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RENAL PRO-APOPTOTIC PROTEINS ARE REDUCED BY GROWTH HORMONE RESISTANCE BUT NOT BY VISCERAL FAT REMOVAL

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Abstract

The growth hormone (GH) receptor knockout (GHRKO) mice are highly insulin sensitive and long-lived. Surgical visceral fat removal (VFR) improves insulin signaling in normal mice and rats and extends longevity in rats. We have previously demonstrated decreased expression of certain pro-apoptotic genes in kidneys of GHRKO mice, and suggested that this may contribute to the increased longevity of these animals. The aim of the present study was to examine the level of the following proteins: caspase-3, caspase-9, caspase-8, bax, bad, phospho-bad (p-bad), bcl-2, Smac/DIABLO, Apaf-1, phospho-p53 (pp53) and cytochrome c (cyc) in male GHRKO and normal (N) mice subjected to VFR or sham surgery, at approximately 6 months of age. The kidneys were collected 2 months after VFR. Results: Caspase-3, caspase-8, bax, bad, Smac/DIABLO, Apaf-1 and pp53 levels were decreased in GHRKO mice as compared to N animals. VFR did not change the level of any of the examined proteins. Conclusion: Decreased renal levels of pro-apoptotic proteins may contribute to extended lifespan due to targeted disruption of GH receptor (Ghr) gene but are apparently not involved in mediating the effects of VFR.

Keywords

apoptosis; caspases; GHRKO mice; kidney; proteins; visceral fat removal

INTRODUCTION

Mice homozygous for the targeted disruption of the growth hormone (GH) receptor/GH binding protein (Ghr) gene (GH receptor/GH binding protein knockout mice; GHRKO; KO) live longer than their normal (N) siblings and are characterized by reduced weight and body size, not detectable level of GH receptor, high level of serum GH, greatly reduced plasma levels of IGF-I and insulin, improved insulin sensitivity, low or normal glucose and improved oxidative stress resistance with reduced oxidative damage (Bartke and Brown-

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Borg 2004; Bartke et al. 2002; Coschigano et al. 2000; Coschigano et al. 2003; Coschigano 2006; Harper et al. 2006; Kopchick and Laron 1999; Liu et al. 2004; Salmon et al. 2005; Sun et al. 2009; Zhou et al. 1997). Furthermore, GHRKO mice have lower incidence and delayed onset of fatal neoplastic diseases (Ikeno et al. 2009).

Surgical visceral fat removal (VFR) is an intervention which has been reported to improve insulin signaling in N mice and rats and extend longevity in rats (Barzilai et al. 1999; Muzumdar et al. 2008; Shi et al. 2007). Therefore, this procedure mimics the effects of calorie restriction (CR). In contrast to the reported beneficial effects of VFR in genetically normal animals, this intervention paradoxically promoted insulin resistance in GHRKO mice (Masternak et al., unpublished data). The beneficial effects of VFR on insulin signaling in N mice and its detrimental impact in KO animals were detected by measuring insulin and glucose tolerance. In N mice, improved insulin signaling after VFR was associated with and likely due to a significant decrease of fasted insulin level and of inhibitory insulin receptor substrate 1 (IRS1) phosphorylation at Serine307 with a concomitant increase of the activating (pY1158) phosphorylation of insulin receptor (Masternak et al., unpublished data). Adipose tissue produces various pro-inflammatory cytokines, including tumor necrosis factor α (TNF α), interleukin-6 (IL-6) and interleukin 1 β (IL-1 β) which may lead to insulin resistance through alterations in insulin sensitivity (Fasshauer and Paschke 2003; Lagathu et al. 2003; Zhang et al. 2001). For this reason, among others, adipose tissue is considered to have important role in the regulation of aging. Therefore, surgical removal of visceral adipose tissue can be expected to improve insulin signaling and potentially have beneficial effects for aging delay. One of adipose tissue hormones - adiponectin is known to improve insulin sensitivity, exert anti-inflammatory effects and promote cell survival. Decreased plasma adiponectin level has been shown to increase insulin resistance and may constitute a risk factor for metabolic syndrome development (Renaldi et al., 2009). Interestingly, Holland et al. (2010) demonstrated that adiponectin may exert anti-apoptotic effects through stimulation of ceramidase activity, and as a result of that, enhanced ceramide catabolism and formation of its anti-apoptotic metabolite – sphingosine-1-phosphate. Moreover, it should be emphasized that pathological changes in kidneys are a very common cause of death in aging mice. Thus, it was of interest to assess the effects of VFR on various renal components of the apoptotic system in GHRKO mice which are characterized by increased plasma levels of adiponectin.

Apoptosis is a physiological process in which cells play an active role in their own death and is involved in the regulation of age-related functional alterations. Two central apoptotic signaling pathways are known: intrinsic (mitochondrial) (involving p53, bax, cytochrome c and caspase-9) - initiated by different factors within the cell (Boatright and Salvesen 2003; Budihardjo et al. 1999; Jin and El-Deiry 2005), and extrinsic (death receptor) pathway (involving caspase-8) (Boatright and Salvesen 2003), whereas caspase-3 is a main effector (executioner) caspase. Smac/DIABLO is a mitochondrial protein that, after a release from mitochondria into cytosol, potentiates apoptosis (Henry-Mowatt et al. 2004; Srinivasula et al. 2000). Anti-apoptotic bcl-2 prevents permeabilization of the outer mitochondrial membrane by inhibiting the action of the pro-apoptotic proteins (Ashkenazi 2008). Disturbed ratio between pro- and anti-apoptotic factors leads to release of cytochrome C into the cytosol through pores in the mitochondrial membrane. Finally, a multi-protein complex, named the apoptosome – consisting of cytochrome C, procaspase-9 and Apaf-1 (apoptotic protease activating factor-1) – is formed (Adrain and Martin 2001; Zou et al. 1999). Formation of the apoptosome leads to activation of the caspase cascade.

We have previously demonstrated reduced renal expression level of certain pro-apoptotic genes in GHRKO vs. N mice (Gesing et al., unpublished data). Therefore, it could be hypothesized that these alterations may contribute to the regulation of longevity in GHRKO

mice. Interestingly, Tao et al. (2008) demonstrated that the double-mutants produced by crossing mice with caspase-3 gene deletion ($\text{casp3}^{-/-}$) and mice harboring the congenital polycystic kidney (cpk) mutation lived longer than control cpk animals. Importantly, changes in gene expression are often not associated with changes in the levels of the corresponding proteins (due to various post-transcriptional and post-translational events).

For all these reasons, it was important to quantify the renal levels of the following apoptosis-related proteins: caspase-3, caspase-9, caspase-8, bax, bad, phospho-bad (pbad; phosphorylation at Ser112), bcl-2, Smac/DIABLO, Apaf-1, phospho-p53 (pp53; phosphorylation at Ser15) and cytochrome c (cyc) in KO and N mice after VFR or sham surgery.

RESULTS

The levels of caspase-3 (Figure 1A), caspase-8 (Figure 1B), bax (Figure 1C), bad (Figure 1D), Smac/DIABLO (Figure 2A), Apaf-1 (Figure 2B) and pp53 (Figure 2C) proteins were decreased in kidneys of KO (pooled KO-sham and KO-VFR) mice as compared to normal (N) (pooled N-sham and N-VFR) animals ($p=0.001$, $p=0.009$, $p=0.002$, $p=0.015$, $p=0.02$, $p=0.047$, $p=0.003$, respectively). In comparisons based only on sham-operated mice, KO-sham mice exhibited decreased caspase-3 (Figure 1A), caspase-8 (Figure 1B), bax (Figure 1C) and pp53 (Figure 2C) protein level in comparison to N-sham animals ($p=0.009$, $p=0.02$, $p=0.001$, $p=0.017$, respectively). Furthermore, bax protein level was diminished in N-VFR as compared to N-sham mice ($p=0.008$) (Figure 1C).

In comparison to N animals, GHRKO mice had increased renal bcl-2 (Figure 3A) and pbad (Figure 3B) protein level in the kidneys ($p<0.001$, $p=0.038$, respectively). KO-VFR mice exhibited the elevated bcl-2 protein level as compared to KO-sham and N-VFR animals (Figure 3A) ($p=0.011$, $p<0.001$, respectively).

No differences in caspase-9 (Figure 4A) and cyc (Figure 4B) protein levels between N and KO mice were detected. Nevertheless, renal caspase-9 protein in KO-sham mice was decreased in comparison to KO-VFR and N-sham animals (Figure 4A) ($p=0.001$, $p=0.002$, respectively).

VFR (pooled N-VFR and KO-VFR) did not change the renal level of the examined proteins as compared to sham surgery (pooled N-sham and KO-sham) (Figures 1–4).

DISCUSSION

Apoptosis is a process involved in the regulation of development and health maintenance of living organisms. As mentioned previously, the decreased expression of certain pro-apoptotic genes (caspase-3, caspase-9 and bax) has been shown in kidneys of GHRKO vs. N mice (Gesing et al., unpublished data) and may contribute to the mechanisms of extended longevity in these knockouts. In the present study, we demonstrated the decreased renal level of caspase-3 and bax protein in KO mice. Furthermore, additional pro-apoptotic proteins (caspase-8, bad, Smac/DIABLO, Apaf-1 and pp53) levels were also decreased in mice with targeted disruption of GH receptor gene as compared to N animals. Therefore, the key novel finding in this study is that altered expression of apoptosis-related genes in GHRKO mice is associated with the corresponding changes of their (and additional) protein products. Thus, reduced levels of pro-apoptotic proteins emerge as possible contributors to the phenotypic characteristics of these animals, including delayed aging, resistance to stress and to age-associated disease and extended longevity.

The increase in caspase-3 expression was reported in various pathological conditions. For example, the elevated renal caspase-3 has been demonstrated in ischemic injury (Faubel and Edelstein 2005; Zhang et al. 2006) and also in mice with polycystic kidney disease (Ali et al. 2000). Moreover, type 2 diabetic *db/db* mice which, among other characteristics, are insulin resistant, have increased renal caspase-3 activity (Ghosh et al. 2009). Therefore, the decrease of caspase-3 protein level as seen in the present study, may be considered – by analogy – as potentially beneficial for KO mice.

In the present study, renal bax protein level was decreased in long-lived KO vs. N mice. This decrease may also be considered beneficial for GHRKO mice and potentially for their extended longevity. Wang et al. (2009) demonstrated that β -catenin/Wnt signaling pathway may promote survival of renal epithelial cells after metabolic stress and this may be associated with inhibition of bax expression. Moreover, a change in the opposite direction, namely an increase of bax expression was detected in renal cortex of diabetic *db/db* mice (Ortiz et al. 1997) and also in kidneys of congenital polycystic kidney disease mice (Ali et al. 2000).

Ghosh et al. (2009) showed increased renal caspase-8 activity in diabetic *db/db* mice. Thus, the decreased protein level of this caspase, observed in the present study, may be – by analogy – beneficial for hypoinsulinemic and highly insulin sensitive GHRKO mice.

Bad protein level was diminished in GHRKO as compared to normal mice. Otherwise, bad protein phosphorylated at Ser112 (pbad) was increased in KOs. Phosphorylation at this site results in the binding of pro-apoptotic bad to 14-3-3 proteins – and through inhibition of bad binding to anti-apoptotic bcl-2 (Zha et al. 1996) may prevent caspases cascade activation. Therefore, the decreased bad as well as increased pbad protein levels observed in the GHRKO mice in the present study, may represent another beneficial effects of GH resistance in these long-lived mutants.

p53 is known as a “guardian of the genome”. However, it is also considered to be a mediator of senescence and a contributor to aging (Campisi 2005; Tyner et al. 2002). DNA damage induces phosphorylation of p53 at Ser15, leading to reduced interaction of p53 with its negative regulator – oncoprotein MDM2 (Shieh et al., 1997). Zhou et al. (2010) showed that p53 promoted renal injury in acute aristolochic acid-induced nephropathy. Moreover, cisplatin-induced nephrotoxicity was abrogated in p53-deficient mice (Wei et al. 2007). Therefore, one could speculate that decreased pp53 protein level in KO mice, as seen in our study, may be beneficial for these long-lived knockouts and may presumably reflect reduced DNA damage in the examined tissue of these mutants.

In the present study, the knockout of the *Ghr* gene increased renal anti-apoptotic bcl-2 protein level. It could be postulated that this increase is beneficial for GHRKOs. Mice deficient for bcl-2 gene have congenital renal hypoplasia, develop polycystic kidney disease and renal failure (Sorenson 2004; Sorenson et al. 1996). Moreover, the results of study by Ortiz et al. (1997) demonstrated that high glucose decreased the expression of the bcl-2 *in vitro* and the expression of this gene in the renal cortex of diabetic *db/db* mice.

As mentioned previously, VFR may improve insulin signaling and extend longevity in genetically normal animals (Barzilai et al. 1999; Muzumdar et al. 2008; Shi et al. 2007) thus resembling some of the effects of calorie restriction (CR). In the present study, VFR did not alter the level of the examined apoptosis-related proteins as compared to sham-operated mice. The obtained results extend our previous observations showing no effects of VFR on the renal expression of apoptosis-related genes in GHRKO and normal mice (Gesing et al., unpublished data).

In summary, decreased renal level of several pro-apoptotic proteins can be hypothesized to contribute to extended longevity in GHRKO mice. In contrast, our findings did not support the hypothesis that alterations in the renal levels of apoptosis-related proteins may contribute to the reported beneficial effects of VFR. Further studies are needed to determine how the levels of apoptosis-related proteins in particular organs can contribute to the regulation of longevity.

MATERIALS AND METHODS

Animals and visceral fat removal (VFR)

Normal and GHRKO mice used in the present study were produced in our breeding colony, developed using animals kindly provided by Dr. J.J. Kopchick (Ohio University). Animals were produced by mating knockout (−/−) males with heterozygous (+/−) females. Normal (+/−) and GHRKO animals (−/−) were separated by phenotypic characteristics. All animal procedures were approved by the Laboratory Animal Care and Use Committee (LACUC) at the Southern Illinois University School of Medicine (Springfield, IL). The mice were housed under temperature- and light-controlled conditions (22 ± 2°C, 12 hr light/12 hr dark cycle) and fed Lab Diet 5001 chow (PMI Nutrition International, Richmond, IN) containing 4.5% fat and 23.4% protein. At the age of approximately 6 months, forty three (43) normal and GHRKO male mice were grouped according to average body weight within the phenotype, and divided into four (4) experimental groups: normal sham-operated (N-sham) (11 animals), normal subjected to visceral fat removal (N-VFR) (11 animals), GHRKO sham-operated (KO-sham) (10 animals), and GHRKO subjected to visceral fat removal (KO-VFR) (11 animals). The animals were anesthetized with ketamine/xylazine, shaved and prepared in the usual sterile fashion. Mice were supplied with ibuprofen in drinking water starting 2 days before and up to 3 days after the surgery. Tap water was available at all times to all animals. In the VFR group, the epididymal fat pads were removed using blunt dissection through a vertical midline incision. Perinephric fat pads were removed via flank incisions. We removed as much epididymal and perinephric fat as was possible without compromising blood supply to the testes and to the adrenals. For sham operations, the abdominal cavity and both sides of the back were incised and the visceral fat was mobilized, but not removed. Two months after VFR or sham operations, the animals were fasted overnight, anesthetized using ketamine/xylazine, bled by cardiac puncture and euthanized by decapitation. Kidneys were rapidly collected, quickly frozen on dry ice and stored at −80°C until processed.

Protein extraction and Western blotting

Total proteins were obtained from tissue homogenates. Approximately 100 mg samples of kidney tissue were homogenized in 1 ml ice-cold T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL), with Protease Inhibitor Cocktail Kit (Pierce Biotechnology, Rockford, IL), Phosphatase Inhibitor Cocktail 1 (Sigma-Aldrich, St. Louis, MO) and Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich, St. Louis, MO)]. After mixing, homogenates were centrifuged at 16 000 *g* for 30 minutes. Protein concentrations were assessed using Pierce BCA (bicinchoninic acid) Protein Assay Kit (Pierce Biotechnology, Rockford, IL) in accordance with manufacturer's protocol.

Western blot procedure was performed using the following primary antibodies: caspase-3, caspase-9 (mouse specific), caspase-8 (mouse specific), bax, bad, phospho-bad (Ser112), bcl-2, phospho-p53 (Ser15), Apaf-1, cytochrome c (all from Cell Signaling Technology, Beverly, MA), Smac/DIABLO (BioVision, Inc., Mountain View, CA), and secondary goat antirabbit antibodies (Calbiochem, La Jolla, CA). Monoclonal anti-β-actin antibody (Sigma-Aldrich Corp., St. Louis, MO) was used, after stripping the membrane, as a control for protein loading.

For Western blotting, protein extracts were mixed with XT Sample Buffer (Bio-Rad Laboratories, Inc., Hercules, CA) and heated in a thermocycler at 99°C for 5 minutes and then cooled to 4°C. Forty micrograms of the protein was separated electrophoretically using Criterion XT Precast Gel (26 wells; Bio-Rad Laboratories, Inc., Hercules, CA) for 90 minutes at 150 V. Subsequently, proteins were wet-transferred for 60 minutes at 100 V onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA) or Immobilon PVDF transfer membranes (Millipore, Billerica, MA) at 4°C. After the transfer, membranes were rinsed briefly in Tris-buffered saline (TBS; pH 7.6) and blocked with 5% nonfat dry milk or 1% BSA (bovine serum albumin) in TBS containing 0.05% Tween 20 (TBST) for 1 h at room temperature. After blocking, membranes were washed with TBST 3 times for 15 minutes each time. Then, the membranes were incubated with the primary antibody specific for the protein of interest and diluted in the appropriate blocking solution at 4°C overnight with shaking. After incubation, the blots were washed three times (15 min each) with TBST and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Horseradish peroxidase activity from secondary antibody was detected using the Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK). Photos of blots were taken with Image Reader LAS-4000 (FujiFilm, Tokyo, Japan) and quantified for statistical analysis using Multi Gauge version 3.0 software (FujiFilm Life Science, Japan). The protein level was expressed as the Arbitrary Unit (AU)/mm². The AU is a unit to measure the emission amount of chemiluminescence material read using LAS. It represents the relative density value accumulated as linear data by a CCD (charge-coupled device) camera in the image surface.

Statistical analysis

The data are expressed as mean $\pm$ Standard Error of the Mean (SEM). To evaluate the effects of the genotype and surgical intervention, two-way analysis of variance (ANOVA) was used. A *t*-test was used to evaluate the effects of surgery within genotypes and genotypes within surgical interventions. A value *p* of 0.05 was used as threshold for statistical significance. All statistical calculations were conducted using SPSS version 17.0 (SPSS, Chicago, IL). All graphs were made using Prism 4.02 (GraphPad Software, San Diego, CA).

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Figure 1.

Caspase-3 (A), caspase-8 (B), bax (C) and bad (D) protein level in the kidney of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice sham-operated (sham) or subjected to visceral fat removal (VFR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a–b – values that do not share the same letter in the superscript are significantly different from each other ($p < 0.05$). * – $p = 0.001$ vs. N mice (the significance for genotype), ** – $p = 0.009$ vs. N mice (the significance for genotype), *** – $p = 0.002$ vs. N mice (the significance for genotype), **** – $p = 0.015$ vs. N mice (the significance for genotype)

Figure 2.

Smac/DIABLO (A), Apaf-1 (B) and pp53 (C) protein level in the kidney of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice sham-operated (sham) or subjected to visceral fat removal (VFR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a–b – values that do not share the same letter in the superscript are significantly different from each other ($p < 0.05$). * – $p = 0.02$ vs. N mice (the significance for genotype), ** – $p = 0.047$ vs. N mice (the significance for genotype), *** – $p = 0.003$ vs. N mice (the significance for genotype)

Figure 3. bcl-2 (A) and pbad (B) protein level in the kidney of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice sham-operated (sham) or subjected to visceral fat removal (VFR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a–b – values that do not share the same letter in the superscript are significantly different from each other ($p < 0.05$). * – $p < 0.001$ vs. N mice (the significance for genotype), ** – $p = 0.038$ vs. N mice (the significance for genotype)

Figure 4. Caspase-9 (A) and cyc (B) protein level in the kidney of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice sham-operated (sham) or subjected to visceral fat removal (VFR). The protein level was expressed as the Arbitrary Unit (AU)/mm². Values are means $\pm$ SEM. a–c – values that do not share the same letter in the superscript are significantly different from each other ($p < 0.05$).

P-values for proteins levels in kidneys of normal (N) and growth hormone receptor/binding protein knockout (GHRKO; KO) mice subjected to visceral fat removal (VFR) or sham surgery (sham).

Table 1

PROTEIN	p value A vs. B	p value C vs. D	p value A vs. C	p value B vs. D	p value AB vs. CD (N vs. KO)	p value AC vs. BD (sham vs. VFR)
Caspase-3	NS	NS	0.009	NS	0.001	NS
Caspase-9	NS	0.001	0.002	NS	NS	NS
bax	0.008	NS	0.001	NS	0.002	NS
Snac/DIABLO	NS	NS	NS	NS	0.02	NS
caspase-8	NS	NS	0.02	NS	0.009	NS
bcl-2	NS	0.003	NS	0.002	<0.001	NS
bad	NS	NS	NS	NS	0.015	NS
pbad	NS	NS	NS	NS	0.038	NS
Apatf-1	NS	NS	NS	NS	0.047	NS
pp53	NS	NS	0.017	NS	0.003	NS
cyc	NS	NS	NS	NS	NS	NS

A represents *N-sham* mice, B represents *N-VFR* mice, C represents *KO-sham* mice, D represents *KO-VFR* mice.
NS, Not significant.