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Caloric Restriction and Growth Hormone Receptor Knockout: Effects on Expression of Genes Involved in Insulin Action in the Heart

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Abstract

Blockade of growth hormone (GH), decreased insulin-like growth factor-1 (IGF1) action and increased insulin sensitivity are associated with life extension and an apparent slowing of the aging process. We examined expression of genes involved in insulin action, IR, IRS1, IRS2, IGF1, IGF1R, GLUT4, PPARs and RXRs in the hearts of normal and GHR^{-/-} (KO) mice fed *ad libitum* or subjected to 30% caloric restriction (CR). CR increased the cardiac expression of IR, IRS1, IGF1, IGF1R and GLUT4 in normal mice and IRS1, GLUT4, PPAR α and PPAR β/δ in GHR-KO animals. Expression of IR, IRS1, IRS2, IGF1, GLUT4, PPAR γ and PPAR α did not differ between GHR-KO and normal mice. These unexpected results suggest that CR may lead to major modifications of insulin action in the heart, but high insulin sensitivity of GHR-KO mice is not associated with alterations in the levels of most of the examined molecules related to intracellular insulin signaling.

Keywords

Caloric restriction; aging; GHR-KO; insulin; fatty acid

INTRODUCTION

Growth hormone (GH) resistance or insensitivity with low circulating levels of insulin-like growth factor-1 (IGF1), insulin and glucose, all of which imply increased insulin sensitivity, are among the key characteristics of long-lived GH receptor/binding protein knockout (GHR-KO) mice (1,2). These knockout mice live approximately 40% longer than their normal siblings (3). Caloric restriction (CR) is known to increase lifespan and delay aging (4). The effects of CR are very similar to key phenotypic characteristics of GHR-KO mice. The similarities include reductions in body weight, plasma levels of insulin, IGF1, glucose and thyroid hormone. Results obtained in animals subjected to CR and in various long-lived mutants suggest that perturbations in the insulin signaling pathway may play a key role in

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extended longevity (5). We have recently described the effects of long-term 30% CR on the expression of genes related to insulin and IGF1 signaling in the liver and skeletal muscle of these animals (6-8). Effects of insulin on protein metabolism in skeletal muscle and the liver are believed to be important in regulating the substrate availability for the heart (9). Insulin resistance is associated with increased risk of chronic heart failure (10). Insulin action through AKT signaling is also responsible for physiological heart growth during postnatal development (11). The study in rat hearts showed that CR restores the cardio-protective effects of preconditioning by increasing tolerance to ischemia (12). In view of the strong dependence of heart functions on insulin actions, it was of interest to include this organ in our ongoing studies of the effects of CR in normal and in long-lived mutant mice.

In the present study, in order to analyze relationships between GH resistance, CR and insulin signaling in the heart, we have examined the effects of GHR-KO and CR on the expression of insulin receptor (IR), insulin receptor substrate 1 (IRS1), insulin receptor substrate 2 (IRS2), IGF1, glucose transporter 4 (GLUT4), peroxisome proliferator-activated receptor γ (PPAR γ), PPAR α , PPAR δ/β (also known as PPAR δ or PPAR β), retinoid X receptor γ (RXR γ), RXR α and RXR β/δ in cardiac muscle.

Insulin action depends on the presence of the IR on the cell surface. Insulin induced intracellular signaling starts by insulin binding to IR and results in autophosphorylation of IR. This action leads to phosphorylation of IRS1 and IRS2, which can bind p85 regulatory subunit of PI 3-kinase and cause activation of this enzyme (13,14). GLUT4 is a regulator of glucose uptake in muscle and has a role in the maintenance of whole body glucose homeostasis (15,16). As a result of activation of the insulin signaling pathway GLUT4 is translocated to the cell surface.

Genes related to IGF1 signaling were also of interest because prolonged longevity of different types of mutant mice, strongly suggests that IGF1 may play a key role in regulation and longevity (17-21).

In the present study we have also analyzed the expression of the PPARs family and the RXRs genes. PPARs are members of the nuclear receptor superfamily that are ligand-dependent transcription factors. These factors are known to regulate the expression of target genes by binding to specific peroxisome proliferator response elements (PPREs) in enhancer sites of regulated genes. PPARs require RXRs to form heterodimers, which allow binding to their specific PPREs (22). There are three known isoforms in the PPAR superfamily, each encoded by a separate gene: PPAR γ , PPAR α and PPAR δ/β . The most explored gene of this superfamily is PPAR γ . PPAR γ is the target receptor for thiazolidinediones (TZDs), drugs used in type 2 diabetic patients as insulin sensitizers (23-26). PPAR α is highly expressed in the liver, skeletal muscle, heart and kidney, where it regulates a wide variety of target genes involved in cellular lipid catabolism and fatty acids β and ω -oxidation and containing PPREs in their promoter regions (27). In the heart, PPAR α activation results in the accumulation of myocardial lipids that leads to other features of diabetic cardiomyopathy (28). However, PPAR α deficient mice have increased levels of total and HDL cholesterol (29).

The function of PPAR β/δ is still unclear, but it is known to play an important role in epidermal maturation and skin wound healing (30, 31) and has a possible function in lipid metabolism (32). Retinoid X receptors have a potential to heterodimerize with many nuclear receptors. PPARs act by forming heterodimers with RXRs, which allows binding to their PPREs. RXR isoforms are encoded by three separate genes: RXR α , RXR β and RXR γ (33).

In this study, expression of genes involved in the initial steps of insulin pathway, PPARs and RXR families were analyzed in the heart muscle of normal and GHR-KO mice fed *ad libitum* (AL) or subjected to long-term CR. The data were also analyzed based on a

comparison of these findings to the expression levels of the same genes in the hearts of young, 3-month old GHR-KO mice, and in GH over-expressing transgenic mice. The results indicate that CR influences genes from the insulin pathway in the heart and potentially retards cardiac aging in these animals. The data also suggest that the high insulin sensitivity of GHR-KO mice is not related to alterations of this molecular pathway in the heart muscle.

MATERIALS AND METHODS

Animals

21-month-old calorically restricted mice—Normal and GHR-KO male mice, eight in each group, were subjected to 30% CR or fed AL starting at 8 weeks of age. The detailed protocol of the experiment was described previously (34,35). Because differences between genotypes and/or after CR treatment tend to be more pronounced in older animals, we continued caloric restriction for the period of 19 months till the animals reached the age of 21 months. The animals were anesthetized using isoflurane; blood was taken by cardiac puncture and the mice were killed by decapitation. Hearts were collected and rapidly frozen on dry ice.

Young GHR-KO mice—The hearts from additional eight GHR-KO and eight normal male mice aged 3 months were collected to examine age-dependent changes.

hGH transgenic mice—The hearts from seven male Mt-hGH transgenic (GH-Tg) mice and eight of their normal male siblings were collected to examine the effects of over-expressed GH on analyzed genes. These animals were produced in our breeding colony derived from transgenic breeders kindly provided by Dr. Thomas Wagner and Jeung Yun (36-38).

Extraction of mRNA and cDNA synthesis

After grinding the heart in liquid nitrogen, 1xPBS was added and total RNA was extracted from a 250 µl aliquot using phenol/chloroform procedure of Chomczynski and Sacchi (39). Using electrophoresis, the quantity and quality of total RNA was analyzed on agarose gel. Afterwards, cDNA was synthesized from 2 µg of total RNA using iScript™ cDNA Synthesis Kit (BIO-RAD) according to the manufacturer's protocol.

Real-Time PCR

The mRNA relative expression was analyzed by real-time PCR (RT-PCR) using the “Smart Cycler” instrument (Cepheid, Sunnyvale, CA) with iQ™ SYBR Green Supermix (BIO-RAD) (6,7). The three steps of the RT-PCR included denaturation at 94°C for 2 min, annealing at 62°C for 30 sec with fluorescence reading, and extension at 72°C for 30 sec. All primers used in the study are listed in table 1. To evaluate the potential of nonspecific products, a melting curve was done for each reaction. All samples were analyzed in a single reaction for each gene to avoid false differences caused by different efficiency in separate PCR reactions.

Two different housekeeping genes were tested for this study, 18S and β-2-microglobulin (B2M). The results indicated that B2M is more appropriate to be used in this study, because its expression was very similar in GHR-KO vs. normal and also in CR vs. AL mice. Additionally, we analyzed the data after normalizing with B2M and 18S and also used raw data of the genes of interest with no normalizing to see if the amount of the matrices used in each sample was similar. This test indicated that raw data were almost identical to data after normalizing with B2M, whereas after normalizing with 18S the results were markedly different. Relative expression from RT-PCR was calculated from the equation $2^{A-B/2^{C-D}}$

(where A = Cycle Threshold [Ct] number for the gene of interest in the first control sample; B = Ct number for the gene of interest in the analyzed sample; C = Ct number for the housekeeping gene in the first control sample; D = Ct number for housekeeping gene in the analyzed sample). The first control was expressed as 1.00 by this equation, and all other samples were calculated in relation to this value. Afterwards, the results in the control group (N-AL) were averaged, and all other outputs were divided by the mean value of the relative expression in the control group to yield the fold change of the genes of interest expression compared to the control group.

Western blots

After crushing the hearts in liquid nitrogen, adding equal volume of PBS and removing 250 μ l for RNA extraction, the remaining homogenate was used for protein extraction (35). Western blotting was performed using PPAR γ (Cell Signalling, Beverly, MA), PPAR α and PPAR δ antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) and protocol described earlier (35). After stripping the membrane, a monoclonal β -actin antibody (Sigma, St. Louis, MO) was used as a control for protein loading.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. To evaluate the effects of the phenotype and diet, two-way ANOVA was used followed by Fisher's PLSD. A t-test was used to evaluate the effects of diet within the phenotypes and phenotypes within diets. $P < 0.05$ was considered significant.

RESULTS

The levels of IR, IRS1 and IRS2 mRNA in the hearts did not differ between N-AL and KO-AL animals (Fig. 1 A, B, C). Caloric restriction increased IR and IRS1 RNA levels in normal animals ($P < .0193$ and $P < .005$, respectively) and IRS1 RNA levels in KO in comparison to the corresponding AL controls ($P < .02$). The level of IR RNA appeared to be higher in KO mice after CR but the difference did not reach statistical significance. IRS2 expression was not affected by phenotype or by CR in either group. Similarly to the findings in 21-month-old animals, IR RNA level also did not differ between young KO and N animals. However, IRS1 and IRS2 RNA levels were increased in young KO mice in comparison to their normal siblings ($P < .01$ and $P < .046$, respectively) (Fig. 2 A, B, C). In contrast to the findings in KO animals, IR and IRS1 RNA levels in short-lived hGH transgenic mice were significantly decreased in comparison to their normal siblings ($P < .0129$ and $P < .02$, respectively) (Fig. 2 D, E). The IRS2 RNA level also appeared to be decreased in hGH mice in comparison to their normal siblings, but it did not reach a significant level ($P < .514$) (Fig. 2 F).

Interestingly, cardiac IGF1 RNA levels did not differ between 21-month-old N-AL and KO-AL mice, but CR increased the level of this molecule in normal animals ($P < .0117$) (Fig. 1 D) with no effect in knockouts. However, the IGF1 RNA level was decreased in the heart tissue of young KO mice ($P < .01$), while it was not changed in hGH transgenic mice in comparison to their controls (3 A, B). IGF1R RNA was increased in KO-AL mice in comparison to N-AL controls ($P < .0374$) (Fig. 1 E). IGF1R RNA was also increased in N-CR animals as compared to N-AL mice ($P < .0427$). Young KO mice were also characterized by elevated level of IGF1R ($P < 0.0024$), while in hGH transgenic mice IGF1R was decreased in comparison to their normal controls ($P < .01$) (Fig. 3 C, D).

Although cardiac GLUT4 RNA levels did not differ between N-AL and KO-AL mice, the GLUT4 protein level in the heart was decreased in the KO-AL as compared to N-AL mice ($P < .0012$) (Fig. 4 A, B). Caloric restriction increased GLUT 4 RNA level in both normal

and KO mice ($P < .046$ and $P < .034$, respectively), but increased GLUT4 protein levels only in KO-CR animals in comparison to KO-AL mice ($P < .0012$). GLUT4 RNA levels did not differ between normal and KO hearts at the age of 3 months, and were decreased in hGH mice in comparison to normal siblings ($P < .0098$) (Fig. 4 C, D).

PPAR γ RNA levels appeared to be elevated in KO-AL in comparison to N-AL and also in both normal and KO animals after CR, but neither the effect of phenotype alone, nor the effects of CR alone reached statistical significance. A combination of GHR disruption and CR lead to a significant increase of PPAR γ mRNA levels (KO-CR vs. N-AL) (Fig 5 A). At the PPAR γ protein level, there was also no significant difference between the phenotypes, but numerical differences suggested a trend opposite to that detected at the RNA level, namely a reduction rather than an increase in KO-AL in comparison to N-AL. PPAR γ protein levels were significantly increased in KO-CR mice in comparison to KO-AL animals ($P < 0.028$) (Fig 5 B). Furthermore, caloric restriction increased PPAR α expression at both RNA and protein levels in KO-CR in comparison to KO-AL mice ($P < 0.0053$ and $P < 0.018$, respectively) (Fig. 5 C, D). The levels of PPAR α protein were decreased in the hearts of KO-AL mice in comparison to normal animals ($P < 0.0119$) with no corresponding changes in PPAR α RNA levels. The effects of phenotype on cardiac PPAR β/δ expression were, in general, opposite at the RNA and protein levels. The PPAR β/δ RNA was increased in KO-AL mice in comparison to their normal siblings ($P < 0.002$), but the protein product level was decreased in KO-AL in comparison to N-AL mice ($P < 0.0023$) (Fig. 5 E, F). Caloric restriction increased the level of PPAR β/δ protein only in KO animals ($P < 0.0147$), while a corresponding trend in the levels of PPAR β/δ RNA was not statistically significant. In young animals PPAR γ RNA levels did not differ between KO and normal mice (Fig. 6A). PPAR α and PPAR β/δ RNA levels were significantly increased in the hearts of young KO mice as compared to normal siblings ($P < 0.0339$ and $P < 0.048$, respectively) (Fig. 6 B, C). For both PPAR α and PPAR β/δ RNA, short living hGH mice showed alterations opposite to those found in KO mice, namely down regulation rather than increase in comparison to their normal siblings ($P < 0.0127$ and $P < 0.023$, respectively) (Fig. 6 E, F). The levels of PPAR γ RNA were also reduced in hGH mice ($P < 0.0023$) (Fig. 6 D).

The levels of RXR γ , RXR α and RXR β/δ mRNA were significantly increased in KO-AL mice in comparison to N-AL animals ($P < .0273$, $P < .0113$, and $P < .0314$, respectively) (Fig. 7 A, B, C). However, the only significant effect of CR on RXR expression was a decrease in RXR γ mRNA levels in KO-CR as compared to KO-AL mice (Fig. 7). RXR α and RXR β/δ RNA levels in young KO mice were also increased in comparison to their normal controls ($P < .0097$ and $P < .0375$, respectively) with no differences in RXR γ RNA levels (Fig. 8 A, B, C). The levels of RXR γ , RXR α and RXR β/δ RNA were significantly decreased in hGH transgenic mice in comparison to their normal siblings ($P < .0177$, $P < .021$ and $P < .024$, respectively) (Fig. 8 D, E, F).

DISCUSSION

In the present study, long-lived GHR/GHBP knockout (KO) mice have been subjected to long-term 30% CR. We have previously reported that this regimen of CR produces the expected reduction in body weight in both KO and normal mice (35). In the same study, CR down regulated circulating IGF1 levels in normal animals, while the extremely low levels of circulating IGF1 in both KO-AL and KO-CR animals were below the detection limits of the employed ELISA kit used in these studies (35). Moreover, CR significantly decreased blood glucose in both phenotypes, with no significant difference between KO and N mice fed *ad libitum* (N-AL 189.4 ± 9.6 , N-CR 139.7 ± 14.1 , KO-AL 155.0 ± 14.1 , and KO-CR 74.8 ± 11.5 , mg/dl) (35). It was also shown that the insulin level was significantly decreased in normal and KO animals after CR, while KO-AL animals had the expected reduction of insulin as

compared to N-AL mice. (N-AL 4.2 ± 0.7 , N-CR 1.4 ± 0.2 , KO-AL 0.9 ± 0.1 , and KO-CR 0.4 ± 0.1 , ng/ml) (35). The genotype (KO vs. N) and CR-induced increase in insulin sensitivity suggested by these findings was supported by the results of homeostasis model analysis (HOMA) (6). Studies in worms, flies and mice (including GHR-KO, Ames dwarfs and Snell dwarfs), as well as widely examined effects of CR in various animal species, strongly indicate insulin signaling as an important pathway in the control of aging (5).

Insulin Receptor

The first gene in the insulin signaling pathway is IR. In the heart muscle tissue, expression of this gene was not affected by the phenotype at either of the ages examined, with no differences between KO-AL and N-AL mice. This resembled our findings in skeletal muscle from the same animals (7). However, in the short-lived animals overexpressing the hGH, IR RNA levels were decreased. This could suggest that in old and young KO animals, IRs in either the heart or the skeletal muscle are unlikely to contribute to enhanced whole-body insulin sensitivity, while up regulation of this molecule in the liver of KO animals (7) can be related to improved whole-body insulin action. However, increased levels of IR in normal animals after CR and a similar trend in KO-CR mice, together with reduced expression of this gene in aged hGH mice, suggest that CR can protect insulin action in the hearts from the effects of aging. We base this suggestion on the fact that RT-PCR used in this study measures relative expression. Thus the findings would seem to imply that CR does not cause an increase in IR RNA level in the hearts of old KO and normal mice, but in both N-AL and KO-AL the IR RNA levels declines with age, resembling the findings in aged hGH transgenic mice. Thus it is tempting to speculate that CR maintains the content of IR at a level similar to that measured in the young mice and perhaps delays aging of the heart in these animals.

Insulin Receptor Substrate 1 and Insulin Receptor Substrate 2

The expression of IRS1 and IRS2 was apparently not affected by the phenotype in old KO animals when compared to normal controls thus resembling the findings on IR expression. However, in the hearts of young KO mice, IRS1 and IRS2 RNA levels were increased in comparison to their normal siblings. This suggests that IR expression is reduced with age in KO-AL to the level measured in N-AL mice, and it is likely the level of these messages continues to decline with age, as observed in the aged hGH mice. Significantly increased levels of IRS1 RNA in N-CR and KO-CR in comparison to their AL siblings can suggest that CR protects the hearts from aging. This suggestion is indirectly supported by the observations in young KO animals and in aged hGH mice.

The effects of CR and phenotype on IR, IRS1 and IRS2 RNA levels in cardiac muscle are opposite to those detected in the skeletal muscle, which was characterized by down regulation of IR, IRS1 and IRS2 after CR in both normal and knockout animals (7). They also differ from the findings in the liver, where no changes were observed after CR (7). We can conclude that the effects of the phenotype and CR on the expression of these insulin-related genes are organ specific and that effects of CR in different organs can be opposite.

Insulin-like Growth Factor-1 and Insulin-like Growth Factor-1-Receptor

The lack of difference in the cardiac expression of IGF1 RNA between long-lived KO and normal animals was surprising in view of the extremely low levels of circulating IGF1 (non-detectable by Elisa kit) and IGF1 mRNA in the liver of KO animals in comparison to their normal siblings (7). Down regulation of IGF1 biosynthesis in these animals related to the disrupted action of GH clearly does not apply to all organs. A previous study which used an independently derived line of GHR (-/-) mutant mice showed that hepatic and kidney IGF1 RNA levels were decreased without changes in IGF1 expression in other organs including

the heart (40). The levels of IGF1 RNA in the hippocampus of Ames dwarf mice do not differ from the levels measured in normal siblings, although Ames dwarf mice are characterized by suppressed hepatic IGF1 expression and circulating IGF1 levels (41). However, the cardiac expression of IGF1 in young KO mice was decreased in comparison to their normal siblings, which is closer to the findings in the skeletal muscle (7). Interestingly, IGF1R RNA levels were elevated in KO-AL mice in comparison to N-AL and also in young, 3-month-old KO mice, while hGH transgenic mice had reduced level of IGF1R message. Because IGF1R can activate IRS1 and IRS2 just like IR, this can explain higher levels of these substrates in young KO mice in which IR RNA levels were not elevated (42). Study of IGF1R transgenic mice showed that over expressed IGF1R can cause enlargement of the heart (42). In GHR-KO mice examined in the present study, elevated level of IGF1R in the cardiac muscle may compensate for the lack of GH action and thus allow normal heart development. In hGH transgenic animals, the decreased IGF1R was likely due to high levels of GH. Moreover, the expression of IRS1 and IRS2 in aged hGH transgenic mice was down regulated, probably because of low level of IR and IGF1R.

Glucose Transporter 4

Increased RNA expression of the GLUT4 gene in normal and KO animals after CR and the corresponding changes of GLUT4 protein in KO-CR animals suggest improved glucose transport in cardiac muscle in animals that had limited availability of food. This effect is the opposite of what we observed in the skeletal muscle of CR animals (7). Results obtained in young KO and normal mice indicate that GLUT4 RNA expression does not change with age. However, GH transgenic animals had significantly reduced levels of GLUT4 RNA, which indicates that an excess of GH can decrease glucose transport in cardiac muscle.

Peroxisome Proliferator-Activated Receptor γ

In the present study, the only significant effect of CR on PPAR γ expression was at the protein level in KO mice. PPAR γ RNA expression was not altered in young KO animals. Thus it is difficult to arrive at any conclusions concerning the possible role of cardiac PPAR γ in mediating the responses to GH resistance or CR. However, these data clearly indicate that cardiac muscle responds differently than skeletal muscle with respect to this gene. In the skeletal muscle, both CR and GHR knockout caused down regulation of PPAR γ gene expression (6). Concerning the possible cardio-protective effects of PPAR γ agonists, decreased level of this nuclear receptor in aged hGH transgenic animals together with a trend for PPAR γ RNA to be higher in old N-CR and KO-CR mice in comparison to their AL controls may imply anti-aging protection in the hearts of CR animals.

Peroxisome Proliferator-activated Receptor α

Chronic enhancement of PPAR α action in the heart was believed to cause myocardial lipid accumulation and other aspects of heart pathology associated with diabetes (27). Thus decreased PPAR α protein level in KO-AL in comparison to N-AL hearts could be related to enhanced insulin sensitivity of these animals, and could be suspected of protecting them from diabetic conditions. However, the levels of PPAR α RNA and protein were increased in KO-CR animals in comparison to KO-AL, and PPAR α RNA level also appeared to be elevated in N-CR mice when compared to N-AL. This indicates that CR may maintain the level of PPAR α in the cardiac muscle of old N and KO animals, thus resembling the situation in the heart of 3-month-old KO mice that have elevated levels of PPAR α RNA in comparison to normal controls. Moreover, the effects of CR on the expression of this nuclear receptor were opposite to the changes detected in aged hGH transgenic mice, in which PPAR α expression was reduced. Perhaps CR maintains the expression of PPAR α RNA in old animals at a level similar to those measured in 3-month-old mice.

Peroxisome Proliferator-activated Receptor β/δ

PPAR β/δ RNA levels were significantly higher while the protein levels were lower in long-lived KO mice in comparison to their normal siblings. This lack of correspondence between the data from Western blot and real-time PCR may reflect regulation at the post-transcriptional level, or differential stability of RNA and/or protein in normal and KO mice. Although, the function of PPAR β/δ in the heart is not known, it was shown that this nuclear receptor is expressed at a level similar to PPAR α , suggesting the possibility that it may control the expression of cardiac genes (27). In our study, PPAR β/δ protein was markedly increased in KO-CR in comparison to KO-AL mice, with a suggestive (though not statistically significant) trend in the same direction at the RNA level. These findings closely resemble the effects of GH resistance and CR on the levels of PPAR α RNA and protein. Similar to PPAR α and PPAR β/δ expression levels in old KO mice, young KO had increased level of PPAR β/δ , while in the short-living hGH animals the level of PPAR α RNA was significantly decreased.

Retinoid X receptors

The RNA expression of RXR γ , RXR α and RXR β/δ was increased in KO-AL mice in comparison to their normal siblings, which can imply increased potential to form heterodimers with PPARs, thus improving the actions of PPARs superfamily receptors in the heart muscle. Alterations in the expression of all three RXRs in young KO vs. young normal mice closely corresponded to the changes observed in the corresponding PPARs. In hGH transgenic mice, RXR γ , RXR α and RXR β/δ were decreased in comparison to their normal siblings, which mimicked the changes observed at the levels of PPARs.

Summary

In our study of genes involved in the initial steps of insulin action and PPARs nuclear receptor superfamily in the heart, we found that both the GHR gene knockout and the CR treatment that extend lifespan in mice serve as regulators of cardiac expression of these genes. Genotype and diet-induced alterations in the message levels and protein products of these genes in the heart were very different from the changes in the expression of the same genes in the skeletal muscle and liver described previously in the same animals. We found significant increase in the expression of analyzed genes in one or both phenotypes after CR. This outcome, taken together with the up regulation in young KO mice and suppression in short-lived hGH animals, suggests that CR may be protecting the hearts of these animals from the aging processes.

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Figure 1.

Expression of genes related to insulin signaling in cardiac muscle tissue of normal (N) and growth hormone receptor/binding protein knockout (GHR-KO) mice fed *ad libitum* (AL) or subjected to 30% caloric restriction (CR). The data from real-time PCR were normalized by housekeeping gene β -2-microglobulin (B2M) and expressed as the relative expression. Means $\pm$ SE. a,b,c – values that do not share the same letter in the superscript are statistically significant ($P < 0.05$)

(A) IR, (B) IRS1, (C) IRS2, (D) IGF1, (E) IGF1R

Figure 2.

Expression of genes related to insulin signaling in cardiac muscle tissue of normal (N) and growth hormone receptor/binding protein knockout (GHR-KO) mice fed *ad libitum* at the age of three months (left column), and of normal (N) and human growth hormone (hGH) transgenic mice fed *ad libitum* at the age of 9 months (right column). The data from real-time PCR were normalized by housekeeping gene β -2-microglobulin (B2M) and expressed as the relative expression. Means $\pm$ SE. a,b – values that do not share the same letter in the superscript are statistically significant ($P < 0.05$)

(A) IR - KO, (B) IRS1 - KO, (C) IRS2- KO, (D) IR-hGH, (E) IRS1-hGH, (F) IRS2- hGH

Figure 3.

Expression of IGF1 and IGF1R mRNA in cardiac muscle of normal (N) and GHR/BP knockout (KO) mice fed *ad libitum* at the age of 3 months old, and of normal and human growth hormone transgenic (hGH) mice at the age of 9 months. The data from real-time PCR were normalized by the housekeeping gene β -2-microglobulin (B2M). Means $\pm$ SEM. a,b – values that do not share the same letter in the superscript are significantly different ($P < 0.05$)

(A) IGF1 - KO (B) IGF1 - hGH, (C) IGF1R - KO, (D) IGF1R - hGH

Figure 4.

Expression of GLUT4 gene in cardiac muscle of normal (N) and GHR/BP knockout (KO) mice fed *ad libitum* (AL) or subjected to 30% caloric restriction (CR). (A) GLUT4 RNA expression, (B) GLUT4 protein level

Expression of GLUT4 mRNA in cardiac muscle of normal (N) and GHR/BP knockout (KO) mice fed *ad libitum* at the age of 3 months, and of normal and human growth hormone transgenic (hGH) mice at the age of 9 months. (C) GLUT4 - KO, (D) GLUT4 - hGH. The data from real-time PCR were normalized by the housekeeping gene β -2-microglobulin (B2M). Equal loading of protein for Western blots was verified using β -actin. Means $\pm$ SEM. a,b,c – values that do not share the same letter in the superscript are significantly different ($P < 0.05$)

Figure 5.

Expression of PPARs superfamily genes in cardiac muscle tissue of normal (N) and GHR/BP knockout (KO) mice fed *ad libitum* (AL) or subjected to 30% caloric restriction (CR). The data from real time PCR were normalized by the housekeeping gene β -2-microglobulin (B2M). Equal loading of protein for Western blots was verified using β -actin. Means $\pm$ SEM. a,b,c – values that do not share the same letter in the superscript are significantly different ($P < 0.05$)

(A) PPAR γ RNA expression, (B) PPAR γ protein level, (C) PPAR α RNA expression, (D) PPAR α protein level, (E) PPAR β/δ RNA expression, (F) PPAR β/δ protein level

Figure 6.

Expression of PPARs superfamily genes in cardiac muscle tissue of normal (N) and growth hormone receptor/binding protein knockout (GHR-KO) mice fed *ad libitum* at the age of three months (left column), and of normal (N) and human growth hormone (hGH) transgenic mice fed *ad libitum* at the age of 9 months (right column). The data from real-time PCR were normalized by housekeeping gene β -2-microglobulin (B2M) and expressed as the relative expression. Means $\pm$ SE. a,b – values that do not share the same letter in the superscript are statistically significant ($P < 0.05$)

(A) PPAR γ - KO, (B) PPAR α - KO, (C) PPAR β/δ - KO, (D) PPAR γ -hGH, (E) PPAR α -hGH, (F) PPAR β/δ - hGH

Figure 7.

Expression of RXR genes in cardiac muscle tissue of normal (N) and GHR/BP knockout (KO) mice fed *ad libitum* (AL) or subjected to 30% caloric restriction (CR). The data from real time PCR were normalized by housekeeping gene β -2-microglobulin (B2M). Means $\pm$ SEM. a,b,c – values that do not share the same letter in the superscript are significantly different ($P < 0.05$). (A) RXR γ , (B) RXR α , (C) RXR β/δ

Figure 8.

Expression of RXR genes in cardiac muscle tissue of normal (N) and growth hormone receptor/binding protein knockout (GHR-KO) mice fed *ad libitum* at the age of three months (left column), and of normal (N) and human growth hormone (hGH) transgenic mice fed *ad libitum* at the age of 9 months (right column). The data from real-time PCR were normalized by housekeeping gene, β -2-microglobulin (B2M) and expressed as the relative expression. Means $\pm$ SE. a,b – values that do not share the same letter in the superscript are statistically significant ($P < 0.05$)

(A) RXR γ - KO, (B) RXR α - KO, (C) RXR β/δ - KO, (D) RXR γ -hGH, (E) RXR α -hGH, (F) RXR β/δ - hGH

Table 1

Sequence of the primers used for real-time PCR. There is a gene bank sequence number by each forward primer.

IR- forward (NM_010568)	5' - ATGAGGCCAACCTTCCTGGAA
IR- backward	5' - ACGGGACATTCTCCATGTCT
IRS1- forward (L24563)	5' - AGCCCCAAAAGCCCAGGAGAATA
IRS1- backward	5' - TTCCGAGCCAGTCTCTTCTCTA
IRS2- forward (AF090738)	5' - AGTAAACGGAGGTGGCTACA
IRS2- backward	5' - AAGCTGCTGAGAAGTCAGGT
GLUT4- forward (AB008453)	5' - ATTGGCATTCTGGTTGCCCA
GLUT4-backward	5' - GGTTCGCGATGATGTAGAGGTA
IGF1- forward (NM_010512)	5' - CTGAGCTGGTGGATGCTCTT
IGF1- backward	5' - CACTCATCCACAATGCCTGT
IGF1-R – forward (AF056187)	5' - ATTGCCTCGGAGTTGGAGAA
IGF1-R - backward	5' - AGCTGCTGCAAGTTCTGGTT
PPAR γ - forward (NM_011146)	5' - CAAAGTAGAACCTGCATCTCC
PPAR γ - backward	5' - CCTTCACAAGCATGAACTCC
PPAR α - forward (NM_011144)	5' - CGAAGCCTACCTGAAGAACTT
PPAR α - backward	5' - ATCTTGCCACAAGCGTCTT
PPAR β/δ - forward (NM_011145)	5' - CTCGAGTATGAGAAGTGCGA
PPAR β/δ - backward	5' - CATCCGTCCAAAGCGGATAG
RXR γ - forward (M84819)	5' - TACCAGAAGTGCCTGGTCAT
RXR γ - backward	5' - TTCCACAGCAAGTTCGGCTT
RXR α - forward (M84817)	5' - GACATACGTGGAGGCAAACA
RXR α - backward	5' - AGGATGACCTGGTCGTCTAG
RXR β - forward (X72017)	5' - CCTCCTCTCACGACTATTAG
RXR β - backward	5' - GGACAGGAGAGACAAGAAGA
B2M- forward (NM_009735)	5' - AAGTATACTCACGCCACCCA
B2M- backward	5' - AAGACCAGTCCTTGCTGAAG