9

Begeleidingsformulier aanvraag dierproef DEC- UM	**DECNR: 2011-095**
Versie 2006 **Nieuw**	**Ontvangen: 03-06-2011**

DEC datum goedkeuring#	Type aanvraag [2]
05-07-2011	Nieuw

VROM/GGONR[3]
IG02-282

LNV/CBDNR[4]
n.v.t.

Hoofdproject	CARIM	NUTRIM	Hersenen en gedrag	GROW	biomaterialen	Ander UM	Geen UM
Deelproject		2					

Financieel beheerder		Budgetnummer	31962174N

Titel van het onderzoek:

Investigation of the role of Glycogen Synthase Kinase-3 bèta (GSK-3β) in acute pulmonary inflammation-associated muscle atrophy

startdatum **01-07-2011** einddatum [9] **31-12-2012** *Duur van de proef[10]: Max. 3 dagen*

	Naam	Tel (+ Tel privé [illegible] VO, VVO en VM)	E-mailadres	Bevoegd-heid[5]	Cap. groep /afdeling
1.Verantwoordelijk onderzoeker (VO)				Art. 9 + 12	
2. Vervanger VO (VVO)				Art. 9	
3. VM GGO[7]				Art. 9	
4. overige uitvoerenden				Art. 9	
5. PI				Art. 9	

Diergroep	**1-4**	**5-8**		
ctrl/exp/sham	sham	exp		
Diersoort	Mus musculus	Mus musculus		
Stam	C57BL/6	C57BL/6		
Construct / mutatie?	MLC-Cre $^{-/-\ OR\ +/-}$/GSK-3β $^{fl/fl}$	MLC-Cre $^{-/-\ OR\ +/-}$/GSK-3β $^{fl/fl}$		
Herkomst (leverancier) *	01	01		
Aantal	40	40		
Geslacht	man	man		
Dieren immuuncompetent?	Ja	Ja		
Leeftijd/gewicht	12 weken	12 weken		
Doel van de proef *	33	33		
Belang van de proef *	01	01		
Toxicologisch onderzoek *	01	01		
Bijzondere technieken *	05/12	05/12		
Anesthesie *	04	04		
Pijnbestrijding *	01	01		
Mate ongerief *	03	04		
Toestand dier einde exp*	01	01		

* VHI-coderingen zie bijlage

Verantwoording

Aanvraag dierproef DEC-UM

Titel: Investigation of the role of Glycogen Synthase Kinase-3 bèta (GSK-3β) in acute pulmonary inflammation-associated muscle atrophy

1. Doel van de proef.

The past years the efficacy of therapies aimed at inhibition of muscle degradation and stimulation of muscle growth based on Insulin-like growth factor I (IGF-I) signalling has been investigated in clinical trials in patients suffering from loss of muscle mass associated with diseases including chronic obstructive pulmonary disease (COPD), chronic heart failure and cancer. The results from these trials were not consistent, and strong adverse side effects have been reported, including insulin resistance. Therefore, identification of signalling steps of the IGF-I pathway that are more specific to muscle maintenance is required to develop more selective pharmacological strategies to prevent muscle loss. Results obtained by our group have revealed an association between GSK-3β activity (one of the essential regulatory molecules downstream of IGF-I signalling) and muscle trophical state in vivo, and a causal role for GSK-3β activity in *in vitro* muscle atrophy experiments. These results now require verification in a mouse model of atrophy to establish causality of GSK-3β activity in muscle atrophy *in vivo*.
Systemic inflammation is commonly observed following COPD disease exacerbation, and a causal role for inflammation in the etiology of muscle atrophy is supported by data from animal studies. *The aim of this proposal is to investigate whether* ***absence of*** *muscular GSK-3β* ***protects against*** *pulmonary inflammation-induced muscle atrophy.* This hypothesis will be addressed in an established mouse model of acute pulmonary inflammation-induced muscle atrophy, in which we will compare the muscle atrophy response of WT and genetically modified mice lacking GSK-3β expression in muscle.

The information that will be obtained from these experiments regarding the potential preservation of muscle mass based on GSK-3β inhibition may provide a platform for a more selective and successful therapeutic approach to prevent muscle atrophy and improve survival and quality of life of patients with chronic organ failure and cancer.

2. Maatschappelijke relevantie en/of wetenschappelijk belang

Weight loss (cachexia) and specifically loss of muscle mass (muscle atrophy) frequently occurs in diseases including chornic obstructive disease (COPD), chronic heart failure and cancer. More than 35% of all causes of death in the Netherlands are a consequence of these diseases (source: http://www.rivm.nl). Loss of muscle mass, is independently of the cause of the primary disease, responsible for muscle weakness and decreased exercise capacity in these conditions, resulting in a decreased quality of life and even increased risk of mortality (see picture). Eventually, 10-20% of patients suffering from these conditions will develop muscle atrophy, and therefore maintenance of muscle mass is an important goal in an integrated therapeutic approach in these diseases to improve quality of life and survival.

3. Alternatieven

Replacement: Since the triggers of skeletal muscle atrophy in COPD originate from the lung, the primary diseased organ in this condition, the mechanisms of muscle atrophy must be studied in a model *in which the interaction between the lung and skeletal musculature is preserved*, i.e. in an intact

organism. Since other animals like Zebrafish or Drosophila lack the proper physiology for addressing the research questions proposed in this project, and investigating causal relationships in humans using genetic manipulation is excluded, the use of mice here is the best option.

Reduction: Power calculations (included) based on data from previous studies by our group allowed us to determine the minimal number of animals required for control and experimental groups separately to find effects on major parameters of muscle atrophy, such as muscle weight

Refinement: The use of genetically modified mice will allow interpretation of the results on a cause-and-effect basis, which can not be accomplished using pharmacological agents that often have non-specific effects. In addition, the model of acute pulmonary inflammation we propose to use here was found to be very reproducible and causes no mortality in our hands, and results in a transient inflammatory response and mild muscle atrophy which are fully reversible.

4. Ethische afweging

Skeletal muscle wasting is responsible for a decreased quality of life and an increased morbidity and mortality in COPD patients (see picture section 2). Prevention or reversal of muscle wasting is therefore expected to strongly improve quality of life and life expectation in these patients. Elucidation of the signaling pathways of muscle wasting may provide a platform for targeted intervention strategies for muscle wasting, and can be accomplished using the mouse model and genetically modified mice described here. As described in section 10a transient discomfort will be experienced initially by the mice during this protocol from the anaesthesia and LPS-instillation, but subsequently only skeletal muscles will be collected for analysis post-mortem. In our opinion, the discomfort of the patients justifies the use of mice as proposed here to develop effective treatment strategies.

Wetenschap

5. Wetenschappelijke onderbouwing

COPD, CHF, and cancer are disease states that are often accompanied by involuntary loss of body weight (cachexia), including muscle mass (muscle atrophy). Muscle atrophy affects 10-20% of these patients and combined, these diseases are responsible for 35% of all deaths in the Netherlands. Muscle atrophy results in decreased quality of life and physical impairment resulting from a reduction in muscle strength and endurance [3-5]. Importantly, skeletal muscle wasting (or loss of fat-free mass) is also associated with increased morbidity and mortality [6]. Therefore, prevention or reversal of muscle atrophy may prove an important therapeutic strategy in the treatment of the systemic consequences of diseases such as COPD, CHF and cancer. Such strategies should be targeted at the modulation of pivotal regulators of the skeletal muscle trophical state, which requires the identification of the regulatory molecules that determine the balance between muscle growth and atrophy.
IGF-I signalling through activation of Akt is known to stimulate muscle growth and suppress muscle atrophy [7], and suppression of GSK-3β activity via this signalling pathway has also been related to muscle growth. In previous work, *in vitro* studies by our lab have shown that inhibition of GSK-3β increases muscle formation [1], and that a reduction in endogenous muscle GSK-3β activity coincides with the initial phases of muscle growth following atrophy in the same mouse model [2]. In addition to these studies, which attribute an important (suppressive) role of GSK-3β in skeletal muscle formation, activation of GSK-3β was also found to suppress cardiac hypertrophy *in vivo* [8]. Skeletal muscle atrophy involves the muscle specific ubiquitin ligases atrogin and MuRF-1. These two atrogenes are induced by a number of atrophic stimuli, including muscle disuse/inactivity and glucocorticoids. An increase in the expression levels of these genes is dependent on the nuclear localization of the FOXO transcription factors, which are inhibited by Akt-mediated phosphorylation [9]. Recent data obtained in cultured muscle cells in our laboratory have revealed that full induction of atrogene expression by atrophy stimuli requires GSK-3β. Therefore, the central hypothesis of this project is that Glycogen Synthase Kinase-3β plays a central role in skeletal muscle atrophy. The experiments proposed here are designed to investigate if muscle atrophy and the induction of atrogin and MuRF-1 expression by pulmonary

inflammation, as we have observed previously (DEC 2007-040, manuscript submitted) are affected by modulation of GSK-3β activity *in vivo*.

GSK-3β activity will be inhibited using genetically modified mice which lack GSK-3β specifically in skeletal muscle. Completion of the studies proposed here are essential to address the role of GSK-3β and potential of therapeutic approaches aimed at GSK-3β inhibition to prevent skeletal muscle atrophy *in vivo*.

1. *van der Velden JL, Langen RC, Kelders MC, Wouters EF, Janssen-Heininger YM, and Schols AM. Inhibition of glycogen synthase kinase-3beta activity is sufficient to stimulate myogenic differentiation. Am J Physiol Cell Physiol 2006;290:C453-62.*
2. *Bernard S, LeBlanc P, Whittom F, Carrier G, Jobin J, Belleau R, and Maltais F. Peripheral muscle weakness in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1998;158:629-34.*
3. *Gosselink R, Troosters T, and Decramer M. Peripheral muscle weakness contributes to exercise limitation in COPD. Am J Respir Crit Care Med 1996;153:976-80.*
4. *Serres I, Gautier V, Varray A, and Prefaut C. Impaired skeletal muscle endurance related to physical inactivity and altered lung function in COPD patients. Chest 1998;113:900-5.*
5. *Schols AM, Slangen J, Volovics L, and Wouters EF. Weight loss is a reversible factor in the prognosis of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1998;157:1791-7.*
6. *Adams GR, and Haddad F. The relationships among IGF-1, DNA content, and protein accumulation during skeletal muscle hypertrophy. J Appl Physiol 1996;81:2509-16.*
7. *Barton-Davis ER, Shoturma DI, and Sweeney HL. Contribution of satellite cells to IGF-I induced hypertrophy of skeletal muscle. Acta Physiol Scand 1999;167:301-5.*
8. *Jiang BH, Zheng JZ, and Vogt PK. An essential role of phosphatidylinositol 3-kinase in myogenic differentiation. Proc Natl Acad Sci U S A 1998;95:14179-83.*
9. *Jiang BH, Aoki M, Zheng JZ, Li J, and Vogt PK. Myogenic signaling of phosphatidylinositol 3-kinase requires the serine-threonine kinase Akt/protein kinase B. Proc Natl Acad Sci U S A 1999;96:2077-81.*

6. Wetenschappelijke beoordeling

The proposed mouse experiments described in this DEC proposal are part of a grant of the Netherlands Astma Foundation. This DEC proposal has been read and approved by the PI (#5 front page).

Proefdier

7. Proefdier keuze

7a. Soort, stam / herkomst / eindbestemming
We propose to use 12 weeks old mice on a C57BL/6 background. The mice are either muscle specific knock-out for GSK-3β (MLC-Cre $^{+/-}$/GSK-3β $^{fl/fl}$) or genotypic control (MLC-Cre $^{-/-}$/GSK-3β $^{fl/fl}$). Mice are bred locally at the IVC-unit at the CPV. After completion of the experimental protocol, mice will be euthanized for collection and isolation of blood and muscle tissue.

7b. Sexe
The major experimental parameter of this proposal is skeletal muscle wet weight, which is strongly determined by the age and gender of the animals.
In addition, as indicated in DEC 2007-040, the response to acute pulmonary inflammation induced by IT-instillation of LPS (the model of this proposal) is strongly determined by the sexe of the animals and attenuated in females. Therefore, we propose to use only male animals, as this will allow us to compare the results to previous experimental data obtained with male animals in this model by our group.

7c. Aantallen
In previous studies we were able to detect a difference (d) between IT-NaCl and IT-LPS (20μg) treated animals of 12.28% in soleus muscle weight. The coefficient of variance (σ) is 9.02% for IT-LPS instillation. As erroneous instillation of LPS in the oesophagus instead of the trachea occurs at a low frequency, which we will only be able to find out after euthanasia, a dropout of 10% is included in the calculations.

- IT-LPS: $n = 15.7x ([\sigma]^2 / [d]^2) = 15.7x ([9.02]^2/[12.28]^2) = 8.5$ → 10% loss: 8.5/0.9 = 9.4 → 10 animals/group

Based on the number of groups with distinct genotype (2), different time points (2) and treatment (2) the total number of animals is 2x2x2x10 = 80 animals

Dierproef

8. Experiment

Mice (MLC-Cre $^{+/-}$/GSK-3β $^{fl/fl}$ or MLC-Cre $^{-/-}$/GSK-3β $^{fl/fl}$) will 24h before the experiment be housed in individual cages to allow registration of food intake throughout the experiment.
Animals will receive either intra-tracheal instillation of NaCl (a) or LPS (20μg) (b), as described in SOP1, and as previously used in DEC2004-112 and DEC2007-040. Every 24h animals and their food will be weighed. Two time points have been included: 48h with maximal atrophy, and 24h when atrophy processes are initiated, which will give important additive information on the mechanisms of atrophy.
At the indicated time points, blood will be collected by cardiac puncture, the lower hind limb muscles soleus (S), gastrocnemius (G) and plantaris (P) will be excised of 12 wks old animals after they are euthanized according to a standardized protocol (SOP2), which is also used in DEC2007-040. These muscles were chosen because they can reproducibly be excised and are very suitable for determination of fiber cross sectional area (CSA, one of the major parameters in this study). In addition to CSA the following parameters will be determined in these muscles:
- mw: muscle wet weight; as a primary parameter of muscle atrophy
- ih: (immuno)histochemistry; to evaluate muscle morphology (the same material as for CSA)
- wb: Western blot; to analyze quantitative changes muscle atrophy-related protein abundance

- rna: RNA analysis; to evaluate mRNA abundance of MuRF1, MAFbx

	IT-NaCl				IT-LPS			
Group	1	2	3	4	5	6	7	8
Genotype	MLC-Cre $^{-/-}$ /GSK-3β $^{fl/fl}$	MLC-Cre $^{+/-}$ /GSK-3β $^{fl/fl}$	MLC-Cre $^{-/-}$ /GSK-3β $^{fl/fl}$	MLC-Cre $^{+/-}$ /GSK-3β $^{fl/fl}$	MLC-Cre $^{-/-}$ /GSK-3β $^{fl/fl}$	MLC-Cre $^{+/-}$ /GSK-3β $^{fl/fl}$	MLC-Cre $^{-/-}$ /GSK-3β $^{fl/fl}$	MLC-Cre $^{+/-}$ /GSK-3β $^{fl/fl}$
h post-IT	24	24	48	48	24	24	48	48
n	10	10	10	10	10	10	10	10

9. Experimentele condities

9a. Anesthesie

General anesthesia will be used by s.c. administration of a mix of ketamine (75mg/kg) and xylazine (3mg/kg). Under anesthesia LPS (20 µg in 0.9% NaCl/mouse) or 0.9% NaCl as vehicle will be instilled intra-tracheally. We do not use isoflurane as mice are kept in a vertical position for 5-10 minutes directly following IT-instillation to allow maximal dispersion of the LPS throughout the lungs. Mice will stay under surveillance under a heatlamp until recovered. (SOP 1).

9b. Pijnbestrijding

Although mice are subjected to discomfort (see 10a), no specific pain is anticipated, and therefore no additional analgesia will be applied.
Due to the use of pentobarbitol for terminal anesthesia, no additional analgesia will be used at the end of the experiment (SOP2).

9c. Euthanasie en Humane eindpunten

For the collection of blood and muscle tissue at the end of the experiment, pentobarbitol (115mg/kg) will be administered i.p.. Cardiac puncture and cervical dislocation will assure death of the animal (SOP 2).

The animals will be checked daily. Attention will be given to grooming, body weight, and food and water consumption will be monitored. In case animals suffer from extreme discomfort (weight loss >20%), which is not expected considering the maximum of 48h that the experiment will last, mice will be euthanized by means of an i.p. pentobarbital injection (115mg/kg) followed by cardiac puncture prior to the end of the protocol.

Zorg

10a. Ongerief

Group	*1-4 (IT-NaCl)*	*5-8 (IT-LPS)*
Individual housing	max 72h; 1x; = 02	max 72h; 1x; = 02
anesthesia	20'; 1x; = 03	20'; 1x; = 03
Terminal anesthesia	1x; = 02	1x; = 02
Development of pulmonary inflammation	n.a.	max 48h; 1x =03
Total discomfort	**03**	**04**

10b. Welzijnsevaluatie
To monitor the health condition and discomfort levels of the animals, animals will be checked and weighed on a daily basis. Furthermore, the food intake will also be monitored daily. We have ample experience with this model, and the estimated discomfort level of the experiments with acute lung inflammation induced muscle atrophy turned out to be correct at the end of the previous experiments.

11. Verzorging en huisvesting

Animals will be housed under standard conditions (food and water ad libitum) in CPV headquarters for the entire duration of the experiment. Euthanasia and collection of muscle tissue will be performed in the CPV operating chambers or in the perating chamber

12. Deskundigheid
All individuals on this proposal have artikel 9 status. IT-instillations will be performed by the VO who has extensive experience with this technique.

13. Standard Operation Procedures (SOP):
SOPs for anaesthesia/IT-instillation and euthanasia/excision of skeletal muscles have been below, and have previously been applied in DEC2004-112 and DEC2006-175, and DEC2007-040 respectively.

SOP 1: **Anaesthesia and intra-tracheal instillation of NaCl and LPS**

Doel
LPS wordt intratracheaal toegediend om een acute (na 1x toediening) of een chronische (na herhaaldelijke toediening) ontsteking in de longen van muizen te induceren. Hiervoor wordt de muis verdoofd met xylazine/ketamine s.c. en in een vertikale opstelling gebracht. Met behulp van een Hamilton naald wordt 20 µg LPS in 50 µl 0,9% NaCl via een canule in de trachea toegediend. De muis blijft vervolgens nog 10 minuten in de vertikale opstelling om een goede verdeling van de vloeistof in de longen te bewerkstelligen.

Benodigdheden
- muizen
- xylazine (20 mg/ml, Sedamun, AUV Cuijck, bestellen via CVP)
- ketamine (100 mg/ml Nimatek, AUV Cuijck, bestellen via CVP)
- steriele 0,9 % NaCl
- E.coli LPS O55:B5 (Sigma, 1 mg/ml opgelost in 0,9% NaCl)
- 1 ml spuiten
- injectienaalden (oranje, G25)
- Canules (blauwe Venflon2, bestellen via AZM)
- Hamilton spuiten (50 µl)
- weegschaal (CPV)
- opstelling voor IT toediening
- tissues
- kleine botte pincet
- wekkertje
- vortex
- oogzalf

Voorbereidingen
Xylazine/Ketamine mix maken verdund in 0,9% NaCl, eindconc. xylazine is 1 mg/ml , eindconc. ketamine is 25 mg/ml
Verdun 400 µl LPS stock (1 mg/ml) met 600 µl 0,9% NaCl, eindconcentratie is 400 µg/ml.
Zet op het CVP de opstelling klaar :

- opstelling IT toediening
- wekkertje
- anaesthesie
- vortex
- schone instrumenten

Werkwijze

1. Weeg de muis.
2. Verdoof de muis met xylazine (3 mg/kg,s.c.) en ketamine (75 mg/kg s.c.) in mix.
3. Vortex LPS oplossing gedurende 5 minuten.
4. Controleer na 5 min of muis verdoofd is.
5. Breng oogzalf aan.
6. Vul Hamilton spuit met 50 µl gevortexede LPS of placebo (0,9% NaCl).
7. Hang de muis loodrecht in de vertikale opstelling.
8. Trek met pincet de tong iets uit de mond om stikken te voorkomen.
9. Trek met linkerhand tong iets naar voren en schuif met rechterhand caule in trachea.
10. Fixeer canule met linkerhand en schuif met rechterhand Hamilton spuit in canule totdat weerstand wordt gevoeld.
11. Injecteer snel en krachtig 25 µl en schuif de Hamilton spuit uit de canule.

SOP2: Euthanasia, blood collection and excising hindlimb muscles

Materials

- Surgical equipment; tweezers, scissors
- 70% ethanol and paper tissues
- Pentobarbital
- Cryovials
- Blood collection vials
- OCT
- 1-methyl butane
- Liquid nitrogen

Method:

1. Euthanize the mouse by i.p. injection of 115mg/kg pentobarbital
2. Total blood will be drawn from the mouse by cardiac puncture.
3. make a circumventing incision in the skin around the ankle joint; make sure not to cut the Achilles tendon
4. Make medial incision in the skin of the hindlimb, and remove the skin up to ± 1cm above the knee joint
5. Remove the upper hindlimb musculature, which covers the lower part of the hindlimb; the gastrocnemius (G) is completely uncovered now
6. Stretch the limb, and cut the Achilles tendon, while holding it with tweezers
7. Pull the muscle down towards the knee joint, while holding the foot
8. Excise the soleus (S) muscle from the G and plantaris (P) complex
9. Excise the GP complex by cutting the 2 tendons connected to the knee joint
10. Excise the P from the G muscle
11. Collect these muscles from both hindlimbs and measure their pared weights
12. For RNA or protein lysate purposes: collect the muscles in cryovials and snap-freeze liquid nitrogen and store at -80°C. For immunohistochemistry: embed the muscles (stretched) in OCT and freeze in 1-methyl butane and store at -80°C
13. Discard the mouse cadaver according to CPV regulations

University Maastricht

Faculty of Health, Medicine and Life Sciences

Dierexperimenten Commissie

DEC

voorzitter
p/a Secretariaat DEC-UM
Postbus 616
NL-6200 MD Maastricht
Telefoon: 04

Aan:

Uw referentie:

Onze referentie :

Maastricht, 29-06-2011

Geachte Onderzoeker,

Uw projectaanvraag: "*Investigation of the role of Glycogen Synthase Kinase-3 bèta (GSK-3β) in acute pulmonary inflammation-associated muscle atrophy*", is op de DEC vergadering van 24 juni 2011 besproken.

De DEC heeft een aantal vragen en opmerkingen:

- De DEC verzoekt de kolommen op het voorblad op te splitsen, je kunt niet 2 coderingen in een kolom gebruiken (bijzondere technieken)
- De DEC verzoekt over punt 9a contact op te nemen met de proefdierdeskundige.

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 **2011-095**, gelieve dit nummer in verdere correspondentie te vermelden.

Hoogachtend,

Voorzitter DEC-UM

Maastricht University

Faculty of Health, Medicine and Life Sciences

Aan

Ons kenmerk	*Doorkiesnummer*	*Maastricht*
		05-07-2011

Project: *Investigation of the role of Glycogen Synthase Kinase-3 bèta (GSK-3β) in acute pulmonary inflammation-associated muscle atrophy.*

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:	**2011-095**
Diersoort:	**muis**
Aantal dieren:	**80**
Einddatum:	**05-07-2015**

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.

Voorzitter DEC-UM

Vicevoorzitter DEC-UM

DEC-UM
Voorzitter DEC-UM

p/a secretariaat DEC-UM

Secretariaat DEC-UM
T (04

Bezoekadres

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