RESEARCH ARTICLE

Growth hormone (GH) receptor knockout mice reveal actions of GH in lung development

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The presence of growth hormone (GH) and GH receptors (GHRs) in the lung suggests it is an autocrine/paracrine target site for pulmonary GH action and/or an endocrine site of pituitary GH action. Roles for GH in lung growth or pulmonary function are, however, uncertain. The possibility that pituitary and/or pulmonary GH have physiological roles in lung development has therefore been investigated in GHR knockout (KO or -/-) mice, using a proteomics approach to determine if an absence of GH-signaling affects the proteome of the developing lung. More than 600 proteins were detected by 2-DE in the lungs of control [GHR (+/+)] and GHR (–/–) mice at the end of the alveolarization period (at day 14 postnatally). Of these, 39 differed significantly in protein content at the $p<0.05$ level [6 were of higher abundance in the GHR (–/–) group, 33 were of lower abundance] and 17 differed at the $p<0.02$ level [5 of higher abundance in the GHR (–/–) group, 12 of lower abundance] and 7 were definitively identified by MS. Vimentin, a protein involved in cellular proliferation, was reduced in content by approximately 75% in the lungs of the GHR (–/–) mice. Three proteins involved in oxidative protection [SH3 domain-binding glutamic acid-rich-like protein, peroxiredoxin 6 (Prdx6), and isocitrate dehydrogenase 1] were also of lower content in the GHR (–/–) lungs (by approximately 88%, 81% and 70%, respectively). Prdx6 is also involved in lipid and surfactant metabolism, as is apolipoprotein A-IV, the lung content of which was reduced by approximately 73% in these mice. Proteasome 26S ATPase subunit 4, a protein involved in the non-lysosomal degradation of intracellular proteins, and electron flavoprotein alpha subunit , involved in intracellular metabolism, were also reduced in content in the lungs of the GHR (–/–) mice (by approximately 70% and 49%, respectively). These results therefore suggest that these proteins are normally dependent upon GH signaling, and that GH is normally involved in early lung growth, oxidative protection, lipid and energy metabolism and in proteasomal activity. These roles may reflect endocrine actions of pituitary GH and/or local autocrine/paracrine actions of GH produced within the lung.

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Abbreviations: GH, growth hormone; **GHR**, GH receptor

1 Introduction

The lung is a target site for growth hormone (GH) action, since the GH receptor (GHR) gene is expressed in pulmonary tissues [1–3]. Indeed, a role for GH in pulmonary function is indicated by the anatomical and biophysical changes in the lung in pathological states of pituitary GH

excess and deficiency. For instance, in acromegaly (see [4] for review) the lungs are large [5], upper airflow is obstructed [6] and the small airways are narrowed [7]. In contrast, a decrease in respiratory muscle strength [8], and a reduction in the maximum inspiratory and expiratory pressure [9], accompanies GH deficiency. Moreover, exogenous GH induces the production of superoxide by alveolar macrophages [10], activates lung neutrophils [11], induces NF-κB production [12], increases lung phosphorylase A activity [13], and stimulates the tyrosine phosphorylation of specific but unidentified proteins in lung epithelial cells [3]. The physiological importance of endogenous GH in lung development is, however, unknown.

Lung development involves cell proliferation, differentiation and survival, and the process of alveolarization, in particular, involves airway branching and widening and angiogenesis of the pulmonary vasculature [14]. Numerous local growth factors, including fibroblast growth factors, sonic hedgehog, and retinoic acid [15] have established roles in lung development and in alveolarization in particular [16–18]. Since the GH gene is widely expressed in Type I and Type II epithelial cells of the rat lung during the period of alveolarization [19], it may also participate in lung development, especially as local GH expression has autocrine/paracrine roles rather than endocrine actions in other tissues [20, 21]. The possibility that pituitary and/or pulmonary GH have physiological roles in lung development has therefore been investigated in GHR knockout (-/-, KO) mice [22], using a proteomic approach to determine if an absence of GH signaling affected the proteome of the developing lung. Actions of GH in skeletal muscle [23] have similarly been determined using a proteomic approach, as have the actions of GH in the livers of mice with deficient GH signaling [24–28].

2 Materials and methods

2.1 Animals

The mice used in these studies were females, housed in the transgenic facility at the Edison Biotechnology Institute and have been described previously [29]. GHR (–/–) mice and their controls (+/+) were obtained from the inbreeding of GHR (+/-) mice. Genotypes were confirmed by RT-PCR of total RNA from the tails of mice collected at autopsy [30]. Animals were housed in a room with a controlled photoperiod of 12-h light:12-h darkness (lights on from 06:00 to 18:00 h) and a temperature 22–23°C. Mice were given free access to a nutritionally balanced diet (Lab Diet: PM1 Feeds, St. Louis, MO, USA) and tap water. All experiments were approved by the University Animal Care and Utilization Committee, and were conducted in accordance with NIH Animal Care guidelines.

2.2 Tissues

GHR (–/–) ($n = 5$) and GHR (+/+) ($n = 6$) mice were killed on postnatal day 14, late in the alveolarization period [31]. Lungs were excised and flash frozen in liquid nitrogen. Protein samples for 2-DE were prepared by mixing the powdered lungs with rehydration buffer (8 M urea, 4% CHAPS, 10 mM DTT, 0.2% Bio-Lytes 3/10, Bio-Rad, Mississauga, Ontario, Canada), which was sonicated twice for 5 s and centrifuged for 10 min at 15 000 rpm at room temperature, to remove any insoluble particles.

2.3 2-DE

The protein content of lung samples in rehydration buffer was measured using the Bio-Rad protein assay after suitable dilution of the samples, and 0.2 mg protein was applied to 11-cm IPG strips, which had a linear pH gradient from 3–10 (Bio-Rad). For IEF, the Bio-Rad protean IEF cell was used at 20°C with fast voltage ramping: step 1: 15 min with an end voltage of 250 V; step 2: 150 min with an end voltage of 8000 V; step 3: 35 000 Vh (approximately 260 min). After IEF, the strips were equilibrated according to the Bio-Rad protocol. The second dimension of 2-DE was carried out using Criterion precast gels (8–16%) (Bio-Rad) in a Criterion Dodeca Cell (Bio-Rad). After separation, the proteins were detected using CBB R-250 (Bio-Rad). To minimize variation in staining, all gels were stained in the same bath. The reproducibility of protein resolution using this 2-DE technique and staining procedure is very high, as detailed previously [32, 33]. In our laboratory, the intensity of staining for a single protein when run on separate gels differs by $<5\%$ and the correlation coefficient (r^2) for the staining intensities of multiple protein samples measured on different gels is >0.97 [32]. This procedure therefore obviates the need for replicate determinations, as previously established [32–36].

2.4 Image analysis

Developed gels were scanned using a GS-800 calibrated densitometer (Bio-Rad). Quantitative analysis of spot intensity was performed using PDQuest 7.1 software (Bio-Rad). The protein spot sensitivity threshold was used to determine significant changes in protein spot size and density. This was based on four parameters: minimum peak volume sensitivity, smallest spot area, largest spot area and the noise filter level. Only spots with relative intensities between 2 and 200 arbitrary units were considered for MS analysis.

2.5 MS

Electrophoretically separated proteins from the spots that demonstrated statistically significant changes in intensity (at the $p<0.02$ level) were excised from the gels and separately pooled. In-gel digestion was performed on a MassPrep Station (Micromass, Manchester, UK), using the method sup-

plied by the manufacturer. Excised gel pieces were destained, reduced with DTT and the cysteine residues reacted with iodoacetamide, before digestion with trypsin (Promega sequencing grade) and extraction. The extract was then analyzed via LC-MS/MS. LC was performed on a Waters CapLC (Milford, MA, USA), using a water/ACN (0.2% formic acid) gradient, on a PicoFrit Capillary column (New Objectives, Woburn, MA, USA) (BioBasic C18, 5-μm particle size, 10 cm × 75 μm id, 15-μm tip). The eluted peptides were then electrosprayed and analyzed on a MicroMass Q-TOF 2, using automated data-dependent MS to MS/MS switching [33]. The resultant MS/MS data were searched against NCBInr and Swiss-Prot databases for identification of the protein. A mass deviation of 0.2 was tolerated and 0 missed cleavage sites were allowed in the searches. The MASCOT (www.matrixscience.com) search engine was used to search the NCBInr protein database for protein identification. The MOWSE scoring algorithm [37] was used for justification of accuracy of protein identification and is incorporated in the MASCOT search engine.

3 Results

Representative electrophoregrams for proteins in the lungs of GHR (+/+) and GHR (–/–) mice are shown in Fig. 1. 2-DE gel analysis detected >600 proteins, of which 39 spots (approximately 7%) differed significantly in protein content at the $p<0.05$ level [6 were of higher abundance in the GHR (–/–) group, 33 were of lower abundance]. Of these proteins, the abundance of 17 were significantly different at the $p<0.02$ level [5 of higher abundance in the GHR (–/–) group, 12 of lower abundance]. These spots were excised from the gels for MS analysis. Of these 17 spots, 14 were sufficiently resolved on the gels to permit accurate excision, and 7 could be definitively identified by MS. The protein identity, the accuracy of protein identification (MOWSE score), and the number of matched peptides for these proteins are summarized in Table 1. Mean spot intensities of proteins from GHR (+/+) and GHR (–/–) lungs are displayed for vimentin [Fig. 2, lung content reduced by 75.0% in the GHR (–/–) group], SH3 domain-binding glutamic acid-rich-like protein

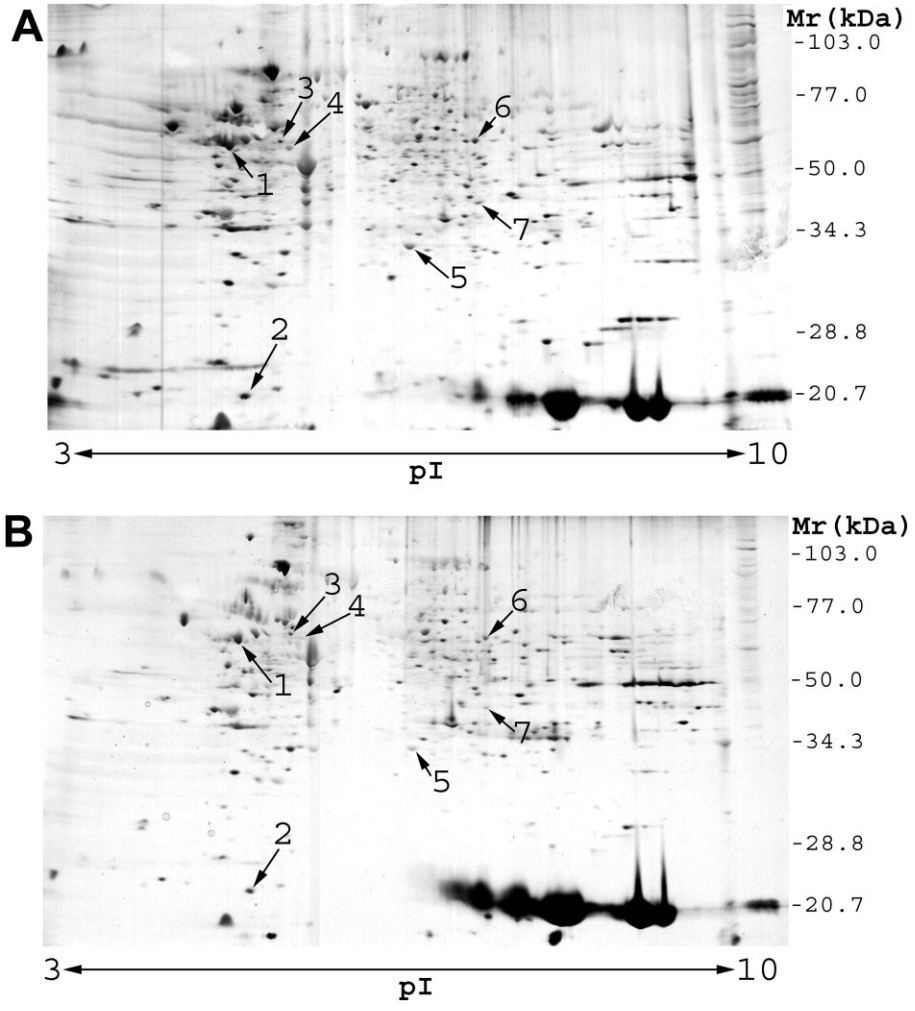

Figure 1. Representative 2-DE of proteins extracted from the lungs of GHR normal (+/+) (A) and GHR (–/–) (B) mice at postnatal day 14. Numbers indicate the seven identified proteins (listed in Table 1) that differed in abundance.

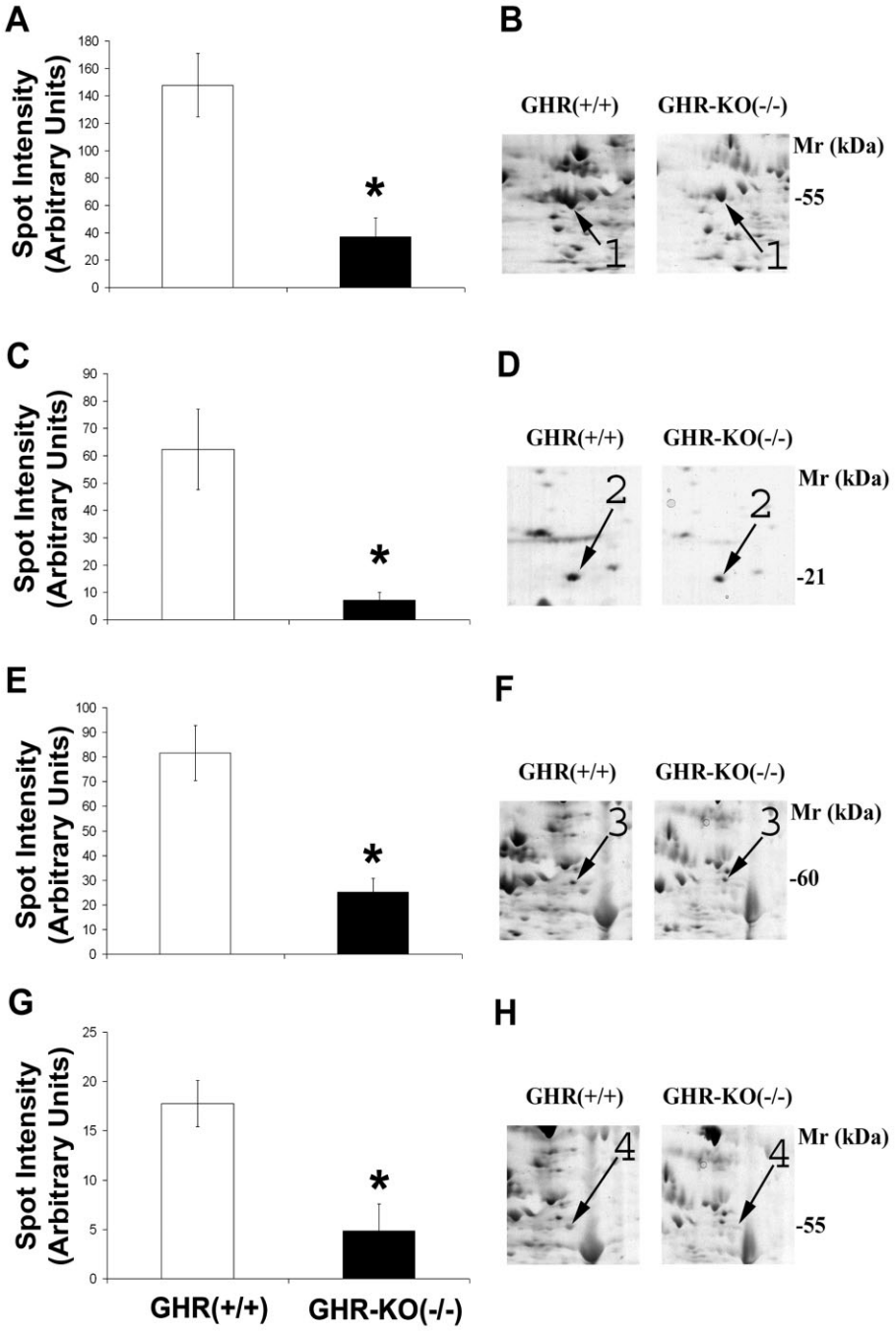

Figure 2. (A, C, E, G) Spot intensity (arbitrary units) for identified proteins in the lungs of GHR normal (+/+) (n=6) and GHR (–/–) mice (n=5) (means ± SEM, asterisks indicate significant differences between the groups, $p<0.02$) and (B, D, F,H) representative 2-DE highlighting individual protein spots (1–4). Protein 1, vimentin (A, B); protein 2, SH3 domain-binding glutamic acid-rich-like protein (C, D); protein 3, proteosome 26S ATPase subunit 4 (E, F); protein 4, apolipoprotein A-IV (G, H).

(Fig. 2, lung content reduced by 88.3%), proteasome 26S ATPase subunit 4 (Fig. 2, lung content reduced by 69.2%), apolipoprotein A IV (Apoa4) (Fig. 2, lung content reduced by 72.7%), peroxiredoxin 6 (Prdx6) (Fig. 3, lung content reduced by 81.0%), isocitrate dehydrogenase 1 (Fig. 3, lung content reduced by 69.6%), and electron transfer flavoprotein alpha subunit (Fig. 3, lung content reduced by 48.6%), along with representative spots from the GHR (+/+) and GHR (–/–) 2-D gels.

4 Discussion

This study demonstrates, for the first time, differences between normal and GHR KO mice in tissue proteome content, in particular differences in the abundance of lung proteins. Since significant differences were seen in the abundance of approximately 7% of the detected proteins, these results suggest that GH signaling is of physiological importance in lung development.

Table 1. Identification of lung proteins

Spot no.	Probability-based MOWSE Score[a)]		Peptides matched	Protein identity (n)
	Threshold	Observed score		
1	29	517	16	Vimentin
2	29	88	2	SH3 domain-binding glutamic acid-rich-like protein
3	30	160	7	Proteasome 26S ATPase subunit 4
4	31	338	9	Apolipoprotein A-IV
5	30	75	2	Peroxiredoxin 6
6	29	139	4	Isocitrate dehydrogenase 1
7	30	86	2	Electron transfer flavoprotein alpha subunit

a) -10 log(p) where p is the probability that the observed match is a random event. Individual scores >29, >30, or >31 indicate identity or extensive homology ($p<0.02$).

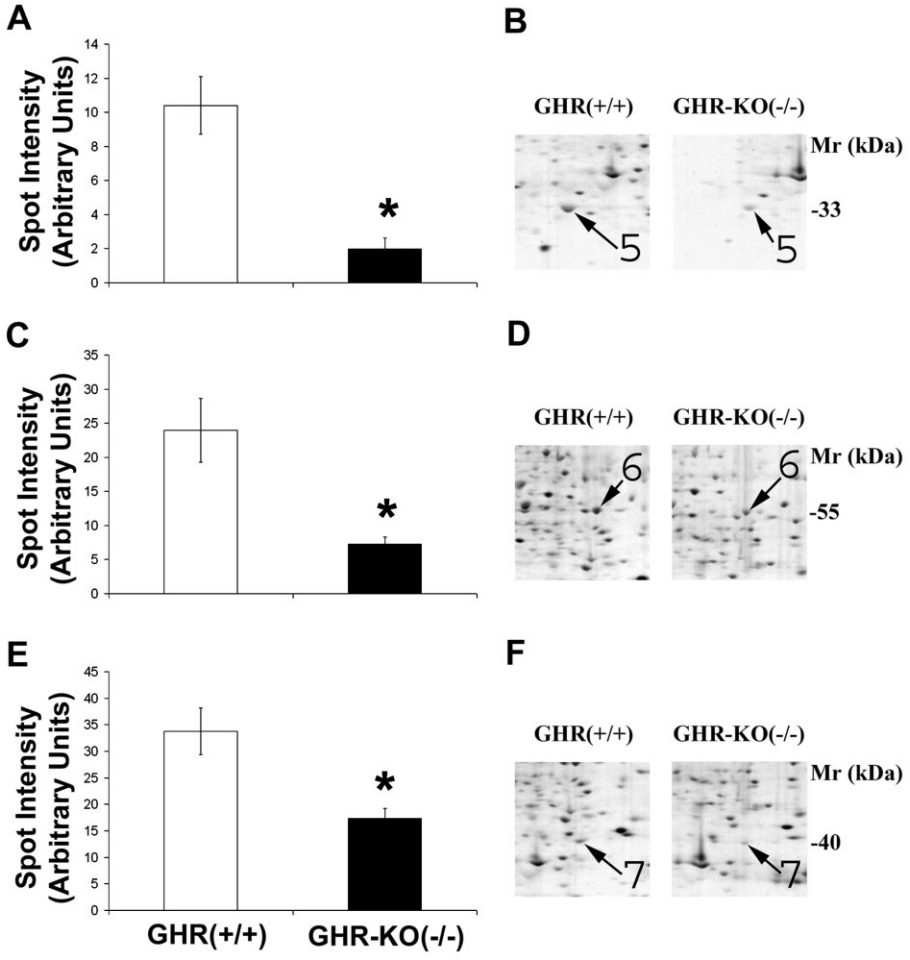

Figure 3. (A, C, E) Spot intensity (arbitrary units) for identified proteins in the lungs of GHR normal (+/+) (n=6) and GHR (–/–) mice (n=5) (means ± SEM, asterisks indicate significant differences between the groups, $p<0.02$) and (B, D, F) representative 2-DE highlighting individual protein spots (5–7). Protein 5, peroxiredoxin 6 (A, B); protein 6, isocitrate dehydrogenase (C, D); protein 7, electron transfer flavoprotein alpha subunit (E, F).

The 7 proteins that were definitively identified in this study include proteins involved in growth (vimentin), proteins involved in oxidative stress (SH3 domain-binding glutamic acid-rich-like protein, Prdx6 and isocitrate dehydrogenase 1), proteins involved in lipid metabolism (Apoa4 and Prdx6), and proteins involved in general metabolism/cell function (electron transfer flavoprotein alpha subunit and proteasome 26S ATPase subunit 4). The decreased content of these proteins in the lungs of GHR (–/–) mice suggests their synthesis is normally dependent upon GH stimulation through the lung GHR.

These proteins were definitively identified by MS, obviating the need to identify them by immunoblotting techniques, which are less sensitive and precise. Indeed, immunoblotting is dependent on the detection of specific epitopes of the proteins, which may undergo different post-translational modifications in different genetic or treatment groups. Actin [33] and myosin light chain 1 [33] measurements in mice are, for instance, discrepant when determined by MS and Western blotting.

Of the proteins identified, vimentin is an intermediate filament expressed in fibroblasts, mesenchymal cells and endothelial cells [38], including those that are responsive to GH action [39]. It is thought to have roles in development, since it promotes cell migration and proliferation [38, 40]. Although null mutation of the vimentin gene results in mice without any obvious phenotypic defect [41], this may reflect the redundancy of growth factors involved in cytoplasmic filament function and the induction of compensatory mechanisms. In the lung, vimentin expression occurs in Type I and Type II epithelial cells [42, 43], and in the endothelia of arteries and veins [44]. The amount of vimentin in the lung proteome is reduced by disease [45], whereas vimentin expression is increased in lung repair following injury [42]. Vimentin is thus thought to be involved in lung growth. The reduced vimentin levels in the lungs of GHR KO mice would thus suggest that they have poorly developed lungs, although this has yet to be determined. This possibility is, however, supported by the reduced amounts of antioxidant proteins in the lungs of GHR KO mice, since deficient antioxidative proteins occur in the proteome of diseased, poorly functioning lung cells [45]. This is the first report to indicate a role for GH in vimentin regulation in the lung, although GH is thought to be involved in lung growth and repair [46] and the autocrine production of GH in human mammary carcinoma cells is accompanied by an induction of vimentin expression [47].

Oxidative stress is particularly important in the lungs, and the postnatal period is when the lung acquires its ability to deal with this stress [48]. One of the antioxidant proteins in the lung is the SH3 domain-binding, glutamic acid-rich-like protein, a member of the SH3BGR family that belongs to the thioredoxin super-family [49]. Thioredoxin is a vital component of the lung antioxidant machinery and *in vivo* delivery of recombinant thioredoxin has been proposed as a therapeutic approach for treating oxidative stress-associated lung disorders [50]. The reduced amount of SH3 domain-binding glutamic acid-rich-like protein in the lungs of the GHR KO mice therefore suggests they would have reduced antioxidant activity. This possibility is also supported by the reduced content of Prdx6 (also known as acidic calcium-independent phospholipase A2), an enzyme involved in oxidative defense against reactive oxygen species [51]. Prdx6 is found predominantly in the lung, specifically within alveolar Type II cells, alveolar macrophages and in bronchiolar epithelium [52]. Its antioxidant role is indicated by the protection against hyperoxic injury that occurs in mouse lungs following the adenoviral overexpression of Prdx6 [53]. Furthermore, antisense oligonucleotide suppression of Prdx6 expression in lung L2 epithelia cells results in increased oxidant sensitivity and apoptosis [54], and Prdx6 (–/–) mice show increased lung injury and mortality in hyperoxia [51].

Isocitrate dehydrogenase 1 is another antioxidant that was reduced in content in the lungs of GHR KO mice. This enzyme produces reduced NADP, which is then used to regenerate reduced glutathione [55], which protects the lungs from oxidative stress [56]. GH has previously been shown to interact with the glutathione-antioxidant system, by inducing glutathione synthesis and by modulating glutathione degradation [57, 58]. In addition, cells genetically engineered to produce human GH were found to overproduce glutathione [59]. The activity of this enzyme has also been shown to be increased by exogenous GH in the liver of chickens [60].The reduced content of isocitrate dehydrogenase 1 in the lungs of GHR KO mice is consistent with the reduced levels of SH3BGR and Prdx6 proteins and a hitherto unknown role for GH in oxidative protection of the developing lung.

In marked contrast, it is generally thought that the longevity of dwarf mice with deficient GH signaling (Ames and Snell mice) reflects increased antioxidant defense and reduced oxidative stress [61, 62]. However, while catalase activity in these dwarf mice is greatly increased in numerous tissues [63–65], glutathione levels are actively decreased in the livers of dwarf mice [63]. Indeed, in these mutant mice, the glutathione level is increased in the liver, brain and muscle following exogenous GH treatment [66]. Tissue levels of antioxidant proteins in dwarf mice and their response to exogenous GH are, therefore, protein specific and possibly also tissue-specific and modified by age [57, 58, 61]. Moreover, in contrast with Ames and Snell mice, catalase activity in the liver and kidneys of GHR KO mice is decreased rather than increased [67]. The longevity in these mice [68] is, therefore, not a result of increased free radical scavenging. Similarly, GH resistance in the Hnf-1 alpha knockout "Laron" mouse [69] is also associated with reduced catalase and glutathione peroxidase activity in the liver and other tissues [70]. These findings are, therefore, consistent with our demonstration of reduced antioxidant protein levels in the lungs of GHR (–/–) mice. There is, however, no evidence of lung dysfunction in GHR KO mice, which live 40% longer than their wild-type siblings [29, 62]. The physiological consequences of having decreased lung proteins involved in antioxidant defense are, thus, not life-threatening. Indeed, without information on the activity of these antioxidative enzymes, the level of antioxidative defense in the lungs of the GHR KO mice is uncertain. In the absence of lung dysfunction, it is thus likely that there is a redundancy of antioxidative defense systems and compensatory mechanisms.

In addition to its role as an antioxidant, Prdx6 is also involved in lung surfactant production. Dipalmitoyl phosphatidylcholine (DPPC) is the major phospholipid constituent of lung surfactant, with 50% of DPPC synthesized

through a phosphatidylcholine (PC) remodeling pathway in Type II pneumocytes that is largely regulated by Prdx6 [71]. Lipid metabolism and the production of surfactant is of major importance in the lung [72], and GH may therefore be involved in this process, especially as the lung content of Apoa4 was also reduced in the GHR KO mice. Apoa4 is primarily involved in the metabolism of triglycerides and high-density lipoproteins [73], and Apoa4 is expressed in the rabbit [74] and mouse [75] lung. The decreased Apoa4 content in GHR KO mice suggests its production is normally dependent upon GH signaling, a possibility supported by the decreased Apoa4 expression in the liver of GH-deficient Snell dwarf mice [28]. Indeed, of 50 hepatic genes in Snell mice that differ from normal mice in transcriptional amount, Apoa4 is the most affected by the lack of GH signaling [28]. The expression of apolipoprotein E is similarly increased by exogenous GH in rat skeletal muscle [76].

In the absence of GHR-mediated GH signaling, the lung contents of 26S ATPase subunit 4 and electron transfer flavoprotein alpha subunit were also reduced, indicating that their production is normally GH dependent. The 26S proteasome complex plays a major role in the ubiquitin-proteasome pathway that mediates the non-lysosomal degradation of intracellular proteins [77, 78]. ATPase subunit 4 is part of the 19S regulatory subunit of the 26S proteasome, and this promotes substrate unfolding and translocation [79]. Proteasome 26S ATPase 4 is widely employed as a 'housekeeper' gene [80], although exogenous GH has previously been found to increase proteasome subunit mRNAs (C-2, C-3, C-5, C-6, C-8 and C-9) in rat skeletal muscle [23] and in rat liver (for C-2, C-3 and C-8) [23]. Proteosomal ATPase has also been identified as a GH-response gene in the rat liver [81]. Electron transfer flavoprotein alpha subunit transfers electrons between several mitochondrial dehydrogenases and the main respiratory chain [82]. It has not previously been thought to be GH regulated.

In summary, the results of this study identify proteins in the lungs of neonatal GHR KO mice that are less abundant than in their normal controls. These results, therefore, suggest that these proteins are normally dependent upon GH signaling, and that GH is normally involved in promoting vimentin-induced lung growth, increasing SH3BGH, Prdx6 and isocitrate dehydrogenase-induced antioxidant protection, increasing Prdx6 and Apoa4 lipid metabolism and increasing lung proteasomal activity. These actions may reflect endocrine actions of pituitary GH and/or local autocrine/paracrine actions of GH produced within the lung [19].

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5 References

[1] Tiong, T. S., Freed, K. A., Herington, A. C., *Biochem. Biophys. Res. Commun.* 1989, *158*, 141–148.

[2] Garcia-Aragon, J., Lobie, P. E., Muscat, G. E., Gobius, K. S., Norstedt, G., Waters, M. J., *Development* 1992, *114*, 869–876.

[3] Batchelor, D. C., Lewis, R. M., Breier, B. H., Gluckman, P. D., Skinner, S. J., *J. Mol. Endocrinol.* 1998, *21*, 73–84.

[4] Colao, A., Ferone, D., Marzullo, P., Lombardi, G., *Endocrinol. Rev.* 2004, *25*, 102–152.

[5] Bartlett, D. Jr., *Respir. Physiol.* 1971, *12*, 297–304.

[6] Trotman-Dickenson, B., Weetman, A. P., Hughes, J. M., *Q. J. Med.* 1991, *79*, 527–538.

[7] Harrison, B. D., Millhouse, K. A., Harrington, M., Nabarro, J. D., *Q. J. Med.* 1978, *47*, 517–532.

[8] Merola, B., Longobardi, S., Sofia, M., Pivonello, R. *et al.*, *Eur. J. Endocrinol.* 1996, *135*, 553–558.

[9] Merola, B., Sofia, M., Longobardi, S., Fazio, S. *et al.*, G., *Eur. J. Endocrinol.* 1995, *133*, 680–685.

[10] Edwards, C. K. 3rd, Arkins, S., Yunger, L. M., Blum, A. *et al.*, *Cell Mol. Neurobiol.* 1992, *12*, 499–510.

[11] Liu, Z., Yu, Y., Jiang, Y., Li, J., *J. Surg. Res.* 2002, *105*, 195–199.

[12] Liu, Z., Yu, Y., Jiang, Y., Li, J., *Ann. Clin. Lab Sci.*. 2002, *32*, 164–170.

[13] Jost, A., Rieutort, M., Bourbon, J., *C. R. Seances Acad. Sci. D.* 1979, *288*, 347–349.

[14] Copland, I., Post, M., *Paediatr. Respir. Rev.* 2004, *5* Suppl. A., S259–S264.

[15] Cardoso, W. V., *Annu. Rev. Physiol.* 2001, *63*, 471–494.

[16] Shenai, J. P, Chytil, F., Stahlman, M. T., *Pediatr. Res.* 1985, *19*, 185–189.

[17] Massaro, G. D., Massaro, D., *Am. J. Physiol.* 1996, *270*, L305–L10.

[18] Massaro, G. D., Massaro, D., *Nat. Med.* 1997, *3*, 675–677.

[19] Beyea, J. A., Olson, D. M., Harvey, S., *Dev. Dyn.* 2005, *232*, 1037–1046.

[20] Waters, M. J., Shang, C. A., Behncken, S. N., Tam, S. P. *et al.*, *Clin. Exp. Pharmacol. Physiol.* 1999, *26*, 760–764.

[21] Sanders, E. J., Harvey, S., *Anat. Embryol.* 2004, *209*, 1–9.

[22] List, E. O., Coschigano, K. T., Kopchick, J. J., *Mol. Genet. Metab.* 2001, *73*, 1–10.

[23] Chrysis, D., Zhang, J., Underwood, L. E., *Growth Horm. IGF Res.* 2002, *12*, 434–441.

[24] Dozmorov, I., Bartke, A., Miller, R. A., *J. Gerontol. A. Biol. Sci. Med. Sci.* 2001, *56*, 872–880.

[25] Dozmorov, I., Galecki, A., Chang, Y., Kresicki, R. *et al.*, *J. Gerontol. A. Biol. Sci. Med. Sci.* 2002, *57*, 899–908.

[26] Miller, R. A., Chang, Y., Galecki, A. T., Kegaley, K. *et al.*, *Mol. Endocrinol.* 2002, *16*, 2657–2666.

[27] Murakami, S., Salmon, A., Miller, N. A., *FASEB J.* 2003, *17*, 1565–1566.

[28] Boylston, W. H., Gerstner, A., DeFord, J. H., Madsen, M. *et al.*, *Aging Cell* 2004, *3*, 283–296.

[29] Zhou, Y., Xu, B. C., Maheshwari, H. G., He, L. *et al.*, *Proc. Natl. Acad. Sci. USA* 1997, *94*, 13215–13220.

[30] Chandrashekar, V., Bartke, A., Coschigano, K. T., Kopchick, J. J., *Endocrinology* 1999, *140*, 1082–1088.

[31] Goncalves, C., Barros, J., Honorio, A., Rodrigues, P., Bairos, V., *Exp. Lung. Res.* 2001, *27*, 533–545.

[32] Sawicki, G., Dakour, J., Morrish, D. W., *Proteomics* 2003, *3*, 2044–2051.

[33] Sawicki, G., Jugdutt, B. I., *Proteomics* 2004, *4*, 2195–2202.

[34] Sawicki, G., Leon, H., Sawicka, M., Sariahmetoglu, C. *et al.*, *Circulation* 2005, in press.

[35] Jugdutt, B. I., Sawicki, G., *Mol. Cell. Biochem.* 2004, *263*, 179–188.

[36] Sawicki, G., Menon, V., Jugdutt, B. I., *J. Cardiac Failure* 2004, *10*, 42–49.

[37] Perkins, D. N., Pappin, D. J., Creasy, D. M., Cottrell, J. S., *Electrophoresis* 1999, *20*, 3551–3567.

[38] Wang, N., Stamenovic, D. J., *Muscle Res. Cell Motil.* 2002, *23*, 535–540.

[39] Tseng, Y. H., Kessler, M. A., Schuler, L. A., *Mol. Cell Endocrinol.* 1997, *128*, 117–127.

[40] Pelosi, G., Fraggetta, F., Nappi, O., Pastorino, U. *et al.*, *Am. J. Surg. Pathol.* 2003, *27*, 1203–1215.

[41] Colucci-Guyon, E., Portier, M. M., Dunia, I., Paulin, D. *et al.*, *Cell* 1994, *79*, 679–694.

[42] Kasper, M., Rudolf, T., Haase, M., Schuh, D., Muller, M., *Virchows Arch. B* 1993, *64*, 271–279.

[43] Koslowski, R., Barth, K., Augstein, A., Tschernig, T. *et al.*, *Histochem. Cell Biol.* 2004, *121*, 509–519.

[44] Kaarteenaaho-Wiik, R., Paakko, P., Herva, R., Risteli, J., Soini, Y., *J. Pathol.* 2004, *203*, 567–574.

[45] Waldburg, N., Kahne, T., Reisenauer, A., Rocken, C. *et al.*, *Pathol. Res. Pract.* 2004, *200*, 147–154.

[46] Fitzgerald, D., Van Asperen, P., O'Leary, P., Feddema, P., *Pediatr. Pulmonol.* 1998, *26*, 241–249.

[47] Mukhina, S., Mertani, H. C., Guo, K., Lee, K. O., Gluchman, P. D., Lobie, P. E., *Proc. Natl. Acad. Sci. USA* 2004, *101*, 15166–15171.

[48] Land, S. C., Wilson, S. M., *Antioxid. Redox. Signal.* 2005, *7*, 92–107.

[49] Mazzocco, M., Maffei, M., Egeo, A., Vergano, A. *et al.*, *Gene* 2002, *291*, 233–239.

[50] Nakamura, T., Makamura, H., Hoshino, T., Ueda, S. *et al.*, *Antioxid. Redox. Signal.* 2005, *7*, 60–71.

[51] Wang, Y., Feinstein, S. I., Manevich, Y., Ho, Y. S., Fisher, A. B., *Free Radic. Biol. Med.* 2004, *37*, 1736–1743.

[52] Kim, T. S., Dodia, C., Chen, X., Hennigan, B. B. *et al.*, *Am. J. Physiol.* 1998, *274*, L750–L761.

[53] Wang, Y., Manevich, Y., Feinstein, S. I., Fisher, A. B., *Am. J. Physiol.* 2004, *286*, L1188–L1193.

[54] Pak, J. H., Manevich, Y., Kim, H. S., Feinstein, S. I., Fisher, A. B.,*J. Biol. Chem.* 2002, *277*, 49927–49934.

[55] Murakami, K., Yoshino, M., *J. Cell Biochem.* 2004, *93*, 1267–1271.

[56] Rahman, I., *Antioxid. Redox. Signal.* 1999, *1*, 425–447.

[57] Brown-Borg, H. M., Rakoczy, S. G., Uthus, E. O., *Ann. NY Acad. Sci.* 2004, *1019*, 317–320.

[58] Brown-Borg, H. M.,Rakoczy, S. G., Uthus, E. O., *Mech. Ageing Dev.* 2005, *126*, 389–398.

[59] Cherbonnier, C., Deas, O., Carbalho, G., Vassal, G. *et al.*, *Br. J. Cancer* 2003, *89*, 1108–1115.

[60] Roseborough, R. W., McMurtry, J. P., Vasilatos-Younken, R., *Comp. Biochem. Physiol.* 1991, *99A*, 207–214.

[61] Hauck, S. J., Bartke, A., *J. Gerontol. A. Biol. Sci. Med. Sci.* 2001, *56*, B153–B162.

[62] Bartke, A., Brown-Borg, H., *Curr. Top. Dev. Biol.* 2004, *63*, 189–225.

[63] Brown-Borg, H. M., Bode, A. M., Bartke, A., *Endocrine* 1999, *11*, 41–48.

[64] Brown-Borg, H. M., Rakoczy, S. G., *Exp. Gerontol.* 2000, *35*, 199–212.

[65] Hauck, S. J., Bartke, A., *Free Radic. Biol. Med.* 2000, *28*, 970–978.

[66] Brown-Borg, H. M., Rakoczy, S. G., *Mech. Ageing Dev.* 2003, *124*, 1013–1024.

[67] Hauck, S. J., Aaron, J. M., Wright, C., Kopchick, J. J., Bartke, A., *Horm. Metab. Res.* 2002, *34*, 481–486.

[68] Coschigano, K. T., Clemmons, D., Bellush, L. L., Kopchick, J. J., *Endocrinology* 2000, *141*, 2608–2613.

[69] Lee, Y. H., Sauer, B., Gonzalez, F., *J. Mol. Cell. Biol.* 1998, *18*, 3059–3068.

[70] Muppala, V., Lin, C. S., Lee, Y. H., *Mol. Pharmacol.* 2000, *57*, 93–100.

[71] Fisher, A. B., Dodia, C., *Am. J. Physiol.* 1997, *272*, L238–L243.

[72] Griese, M., Beck, J., Feuerhake, F., *Am. J. Physiol.* 1999, *277*, L901–L909.

[73] Bai, H., Saku, K., Liu, R., Oribe, Y. K. *et al.*, *Eur. J. Clin. Invest.* 1996, *26*, 1115–1124.

[74] Lenich, C., Brecher, P., Makrides, S., Chobanian, A., Zannis, V. I., *J. Lipid Res.* 1988, *29*, 755–764.

[75] Srivastava, R. A., Pfleger, B. A., Schonfeld, G., *Biochim. Biophys. Acta.* 1991, *1090*, 95–101.

[76] Tollet-Egnell, P., Parini, P., Stahlbert, N., Lonnstedt, I. *et al.*, *Physiol. Genomics.* 2002, *16*, 261–267.

[77] Mason, G. G., Murray, R. Z., Pappin, D., Rivett, A. J., *FEBS Lett.* 1998, *430*, 269–274.

[78] Naujokat, C., Hoffmann, S., *Lab. Invest.* 2002, *82*, 965–980.

[79] Braun, B. C., Glickman, M., Kraft, R., Dahlmann, B., *Nat. Cell. Biol.* 1999, *1*, 221–226.

[80] Szabo, A., Perou, C. M., Karaca, M., Perreard, L. *et al.*, *Genome. Biol.* 2004, *5*, R59.

[81] Tollet-Egnell, P., Flores-Morales, A., Odeberg, J., Lundeberg, J., Norstedt, G., *Endocrinology* 2000, *141*, 910–921.

[82] Purevjav, E., Kimura, M., Takusa, Y., Ohura, T., *Eur. J. Clin. Invest.* 2002, *32*, 707–712.

