

Deletion of growth hormone receptor gene but not visceral fat removal decreases expression of apoptosis-related genes in the kidney—potential mechanism of lifespan extension

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Abstract Mice homozygous for the targeted disruption of the growth hormone (GH) receptor (Ghr) gene (GH receptor knockout; GHRKO; KO) are hypoinsulinemic, highly insulin sensitive, normoglycemic, and long-lived. Visceral fat removal (VFR) is a surgical intervention which improves insulin signaling in normal (N) mice and rats and extends longevity in rats. We have previously demonstrated decreased expression level of certain pro-apoptotic genes in skeletal muscles and

suggested that this may contribute to the regulation of longevity in GHRKO mice. Alterations in apoptosis-related genes expression in the kidneys also may potentially lead to lifespan extension. In this context, we decided to examine the renal expression of the following genes: caspase-3, caspase-9, caspase-8, bax, bad, bcl-2, Smac/DIABLO, Apaf-1, p53, and cytochrome c1 (cyc1) in male GHRKO and N mice subjected to VFR or sham surgery, at approximately 6 months of age. The kidneys were collected 2 months after VFR. As a result, caspase-3, caspase-9, and bax expressions were decreased in KO mice as compared to N animals. Expressions of Smac/DIABLO, caspase-8, bcl-2, bad, and p53 did not differ between KOs and N mice. VFR did not change the expression of the examined genes in KO or N mice. In conclusion, endocrine abnormalities in GHRKO mice result in decreased expression of pro-apoptotic genes and VFR did not alter the examined genes expression in N and KO mice. These data are consistent with a model in which alterations of GH signaling and/or insulin sensitivity lead to increased lifespan mediated by decreased renal expression of pro-apoptotic genes.

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Introduction

Growth hormone (GH) receptor/GH binding protein knockout mice (GHRKO; KO) are GH resistant due

to targeted disruption of the GH receptor/GH binding protein (Ghr/bp) gene (Zhou et al. 1997). These mice are insulin sensitive and live longer than their normal (N) siblings, and are characterized by reduced weight and body size, not detectable concentration of GH receptor, high level of serum GH, greatly reduced plasma levels of IGF-I and insulin, and low or normal glucose (Bartke and Brown-Borg 2004; Bartke et al. 2002; Coschigano 2006; Coschigano et al. 2000; 2003; Harper et al. 2006; Kopchick and Laron 1999; Liu et al. 2004; Zhou et al. 1997). Moreover, GHRKO mice have improved oxidative stress resistance and reduced oxidative damage (Bartke and Brown-Borg 2004; Salmon et al. 2005; Sun et al. 2009) with lower incidence and delayed onset of fatal neoplastic diseases (Ikeno et al. 2009).

Visceral fat removal (VFR) is a surgical 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), thus mimicking the effects of calorie restriction (CR). It is known that adipose tissue, through the production of pro-inflammatory cytokines (tumor necrosis factor- α , interleukin-6, interleukin-1 β) may cause the alterations in insulin sensitivity leading to the insulin resistance (Fasshauer and Paschke 2003; Lagathu et al. 2003; Zhang et al. 2001). Moreover, some of these cytokines could induce apoptosis (Murata et al. 2010; Sishi and Engelbrecht 2011). In contrast, Holland et al. (2011) have recently demonstrated that adiponectin, an adipose tissue hormone which is known to improve insulin sensitivity, exert anti-inflammatory effects, and promote cell survival, may exert anti-apoptotic effects as well. These effects are caused by stimulation of ceramidase activity, and as a result of that, enhanced ceramide catabolism and formation of its anti-apoptotic metabolite—sphingosine-1-phosphate (Holland et al. 2011). Interestingly, the long-living GHRKO mice have been shown to have increased plasma levels of adiponectin (Berryman et al. 2004). Thus, it is not clear what over-riding effect VFR might have in the GHRKO mice.

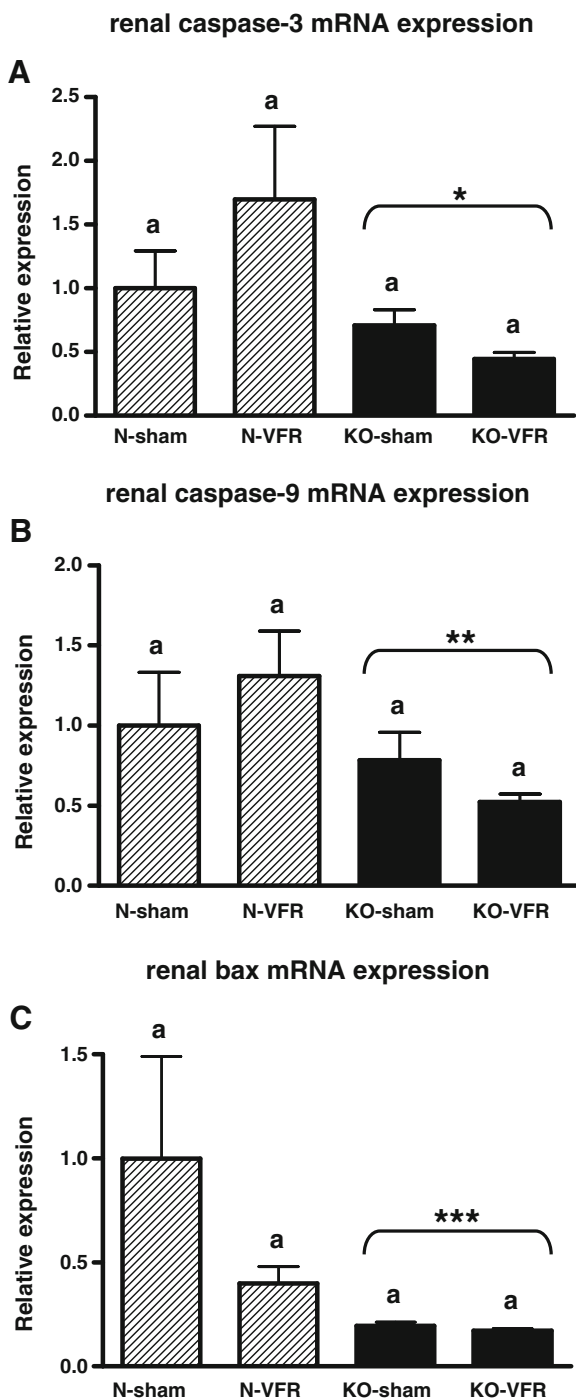
Apoptosis is involved in the regulation of age-related functional alterations and longevity. Two central apoptotic signaling pathways are known: intrinsic (mitochondrial) (involving p53, bax, cytochrome c, and caspase-9, among others)—initiated by different factors within the cell (Boatright and Salvesen 2003;

Budihardjo et al. 1999; Jin and El-Deiry 2005), and extrinsic (death receptor) pathway (involving caspase-8) (Boatright and Salvesen 2003). In spite of its well-known benefits, leading to removal of abnormal cells, the role of apoptosis in longevity regulation is still unclear. Deletion of GH receptor decreased the expression level of some (caspase-3, caspase-9, bax, and Smac/DIABLO) but not all apoptosis-related genes in skeletal muscles, and we hypothesized that these alterations may play a role in the regulation of longevity in these knockout mice (Gesing et al. 2011). Interestingly, the double mutants produced by crossing mice with caspase-3 gene deletion (casp3 $^{-/-}$) and mice harboring the congenital polycystic kidney (cpk) mutation lived longer than control cpk animals (Tao et al. 2008). As apoptosis appears to increase in the normal aging kidney (Miyazawa et al. 2009) and pathological changes in kidneys are a very common cause of death in aging mice (Barton et al. 2000; Doi et al. 1988; Zheng et al. 2004), we decided to continue the study of apoptotic processes in the kidney of long-lived GHRKO mice. In the present study, we have examined the renal expression levels of the following apoptosis-related genes: caspase-3, caspase-9, caspase-8, bax, bad, bcl-2, Smac/DIABLO, Apaf-1, p53, and cytochrome c1 (cyc1) in KO and N mice after VFR or sham surgery.

Materials and methods

Animals, visceral fat removal, and assessment of blood chemistry

The 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 at the Southern Illinois University School of Medicine (Springfield, IL). The mice were housed under temperature- and light-controlled conditions ($22\pm 2^\circ\text{C}$, 12 h light/12 h 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 approxi-



mately 6 months, 43 normal and GHRKO male mice were grouped according to average body weight within the phenotype, and divided into four experimental groups: normal sham-operated (N-sham; 11 animals), normal subjected to visceral fat removal (N-

Fig. 1 Caspase-3 (A), caspase-9 (B), and bax (C) mRNA expression 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 data from RT-PCR were normalized by the housekeeping gene β 2-microglobulin and expressed as the relative expression. Values are means \pm SEM. *a*—values that share the same letter in the superscript are not significantly different. * $p=0.027$ vs. N mice (the significance for genotype), ** $p=0.043$ vs. N mice (the significance for genotype), *** $p=0.042$ vs. N mice (the significance for genotype)

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 or 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, and fasting glucose levels were measured in blood collected from the tail vein using OneTouch Ultra glucometer (Life Scan, Milpitas, CA). Next, the mice were 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. Plasma obtained from blood collected by cardiac puncture was used for assessment of insulin using Rat/Mouse Insulin ELISA (Linco Research Inc., St. Charles, MO) following manufacturer's protocols.

RNA extraction and complementary DNA transcription

The RNA was extracted from the homogenates of kidneys using guanidinium thiocyanate-phenol-chloroform method based on Chomczynski–Sacchi procedure (1987). RNA quantity and quality were analyzed on 1.5% agarose gel using electrophoresis. Potentially contaminating residual genomic DNA was

eliminated using deoxyribonuclease I (Promega, Madison, WI). Reverse transcription was performed and complementary DNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) in accordance with manufacturer's instruction.

Real-time polymerase chain reaction

The real-time polymerase chain reaction (RT-PCR) was carried out using the Smart Cycler instrument (Cepheid, Sunnyvale, CA) with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). The three steps of the PCR included: denaturation at 94°C for 2 min, annealing at 62°C for 30 s with fluorescence reading, and extension at 72°C for 30 s. In addition, a melting curve was done for each reaction to evaluate the potential of nonspecific products. β_2 -Microglobulin (B2M) was used as a housekeeping gene; it has been previously validated in our laboratory as the most appropriate gene for normalizing the data (Masternak et al. 2006). The gene expression was assessed by measurement of steady state levels of mRNA. 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, and 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. Then, the results in the control group (N-sham) 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 expression of genes of interest compared to the control group.

For RT-PCR the following primers were used [gene bank sequence number by each forward primer]:

B2M: forward [NM_009735]: 5'-aagtatactcaccacacca, backward: 5'-aagaccagtccttctgaag;
 caspase-3: forward [NM_009810]: 5'-tgcagcatgctgaagctgta, backward: 5'-gagcatggacacaatacacg;
 caspase-9: forward [NM_015733]: 5'-agcagagagtagtgaagctg, backward: 5'-acacagacatcatgagctcc;
 caspase-8: forward [AJ007749]: 5'-accgagatctctggaatgga, backward: 5'-tgettcccttctctctct;
 bax: forward [NM_007527]: 5'-ccaccagctctgaaagagatc, backward: 5'-cagctcttctgtggagcagc;
 bad: forward [NM_007522]: 5'-agagtatgttccagatcccag, backward: 5'-gtcctcgaaaagggctaagc;
 bcl-2: forward [NM_009741]: 5'-tgggatgcctttgtggaact, backward: 5'-gagacagccaggagaaatca;
 Smac/DIABLO: forward : [NM_023232] 5'-aagagctgcaccagaaagca, backward: 5'-tctgactgccaatggcagga;

Table 1 Relative gene expression (as percent deviation from control group—N-sham) in kidneys of normal (N) and growth hormone receptor/binding protein knockout (KO) mice sham-operated (sham) or subjected to visceral fat removal (VFR)

Gene	N-sham (A)	N-VFR (B)	KO-sham (C)	KO-VFR (D)	<i>p</i> value, A vs. B	<i>p</i> value, C vs. D	<i>p</i> value, A vs. C	<i>p</i> value, B vs. D	<i>p</i> value, AB vs. CD (N vs. KO)	<i>p</i> value, AC vs. BD (sham vs. VFR)
Caspase-3	100±29%	169±57%	70±12%	44±5%	NS	NS	NS	NS	0.027	NS
Caspase-9	100±33%	131±28%	78±17%	52±5%	NS	NS	NS	NS	0.043	NS
bax	100±49%	40±8%	19±2%	17±1%	NS	NS	NS	NS	0.042	NS
Apaf-1	100±12%	83±11%	203±51%	197±17%	NS	NS	NS	0.034	<0.001	NS
cyc1	100±10%	92±10%	207±39%	192±19%	NS	NS	0.008	0.012	<0.001	NS
Smac/DIABLO	100±28%	94±13%	84±14%	67±5%	NS	NS	NS	NS	NS	NS
caspase-8	100±10%	92±8%	120±14%	108±9%	NS	NS	NS	NS	NS	NS
bcl-2	100±44%	77±16%	83±24%	59±10%	NS	NS	NS	NS	NS	NS
bad	100±28%	99±16%	89±16%	59±5%	NS	NS	NS	NS	NS	NS
p53	100±26%	115±19%	123±28%	107±12%	NS	NS	NS	NS	NS	NS

Values are means±SEM

NS no significant

Apaf-1: forward [AF064071]: 5'-tacaacgctctgcta
cacga, backward: 5'-cacacagcactgtccttaca;
p53: forward [AF151353]: 5'-tcacagtggat
cagcct, backward: 5'-acacteggggcttcaact;
cytochrome c1: forward [NM_025567]: 5'-tacaag
caggtgtgctcttc, backward: 5'-atcattaggccatctgga.

Statistical analysis

The data are expressed as mean±standard error of the mean (SEM). To evaluate the effects of the genotype and surgical intervention, two-way analysis of variance, was used. A *t* test was used to evaluate the effects of surgery within genotypes and genotypes within surgical interventions. A value of $p < 0.05$ was considered significant. All statistical calculations were conducted using SPSS version 17.0 (SPSS, Chicago, IL) with $\alpha = 0.05$. All graphs were made using Prism 4.02 (GraphPad Software, San Diego, CA).

Results

Expression of caspase-3 gene was decreased in kidneys of KO as compared to normal (N) mice ($p = 0.027$) (Fig. 1A, Table 1). Similarly, the caspase-9 (Fig. 1B, Table 1) and bax (Fig. 1C, Table 1) mRNA levels were decreased in KOs in comparison to N animals ($p = 0.043$, $p = 0.042$, respectively). In contrast, the mRNA levels of Apaf-1 (Fig. 2A, Table 1) and cycl1 (Fig. 2B, Table 1) were increased in knockouts as compared to N mice ($p < 0.001$ both). Moreover, the level of Apaf-1 mRNA was up-regulated in KOs subjected to VFR (KO-VFR) in comparison to N mice after VFR ($p = 0.034$) (Fig. 2A, Table 1). Additionally, there was increased cycl1 expression in KO-sham and KO-VFR mice, in comparison to the corresponding N mice ($p = 0.008$, $p = 0.012$, respectively) (Fig. 2B, Table 1). Expression of Smac/DIABLO (Fig. 3A, Table 1), caspase-8 (Fig. 3B, Table 1), bcl-2 (Fig. 3C, Table 1), bad (Fig. 3D, Table 1), and p53 (Fig. 3E, Table 1) in KO mice did not differ from values measured in N animals. VFR did not change the expression of the examined genes in the kidneys of KO or N mice (Figs. 1, 2, and 3, Table 1).

Glucose levels were increased in GHRKO mice after VFR in comparison to sham-operated knockouts

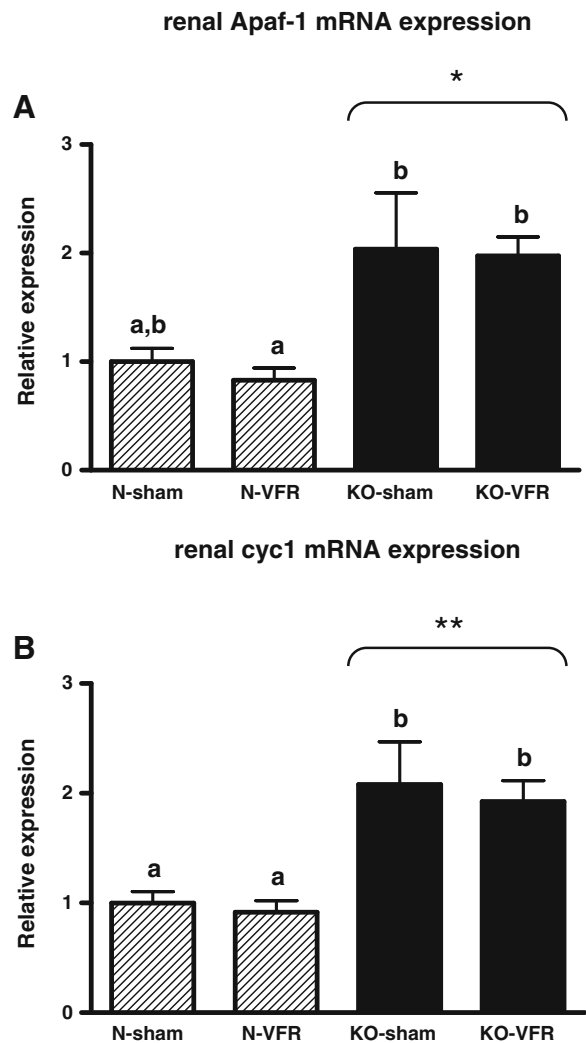


Fig. 2 Apaf-1 (A) and cycl1 (B) mRNA expression in the kidney of normal (N) and growth hormone receptor/binding protein knockout (KO) mice sham-operated (sham) or subjected to visceral fat removal (VFR). The data from RT-PCR were normalized by the housekeeping gene $\beta 2$ -microglobulin and expressed as the relative expression. Values are means±SEM. *a, b*—values that do not share the same letter in the superscript are significantly different ($p < 0.05$). * $p < 0.001$ vs. N mice (the significance for genotype), ** $p < 0.001$ vs. N mice (the significance for genotype)

($p = 0.009$), while in normal mice, there was a trend in the opposite direction, i.e., reduction of glucose after VFR (Fig. 4A). Moreover, there was a significant genotype/VFR interaction for glucose levels ($p = 0.016$) (Fig. 4A). Interestingly, fasting insulin levels were significantly decreased after VFR in normal mice ($p = 0.019$) (Fig. 4B).

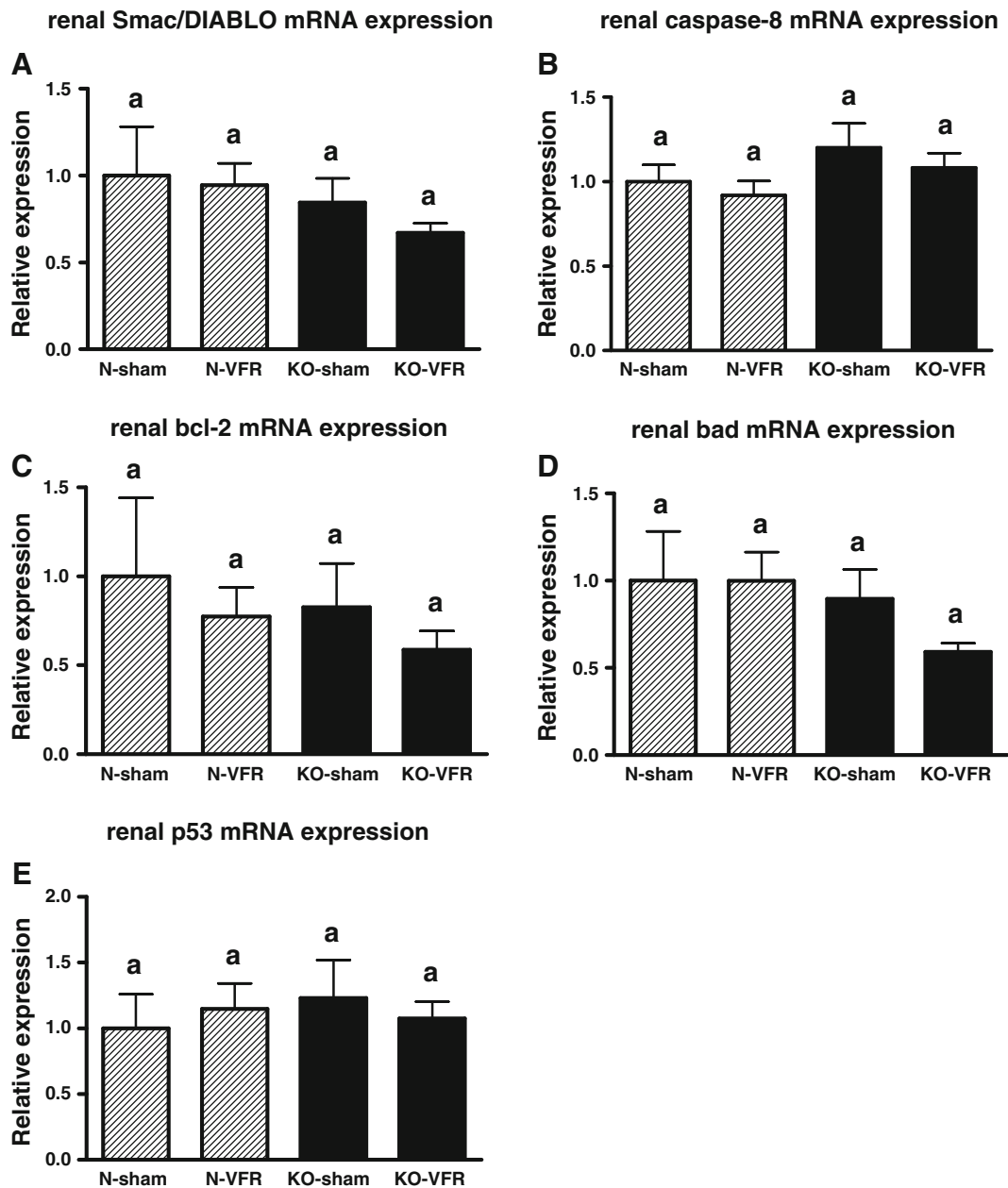


Fig. 3 Smac/DIABLO (A), caspase-8 (B), bcl-2 (C), bad (D), and p53 (E) mRNA expression in the kidney of normal (N) and growth hormone receptor/binding protein knockout (KO) mice sham-operated (*sham*) or subjected to visceral fat removal (*VFR*). The data from RT-PCR were normalized by the

housekeeping gene β 2-microglobulin and expressed as the relative expression. Values are means \pm SEM. *a*—values that share the same letter in the superscript are not significantly different

Discussion

The kidneys are organs which are strongly affected by aging (Percy et al. 2008; Yang and Fogo 2010) that may cause increased glomerulosclerosis, interstitial

fibrosis, tubular atrophy, and a loss of functional renal tissue, involving the number of nephrons and also glomeruli and tubules in the cortex. These histologic features may lead, among others, to increased renal vascular resistance and reduced renal plasma flow.

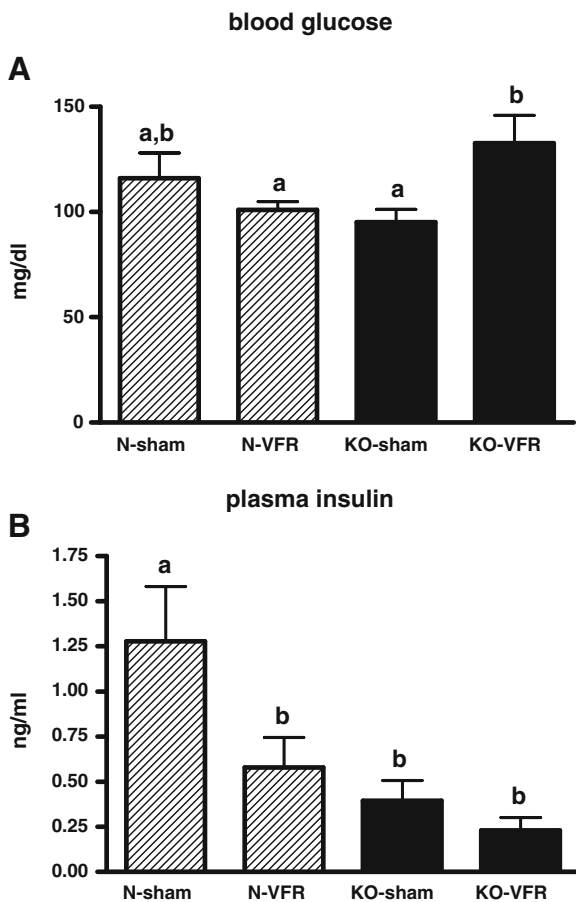


Fig. 4 Blood glucose (A) and plasma insulin (B) level in normal (*N*) and growth hormone receptor/binding protein knockout (*KO*) mice sham-operated (*sham*) or subjected to visceral fat removal (*VFR*). Values are means \pm SEM. *a*, *b*—values that do not share the same letter in the superscript are significantly different ($p < 0.05$)

The process of apoptosis that is involved in the regulation of development and health maintenance, has not been, as yet, completely examined in long-lived GHRKO mice. It is known that the level of apoptosis increases during aging. Miyazawa et al. (2009) showed that protein level of cytochrome c, the leakage of which from mitochondria to the cytoplasm is considered as one of markers of apoptosis, increased 3.5-fold in kidney of 24-month-old compared to 2-month-old C57BL/6J mice. Thus, the suppression of apoptosis would seem to be beneficial physiological effect in the context of prolonged lifespan.

As was mentioned above, we observed decreased expression of certain pro-apoptotic genes (caspase-3,

caspase-9, bax, and Smac/DIABLO) in skeletal muscles of *KO*s that may contribute to the regulation of longevity in these knockouts (Gesing et al. 2011). For all the abovementioned reasons, it was of interest to assess the expression of apoptosis-related genes in kidneys of GHRKO mice.

In the present study, the expression level of main effector (executioner) caspase—caspase-3—was decreased in kidneys of GHRKO mice as compared to normal animals. This change may be considered as potentially beneficial for longevity extension in *KO*s because a change in the opposite direction, an increase in caspase-3 expression was reported in various pathological conditions. The increased expression of caspase-3 has been reported in ischemic injury to kidney (Faubel and Edelstein 2005; Zhang et al. 2006). Moreover, the renal level of caspase-3 expression was markedly increased in polycystic kidney disease mice (Ali et al. 2000). The decreased caspase-3 expression in insulin-sensitive GHRKO mice may correspond to the observation that type 2 diabetic *db/db* mice with a point mutation in the leptin receptor gene, leading to insulin resistance, among others (Moiien-Afshari et al. 2008), have increased renal caspase-3 activity (Ghosh et al. 2009). Interestingly, the caspase inhibitor IDN-8050 slowed the disease progression in the Han:SPRD rat model of polycystic kidney disease (Tao et al. 2005).

In our present study, the mRNA level of caspase-9 was decreased in GHRKO mice. This change in caspase-9 expression also may be regarded as beneficial for these insulin-sensitive mice and consistent with the results of the study performed by Mishra et al. (2005) in which the authors demonstrated that high glucose increased caspase-9 activity in human mesangial cells.

The targeted disruption of the *Ghr* gene leads to decreased bax expression. It could be speculated that this change may be beneficial for GHRKO mice. It has been shown that β -catenin/Wnt signaling pathway may promote survival of epithelial cells in kidney after metabolic stress and this beneficial effect seems to be associated with inhibiting bax expression (Wang et al. 2009). In agreement with our results, the opposite—increased bax expression—was observed in renal cortex of diabetic *db/db* mice (Ortiz et al. 1997) and also in cystic kidneys of congenital polycystic kidney disease mice (Ali et al. 2000).

Unexpectedly, our study showed increased renal Apaf-1 expression in KO mice. This change seems to be difficult to explain in the context of results obtained in the present study for other apoptosis-related genes. Nevertheless, it could be hypothesized that this up-regulation of Apaf-1 expression may have some beneficial implication in KO mice. It is known that Apaf-1 null mice have many developmental defects, and embryos lacking Apaf-1 show severe craniofacial deformation and retention of interdigital webs (Hickman and Helin 2002).

The *cyc1* gene expression also was up-regulated in the kidneys of GHRKOs. However, it was previously reported that cytochrome c release may occur without loss of membrane potential, which normally precipitates opening of the permeability transition pores (Gross et al. 1999). Furthermore, cytochrome c is also involved in the electron transport chain. Thus, interpretation of the findings concerning cytochrome c gene expression is complicated by its multiple roles in physiological processes.

As mentioned previously, VFR may 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). These alterations resemble the effects of calorie restriction (CR). In our present study, VFR did not alter the expression of the examined apoptosis-related genes in GHRKO and normal mice. These results seem to be consistent with our previous observation that CR did not affect apoptosis-related genes expression in GHRKO mice (Gesing et al. 2011).

After VFR, glucose levels were increased in GHRKO mice in comparison to sham-operated knock-outs. Furthermore, VFR caused a decrease of fasting insulin levels in normal mice. These preliminary results show that VFR may promote insulin resistance in GHRKO mice, although it had the expected beneficial effect on insulin signaling in normal mice. Therefore, one could speculate that the role of visceral fat in the regulation of insulin signaling in the N mice and mice with deletion of growth hormone receptor may be different and needs further analysis.

In summary, endocrine abnormalities in GHRKO mice result in decreased expression of pro-apoptotic genes and VFR did not alter the examined genes expression in N and KO mice. These data are consistent with a model in which alterations of GH signaling and/or insulin sensitivity lead to increased lifespan mediated by decreased renal expression of

pro-apoptotic genes. Nevertheless, further studies are still needed to determine how the expression levels of apoptosis-related genes in particular organs can contribute to the regulation of longevity.

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