteins. Anal Biochem. 1983;132(1):89-95.

University Maastricht

Faculty of Health, Medicine and Life Sciences

Dierexperimenten Commissie

DEC

Aan:

voorzitter
p/a Secretariaat DEC-UM
Postbus 616
NL-6200 MD Maastricht
Telefoon: 043

Uw referentie: Onze referentie : Maastricht, 01-03-2011

Geachte Onderzoeker,

Uw projectaanvraag: "*Regulated sugar for my sweetheart: Glycogen-targeting of AMP-Activated protein kinase (VIDI project)*", is op de DEC vergadering van 25 februari 2011 besproken.

De DEC heeft een aantal vragen en opmerkingen:

- Bij punt 4 verzoekt de DEC het woord "Instrumental" te verwijderen.
- Punt 7c- De DEC gaat niet akkoord met een uitvalspercentage van 25% voor het cannuleren van de aorta. De DEC is van mening dat deze methode verfijnd kan worden.
- Gezien de verschillende uitkomstparameters, benoemd in experiment 1 t/m 6 (bij punt 7c), verzoekt de DEC de variatie en de daarop gebaseerde berekening van de groepsgroottes, per experiment te specificeren. Voorts dient gerekend te worden met 6,13 waarnemingen per groep, in plaats van dieren. Er kunnen 6 waarnemingen gedaan worden met de cellen afkomstig uit 1 dier. Daarmee zou het aantal aangevraagde dieren door 6 gedeeld kunnen worden. De DEC verzoekt de berekeningen aan te passen.
- De DEC stelt het op prijs, dat gewaarborgd wordt, dat tijdens het openen van de borst het dier geen pijnsensatie ondergaat en adviseert eventuele cervicale dislocatie toe te passen voordat de borst geopend wordt.
- De DEC verzoekt te onderbouwen waarom andere vormen van anesthesie niet mogelijk zijn.

Conclusie:
Het project wordt aangehouden.

Gelieve eventuele vragen te beantwoorden in **een brief en indien noodzakelijk Uw project aan te passen en duidelijk de aanpassingen grijs te markeren.**
Uw project staat bij de DEC geregistreerd onder nummer **2011-028**, gelieve dit nummer in verdere correspondentie te vermelden.

Hoogachtend

Voorzitter DEC-UM

Maastricht, 1 maart 2011

Beste leden van de DEC commissie van de Universiteit Maastricht,

Hartelijk dank voor uw constructieve opmerkingen op DEC project 2011-028 "*Regulated sugar for my sweetheart: Glycogen-targeting of AMPK-activated protein kinase (VIDI project)*". Ik zal ze puntsgewijs behandelen.

• Deze voorgestelde wijziging is doorgevoerd.

• Cardiomyocyten uit het muizehart worden verkregen via Langendorf perfusie met collagenase-houdend medium. De moeilijkste stap in deze procedure is het canuleren van de muize-aorta, vanwege de fragiliteit van de aorta en de zeer kleine diameter. Dit vereist microchirurgie en een zeer vaste hand. Deze stap moet ook snel gebeuren om het hart na de verwijdering uit de muis zo kort mogelijk aan zuurstofarme condities bloot te stellen. In 25% van de gevallen scheurt de aorta tijdens de canulatie, wat het onmogelijk maakt om het hart te perfunderen voor de isolatie van cardiomyocyten. Aangezien wij deze methode al jaren toepassen valt er geen winst meer te behalen uit verdere optimalizatie van de procedure (en daadoor terugdringen van het uitvalspercentage). Tevens zijn wij een van de weinige groepen in de wereld die rapporteren over metabole metingen met muize-cardiomyocyten. Dit geeft indirect doch duidelijk aan dat het moeilijk is om voldoende cardiomyocyten uit het muizehart te isoleren voor metabole metingen. Deze argumenten heb ik toegevoegd (in grijze markering) aan onderdeel 7c (pag. 7).
Momenteel onderwijzen wij deze methode van muize-cariomyocyt isolatie ook aan andere groepen binnen CARIM Beide groepen hebben na maandenlang oefenen de methode nog steeds niet zo goed in de vingers in vergelijking met mijn groep. Dus zij komen bij lange na niet in de buurt van een uitvalspercentage van 25%.

• We hebben nu de experimenten uitgesplitst, en aparte groepsgrootte berekeningen uitgevoerd voor de metabole flux experimenten (experiment 1 & 5), metaboliet concentratie experimenten (experiment 1 & 5) en eiwit translocatie/fosforylatie experimenten (experiment 3 & 4). Zie hievoor grijs gemarkeerde text in onderdeel 7c, laatste alinea (pag. 7-8).
- Wat betreft de opmerking dat de 6 experimenten kunnen worden uitgevoerd met 1 cardiomyocyt-isolatie, wil ik reageren dat dit onmogelijk is. Per muize-strain willen 2 dieten doorvoeren en van de cardiomyocyt-isolaties dan 6 experimenten doen met 2 AMPK-stimuli met een gemiddelde groepsgrootte van 9. Dit brengt het totaal aantal dieren op (2 * 6 * 2 * 9) = 216 (zie opmerking in grijze markering, onderdeel 7c, laatste alinea, pag. 7-8). Blijkbaar ontstond bij de commissie de indruk dat de 6 experimenten tegelijk binnen 1 muize-cardiomyocyt isolatie kunnen worden uitgevoerd. Mogelijk heeft de zinsnede in onderdeel 8 (pag. 9) tot deze verwarring bijgedragen. Deze zinsnede bevat de mededeling dat vanuit 1 muizehart 6 cardiomyocyt incubaties worden doorgevoerd. Deze 6 incubaties worden niet gebruikt voor de 6 verschillende experimenten, maar worden echter gebruikt ten bate van 1 experiment (namelijk om 1 meting te verkrijgen voor het effect van 1 AMPK-stimulus op 1 bepaalde parameter). Om in iets meer detail te treden, wordt het effect van AMPK-stimulatie gerelateerd aan een controle conditie (niet-gestimuleerd). Een AMPK-stimulatie meting zonder controle meting zegt niets. Ook moet er gecorrigeerd worden voor de achtergrond. Dus daarom bevatten de 6 incubaties: (i) een duplo achtergrond meting, (ii) een duplo controle meting en (iii) een duplo AMPK-stimulus meting ten bate van 1 experiment. Dit is verder duidelijk gemaakt door de toevoeging van 3 nieuwe figuren, die schematisch de indeling van het aantal experimenten en het aantal incubaties per experiment laten zien. Tevens wil ik ook

opmerken dat het niet mogelijk is om vanuit 1 cardiomyocyt-incubatie 6 verschillende experimenten te verrichten, omdat voor elk van de 6 experimenten de cardiomyocyten verschillend worden opgewerkt. Zie hievoor de meer gedetailleerde beschrijving per experiment (onderdeel 8, pag. 9). Deze argumenten heb toegevoegd (in grijze markering) aan onderdeel 8 (pag. 9). De nieuwe figuren staan weergegeven op pag. 10-12.

- Dislocatie zal in een gedeelte van het aantal dieren de hartperfusie nadelig beïnvloeden, aangezien de aorta door het strekken beschadigd kan raken, waardoor er scheuring van de aortaboog en de carotic arteries kan plaatsvinden. Dit zal dan tot gevolg hebben dat canulering van de aorta niet meer mogelijk is.

- Naar mijn mening is pentobarbital een goed verdovingsmiddel, dat de cardiomyocyten minimaal beinvloedt. Andere middelen, indien voorgesteld door de commissie, zullen getest moeten worden voor verdoving en effect op de cardiomyocyten. Bij gebleken geschiktheid zullen ze dan pentobarbital vervangen (zie opmerking in grijze markering, onderdeel 14, pag. 14).

Hierbij hoop ik de vragen voldoende duidelijk beantwoord te hebben, wat het hopelijk mogelijk maakt om dit DEC project goed te keuren.

Hoogachtend,

Maastricht University

Faculty of Health, Medicine and Life Sciences

Aan:

Ons kenmerk	*Doorkiesnummer*	*Maastricht*
		17-03-2011

Project: *Regulated sugar for my sweetheart: Glycogen-targeting of AMP-Activated protein kinase (VIDI project).*

Verantwoordelijk onderzoeker (VO):

Hierbij delen wij U mede dat voornoemd project aan de ethische toetsingscriteria voor proefdiergebruik voldoet.
De DEC maakt geen bezwaar tegen uitvoering van dit project zoals aangevraagd en geeft een **positief advies**.

Projectnummer:	**2011-028**
Diersoort:	**muis**
Aantal dieren:	**648**
Einddatum:	**17-03-2015**

Uw project staat bij de DEC en CPV geregistreerd onder bovenstaand nummer. Gelieve dieren, die voor dit project bestemd zijn, ook onder dit nummer aan te vragen.

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