Growth Hormone & IGF Research 42–43 (2018) 52–57

Contents lists available at ScienceDirect

Growth Hormone & IGF Research

journal homepage: www.elsevier.com/locate/ghir

Standardizing protocols dealing with growth hormone receptor gene disruption in mice using the Cre-lox system

Silvana Duran-Ortiz[a,b,c], Stephen Bell[a], John J. Kopchick[a,c,d,*]

[a] Edison Biotechnology Institute, Heritage College of Osteopathic Medicine, Ohio University, Athens, OH 45701, United States
[b] Department of Biological Sciences, College of Arts and Sciences, Heritage College of Osteopathic Medicine, Ohio University, Athens, OH 45701, United States
[c] Molecular and Cellular Biology Program, Heritage College of Osteopathic Medicine, Ohio University, Athens, OH 45701, United States
[d] Department of Biomedical Sciences, Heritage College of Osteopathic Medicine, Ohio University, Athens, OH 45701, United States

ARTICLE INFO

Keywords:
Growth hormone
GHRKO mice
GHR−/− mice
iC-GHRKO
aGHRKO
Tamoxifen
Cre-lox

ABSTRACT

Objective: Mice and humans with reduced growth hormone (GH) action before birth are conferred positive health- and life-span advantages. However, little work has been performed to study the effect of conditional disruption of GH action in adult life. With this as our objective, we sought to elucidate a reproducible protocol that allows generation of adult mice with a global disruption of the GH receptor (*Ghr*) gene, using the tamoxifen (TAM)-inducible Cre-lox system, driven by the ROSA26 enhancer/promoter. Here we report the optimum conditions for the gene disruption.

Design: Six month old mice, homozygous for the ROSA26-Cre and the *Ghr*-floxed gene, were injected, once daily for five days with four distinct TAM doses (from 0.08 to 0.32 mg of TAM/g of body weight). To evaluate the most effective TAM dose that leads to global disruption of the GHR, mRNA expression of the *Ghr* and insulin growth factor-1 (*Igf1*) genes were assessed in liver, adipose tissue, kidney, and skeletal and cardiac muscles of experimental and control mice. Additionally, serum GH and IGF-1 levels were evaluated one month after TAM injections in both, TAM-treated and TAM-untreated control mice.

Results: A dose of 0.25 mg of TAM/g of body weight was sufficient to significantly reduce the *Ghr* and *Igf1* expression levels in the liver, fat, kidney, and skeletal and cardiac muscle of six-month old mice that are homozygous for the *Ghr* floxed gene and Cre recombinase. The reduction of the *Ghr* mRNA levels of the TAM-treated mice was variable between tissues, with liver and adipose tissue showing the lowest and skeletal and cardiac muscle the highest levels of *Ghr* gene expression when compared to control mice. Moreover, liver tissue showed the 'best' *Ghr* gene disruption, resulting in decreased total circulating IGF-1 levels while GH levels were increased versus control mice.

Conclusion: The results show that in mice at six months of age, a total TAM dose of at least 0.25 mg of TAM/g of body weight is needed for a global downregulation of *Ghr* gene expression with a regimen of 100 μL intraperitoneal (ip) TAM injections, once daily for five consecutive days. Furthermore, we found that even though this system does not achieve an equivalent disruption of the *Ghr* between tissues, the circulating IGF-1 is > 95% decreased. This work helped to create adult mice with a global GHR knockdown.

1. Introduction

Studies in worms, fruit flies and mice have shown that a reduction of growth hormone (GH) and insulin growth factor -1 (GH/IGF-1) levels have positive health benefits and often results in increased longevity [1]. Besides the increased life-span, mice that have a germline mutation in the GH receptor (*Ghr*) gene, namely, GHR knockout (GHRKO) or GHR gene disrupted (GHR−/−) mice have also shown health benefits. These mice are dwarf, obese, with low circulating IGF-1 and insulin levels, display high insulin sensitivity, have low rates of cancer, and are resistant to obesity induced Type 2 diabetes [2]. Furthermore, the GHR −/− mice show a similar phenotype to humans that have a condition

Abbreviations: GH, growth hormone; GHR, growth hormone receptor; GHRKO, growth hormone receptor knockout; GHR−/−, growth hormone receptor disrupted; IGF-1, insulin-like growth factor 1; Subq, subcutaneous; quad, quadriceps; TAM, tamoxifen; iC-GHRKO, inducible heart-specific growth hormone receptor knockout; aGHRKO, global adult growth hormone receptor knockout; ip, intraperitoneal.

* Corresponding author at: Department of Biomedical Sciences, Ohio University, Athens, OH 45701, United States.
E-mail addresses: sd504111@ohio.edu (S. Duran-Ortiz), bells1@ohio.edu (S. Bell), kopchick@ohio.edu (J.J. Kopchick).

https://doi.org/10.1016/j.ghir.2018.08.003
Received 10 May 2018; Received in revised form 19 July 2018; Accepted 27 August 2018
Available online 29 August 2018

called Laron Syndrome (LS) in which the patients are GH resistant with low levels of IGF-1, high levels of GH, and are obese, dwarf, and resistant to the development of diabetes and cancer [3,4]. Therefore, it has been hypothesized that impaired GH induced signaling could positively influence longevity and overall health of both humans and animals [5]. It is important to note that besides LS patients, other individuals with GH deficiencies due to Prop1 mutations or isolated GH deficiency, have shown reduced or unaltered longevity [6–10]. Nonetheless, both LS and GH-deficient humans are protected from age-related diseases [8,11].

Since mouse and human studies have indicated that decreased GH action could increase health- and life span, drugs that could decrease GH have been proposed as an anti-aging medication. In fact, a 2013 workshop held in Erice, Italy comprising of leading experts in the field of aging research reached the conclusion that the most promising strategy to extend health- and life-span was "pharmacological inhibition of the GH/IGF-1 axis" [12].

On the other hand, as humans age, their circulating levels of GH decrease such that by 60 years of age, the amounts of GH circulating in the blood are greatly reduced [13]. This phenomenon is known as somatopause since the decreased skin thickness, increased fat deposits, and loss of lean mass in older humans has been attributed to the reduced circulating GH [13,14]. It has been suggested that GH treatment for aging patients may improve their body composition and possibly health markers [15]. Nevertheless, concerns exist with regards to elevated GH levels in both humans and mice, which have been shown to cause an increased risk of developing cancer and diabetes mellitus [16,17].

Most of the physiological studies that address the effects of GH resistance are performed in LS patients and in GHRKO mice, where decreased GH action occurs since birth. Besides Laron syndrome patients, a Dutch cohort between 85 and 100 years of age with polymorphisms that decrease the GH/IGF-1 axis have also shown increased lifespan, while the Ashkenazi Jewish centenarians with exceptional longevity have mutations in the IGF-1 receptor gene, resulting in a decrease in circulating IGF-1 [18–21]. Therefore, mice and human studies support the notion that opposed to the popular belief that GH is 'good' for the elderly as stated above, reduced GH action in adults may actually result in increased health and longevity. But, few data exists related to the hypothesis that reducing GH action in adult life may be beneficial in terms of health- and perhaps life-span [22–24]. Thus, we and others have set out to study the effects of disrupting GH action in adult life in order to elucidate the physiological and molecular implications of this disruption [22,23,25,26]. Fortunately, molecular mechanisms to conditionally disrupt or introduce specific genes at a specific time in development have been established [27].

One of the technologies to disrupt specific genes in a time and tissue specific controlled manner is the tamoxifen (TAM) inducible Cre-lox system. This method involves the expression of the bacteriophage Cre recombinase driven by a specific promoter/enhancer. Cre recombinase can recognize 34 bp LoxP sites (placed flanking the gene that will be ablated also known as the 'floxed' gene) and induce recombination between these sites. Therefore, mice that express both the Cre recombinase and the floxed gene are capable of gene disruption [28]. The main advantage of this system is that it allows the conditional disruption of genes within specific tissues and at a specific desired age [28]. However, a peculiarity of the TAM inducible Cre-driver system is that the TAM dose that is effective to induce the recombination to ablate the target gene must be determined experimentally to 'fit' the specific characteristics of the mouse model [29]. According to Jackson laboratory indications, the use of different promoters/enhancers, and the age at which recombination is induced can play a role in the TAM dosage required for recombination to occur [30]. Therefore, the goal of this study was to determine the maximal conditions for generating adult mice with GHR disrupted globally at six months of age using the TAM-inducible Cre-lox technique, these mice will be referred as adult-onset-6 months GHR knock down (a6mGHRKD) mice.

2. Methodology

2.1. Mouse housing and breeding

Mice carrying a "floxed" *Ghr* allele flanking exon four of the *Ghr*, were generated by the Knockout Mouse Project and have been previously described (20, 21). Mice that express an inducible ubiquitous Cre recombinase gene driven by the ROSA26 gene promoter/enhancer (ROSA26-Cre-ERT2) [B6.129Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J mice] were purchased from The Jackson Laboratory (22–24). C57BL/6 mice were bred to homozygosity for both the floxed *Ghr* and the Cre alleles as described [23]. Mice were housed at 22 °C under a 14-h light, 10-h dark cycle, 3–4 mice per cage, with ad libitum access to water and standard laboratory chow (ProLab RMH 3000). All experiments were approved by the Ohio University Institutional Animal Care and Use Committee.

2.2. TAM treatment regimen

Mice homozygous for Cre and LoxP sites were used to determine the minimum TAM dose that will result in *Ghr* recombination. Five separate groups of male and female mice (six mice per group) were used. Once the animals reached six months of age, four of the groups were treated with varying doses of TAM, while one of the groups was injected with peanut oil (vehicle) as a control (Table 1). To induce *Ghr* gene disruption, mice received ~100 μL ip injections of TAM dissolved in peanut oil, once per day, over five consecutive days.

2.3. Global Ghr disruption

Ghr and Igf1 gene expression of liver, kidney, subcutaneous (subq) adipose tissue, quadriceps (quad) skeletal muscle, and heart was measured in TAM-treated and control mice. Mice were sacrificed one month after TAM or peanut oil injections using CO_2, dissected and organ harvested. Collected organs were snap frozen in liquid nitrogen and stored at −80 °C. At the time of dissection, blood collection was performed via bleeding from the retro-orbital sinus.

For RNA isolation, frozen tissues were homogenized using a Precellys 24-Dual homogenizer, and RNA isolation was performed using the Thermo Scientific™ GeneJET RNA Purification Kit following manufacturer's instructions. cDNA synthesis employed the Maxima First Strand cDNA Synthesis kit. RT-quantitative PCR (RTqPCR) to evaluate *Ghr* and *Igf1* gene expression was performed using Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific). Two references target genes were used for normalization: *Hprt*: Forward: 5′-ATCAGTCAACGGGGGACATA-3′ Reverse 5′-AGAGGTCCTTTTCACC AGCA-3′, and *Rpl38:* Forward: 5′- CGCGTCGCCATGCCTCGGAA-3′ Reverse 5′- ACTTGGCATCCTTCCGCCGGG -3′. Data analysis was performed using qBasePlus software. Additionally, serum levels of GH and IGF-1 were evaluated using the mouse/rat Elisa kits from Alpco.

Table 1
TAM concentrations used to standardize the ablation of the *Ghr* gene in male and female mice of six months of age.

TAM concentration (mg/g of body weight)	N
0 (control)	6
0.08	6
0.16	6
0.25	6
0.32	6
Total mice	30