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The dwarf mutation decreases high dose insulin responses in skeletal muscle, the opposite of effects in liver

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Abstract

The in vivo status of the proximal components of the insulin signaling system was investigated in skeletal muscle of Ames ($Prop1^{df}/Prop1^{df}$) dwarf mice. The insulin-stimulated phosphorylation of the insulin receptor (IR) was reduced by 55% in Ames dwarf mice, while IR receptor protein content was not altered. Insulin-stimulated phosphorylation of IRS-1 and IRS-2 were decreased by 79 and 51%, respectively, while IRS-1 and IRS-2 protein levels were decreased by 66 and 43%. In addition, insulin-stimulated association of IRS-1 and IRS-2 with the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase was significantly reduced (by 80 and 41%, respectively), whereas insulin-stimulated PI 3-kinase activity was reduced by 66%. However, insulin-stimulated phosphorylation of Akt was slightly reduced (by 20%), suggesting that the attenuation of insulin signaling downstream PI 3-kinase may involve other signaling molecules. Our current results demonstrate that the *Prop1* mutation decreases high dose insulin responses in skeletal muscle. This alteration is remarkable because these animals are hypersensitive to insulin and display an augmented response to insulin in liver at the same signaling steps. Reduced response to insulin in skeletal muscle could be important for the control of glucose homeostasis in these animals and could have implications in their extended longevity.

Keywords: Insulin sensitivity; Insulin signaling; Aging; Insulin receptor substrate; Growth hormone

1. Introduction

The actions of insulin are initiated through a specific transmembrane tyrosine kinase receptor, the insulin receptor (IR) (Saltiel and Kahn, 2001). The IR tyrosine kinase is activated upon insulin binding, resulting in the phosphorylation of several intracellular proteins including insulin receptor substrate (IRS)-1 and IRS-2 (Saltiel and Kahn, 2001). Phenotypes of IRS-1- and IRS-2-knockout-mice suggest that these proteins have a crucial role in the coordination of the effects of insulin on peripheral metabolism (Araki et al., 1994; Tamemoto et al., 1994; Whiters et al., 1998). The phosphorylated tyrosines in IRS-1 and IRS-2 act as docking sites for various signaling molecules including phosphatidylinositol (PI) 3-kinase (Shepherd et al., 1998; Saltiel and Kahn, 2001). The binding of tyrosine phosphorylated IRS-1 and IRS-2 to the p85 regulatory subunit of PI 3-kinase results in the activation of this enzyme (Shepherd et al., 1998; Saltiel and Kahn, 2001). Activation of PI 3-kinase has been demonstrated to be a pivotal event in the metabolic actions of insulin and IGF-1 (Shepherd et al., 1998). One downstream target of the 3-phosphoinositides generated by PI 3-kinase is the serine/threonine kinase Akt (Coffer et al., 1998). Akt appears as a critical mediator of many insulin actions (Coffer et al., 1998; Saltiel and Kahn, 2001). Moreover, recent studies have shown that Akt is a physiological mediator of insulin action (Cho et al., 2001). Akt is activated by phospholipid binding and phosphorylation at two regulatory sites, Thr308 and Ser473 (Alessi et al., 1996).

Studies in *Caenorhabditis elegans* demonstrated that disruption of the *daf-2* signaling pathway extends lifespan (Kimura et al., 1997). Similarities among the *daf-2* pathway, insulin-like signaling in flies and yeast, and the mammalian insulin-like growth factor 1 (IGF-1) signal-

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ing cascade raise the possibility that modifications to insulin/IGF-1 signaling could also extend lifespan in mammals (Bartke, 2001; Clancy et al., 2001; Fabrizio et al., 2001; Tatar et al., 2001). In fact, a major (45–65%) extension of life span has been reported in Ames dwarf mice which are deficient in GH, prolactin (PRL) and thyrotropin (TSH) (Brown-Borg et al., 1996), and a comparable life extension has been demonstrated in Snell dwarf mice which have the same hormone deficiencies (Flurkey et al., 2001, 2002), and in animals with GH resistance (Coschigano et al., 2000), while a smaller but statistically significant extension of life span was demonstrated in mice with isolated GH deficiency when their tendency to obesity was counteracted by a low fat diet (Flurkey et al., 2001).

Phenotypic characteristics of Ames dwarf mice are related to primary deficiency of GH, PRL and TSH (Bartke, 1964, 1979; Andersen et al., 1995). These endocrine changes lead to suppression of peripheral IGF-1 levels and to a state of enhanced sensitivity to insulin as determined in an insulin tolerance test (Borg et al., 1995; Dominici et al., 2002). In a previous study, we have investigated the insulin signaling system in liver of these animals and found that it was enhanced, as demonstrated by an up-regulation of the IR, IRS-1 and IRS-2 in this tissue (Dominici et al., 2002). In a study published at approximately the same time, up-regulation of IRS-1 and IRS-2 was found in liver of young Snell dwarf mice (Hsieh et al., 2002a,b). In aged Snell dwarfs the IRS-1 pool level increased further while the IRS-2 pool level showed a remarkable decrease (Hsieh et al., 2002a,b). These findings would seem to challenge the theory that attenuation of insulin signaling may be related to an increase of longevity in mammals. However, plasma insulin levels in Ames dwarf mice are reduced when measured in the fed or fasted state (Borg et al., 1995; Dominici et al., 2002) or after a glucose challenge (Dominici et al., 2002). Thus, to gain further insight of the state of insulin signaling in this mouse model of longevity, we have examined the first steps of insulin action in skeletal muscle of these animals. The in vivo phosphorylation of the IR, IRS-1 and IRS-2, the association of IRS-1 and -2 with the p85 regulatory subunit of PI 3-kinase, the phosphotyrosine-derived activity of PI 3-kinase as well as the phosphorylation level of Ser473 of Akt after insulin stimulation were evaluated in this tissue.

2. Materials and methods

2.1. Materials

The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Hercules, CA, USA). HEPES, Tris, phenylmethylsulfonyl fluoride (PMSF), aprotinin, ATP, Triton X-100, Tween 20, porcine insulin, bovine serum albumin (fraction V) (BSA), phosphatidylinositol, and polyvinylidene difluoride (PVDF) membranes were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Protein A-Sepharose 6 MB was from Pharmacia (Upsala, Sweden). ^{125}I-protein A (30 μCi/μg) was purchased from ICN Biomedicals (Costa Mesa, CA, USA). [γ-^{32}P]ATP was from Dupont-NEN (Boston, MA, USA). The monoclonal anti-phosphotyrosine antibody (αPY, PY99) and the polyclonal anti-insulin receptor β-subunit antibody (αIR, C-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The anti-rat carboxy-terminal IRS-1 antibody (αIRS-1), the anti-rat carboxy-terminal IRS-2 antibody (αIRS-2) and the antibody to the p85 subunit of PI-3-kinase (αp85) were from Upstate Biotechnology (Lake Placid, NY, USA). The phosphoplus Akt (Ser473) antibody kit was purchased from New England Biolabs (Beverly, MA, USA).

2.2. Animals

Female Ames dwarf mice (*Prop1*^{df}/*Prop1*^{df}), 4–5 months of age produced in our breeding colony were used. Mice were produced in a closed colony with a heterogeneous genetic background. Their normal (+/+ or +/*Prop1*^{df}) littermates were used as controls. We are not aware of any evidence that animals heterozigous for Ames dwarfism (+/*Prop1*^{df}) may differ from homozygous (+/+) normal animals. Mice were housed in groups of 4–5 per cage in plastic "shoe box type" cages with wood chips in a room with a controlled photoperiod of 12 h light:12 dark cycle (lights on from 06:00 to 18:00 h) and a temperature of 22±2 °C. Each cage was equipped with individual filter top (microisolator unit). Sentinel animals were housed in the same room and used for testing for antibodies to all major murine pathogens. The results of the test were uniformly negative. Animals were given free access to a nutritionally balanced diet (Rodent Laboratory Chow 5001; not autoclaved; 23.4% protein, 4.5% fat, 5.8% crude fiber; LabDiet, PMI Feeds, Inc., St. Louis, MO, USA) and tap water. All animal studies were approved by the Southern Illinois University Animal Care and Use Committee.

2.3. Measurement of glucose and hormone concentrations

Fasting serum insulin concentration was determined using a solid phase radioimmunoassay kit from DPC (Diagnostic Products Inc., Los Angeles, CA, USA). Serum glucose was measured with the glucose oxidase procedure (Trender; Sigma Chemical Co.). Growth hormone and prolactin serum concentrations were measured by radioimmunoassay (RIA) as previously described (Dominici et al., 1998, 2002). Plasma concentrations of triiodothyronine (T_3) and thyroxine (T_4)

were determined by commercial RIA kit (ICN Biomedicals). Serum IGF-1 levels were determined after acid–ethanol extraction using a human IGF-1 RIA kit as described (Chandrashekar and Bartke, 1993) All RIA measurements were performed in duplicate within the same assay. Variations between duplicate samples of less than 5% were considered acceptable. The sensitivity for these assays were: GH, 2 ng/ml; PRL, 2 ng/ml; T3, 25 ng/dl; T4, 1 μg/dl; Insulin, 3 μIU/ml; IGF-1, 2 ng/ml.

2.4. Insulin administration and tissue homogenization

Female dwarf mice and their normal siblings were starved overnight, and 15 min before the experiment they were anesthetized with isoflurane (Baxter Pharmaceutical Products Inc., Deerfield, IL, USA) as previously described (Dominici et al., 2002). After anesthesia was induced, the portal vein was exposed and 10 IU of porcine insulin per kg body weight in normal saline (0.9% NaCl) in a final volume of 0.1 ml was injected via this vein. Ames dwarf mice and normal mice were injected with diluent to obtain data under basal conditions. Approximately 3 min after injection, hindlimb skeletal muscle was removed, coarsely minced, and homogenized in 10 volumes of solubilization buffer A [1% Triton, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF, and 0.1 mg/ml aprotinin] at 4 °C as described previously (Dominici et al., 1999). Muscle extracts were centrifuged at $100\,000 \times g$ for 1 h at 4 °C to eliminate insoluble material, and protein concentration was measured using the Bradford method (Dominici et al., 1999, 2002).

2.5. Immunoprecipitation and immunoblotting

Equal amounts of muscle protein were incubated at 4 °C overnight with αIR, αIRS-1 or αIRS-2 (4 μg/ml final concentration for all antibodies). Immune complexes were collected by incubation with protein A-Sepharose, washed with solubilization buffer A, boiled in Laemli sample buffer, and stored at −70 °C until needed for electrophoresis. Resolution of proteins by SDS-PAGE and Western transfer of proteins to PVDF membranes was performed as previously described (Dominici et al., 1999, 2002). Membranes were blocked by incubation with a blocking buffer composed of Tris-buffered-saline–Tween 20 (TBS–T) buffer [10 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.02% Tween 20] containing either 3% BSA (for phosphotyrosine detection) or 5% nonfat dry milk (for protein detection). The membranes were then incubated for 4 h at room temperature with αPY (1 μg/ml), αIR (1 μg/ml), αIRS-1 (1 μg/ml) or αIRS-2 (1.5 μg/ml). Finally, membranes were incubated with ^{125}I-protein A as described (Dominici et al., 2002), and subjected to autoradiography. The intensities of the bands were quantitated by optical densitometry. The amount of the p85 subunit of the PI 3-kinase in αIRS-1 or αIRS-2 immunoprecipitates was evaluated by stripping the membranes and reblotting as previously described (Dominici et al., 2002). To determine the abundance of p85 in skeltal muscle, equal amounts of solubilized proteins (100 μg) were denatured by being boiled in reducing sample buffer, resolved by SDS-PAGE, and subjected to immunoblotting with αp85 (1:2000 dilution). Bound antibodies were detected by incubation with ^{125}I-protein A as described.

2.6. PI 3-kinase activity

Skeletal muscle of normal and Ames dwarf mice which had been injected with or without insulin were removed and homogenized in lysis buffer as described (Dominici et al., 1999). Lysates were immunoprecipitated with αPY (5 μg). PI 3-kinase was measured as described previously (Dominici et al., 1999, 2000, 2002).

2.7. Akt phosphorylation assay

Western blot analysis of Ser473 of Akt in total muscle extracts was carried out using the Phosphoplus Akt antibody kit (New England Biolabs Inc.) according to the manufacturer's protocol. Membranes were reprobed with a rabbit anti-Akt antibody (provided in the kit). Band intensities were quantitated by densitometric analysis.

2.8. Statistical analysis

Results are presented as means ± S.E.M. Experiments were performed by analyzing all groups of animals in parallel. Data were analyzed with one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test using GRAPHPAD INSTAT version 3.00 for Windows 95 by GraphPad Software, Inc. (San Diego CA, USA). Student's t test was used when the values of two groups were analyzed. The level of significance was set at $P < 0.05$.

3. Results

3.1. Animal characteristics

In the fasted state, the Ames dwarf mice displayed a moderate reduction in blood glucose levels, while circulating insulin levels exhibit a more profound reduction (Table 1). In agreement with previous reports, the concentrations of GH, IGF-1, PRL and thyroid hormones in Ames dwarf mice were extremely low and could not be detected (Table 1).

Table 1
Characteristics of Ames dwarf mice and their normal littermates

	Normal	Ames dwarf
Body weight (g)	37±5 (14)	16±3* (13)
Glucose (mg/dl)	155±17 (6)	113±19* (6)
Insulin (μIU/ml)	22±4 (6)	13±4* (6)
GH (ng/ml)	14±7 (12)	ND (12)
IGF-1 (ng/ml)	267±44 (6)	ND (6)
PRL (ng/ml)	5±2 (12)	ND (12)
T_3 (ng/dl)	88±12 (6)	ND (6)
T_4 (μg/dl)	3.8±0.4 (6)	ND (6)

Values are mean±S.E.; (numbers of mice in parentheses). Blood glucose and insulin concentrations were measured after an overnight fast. Data on GH, IGF-1, T_3 and T_4 levels were presented previously (Dominici et al., 2002). *, Significantly different from normal mice ($P < 0.01$); ND: non-detectable.

3.2. Tyrosine phosphorylation and protein levels of IR

For analysis and presentation, the value of insulin-stimulated normal mice was set as 100%. After in vivo insulin stimulation the extent of IR phosphorylation as determined by immunoblotting with an anti-phosphotyrosine antibody was reduced by 55% in skeletal muscle of Ames dwarf mice ($P < 0.01$) relative to normal mice (Fig. 1, A and B). IR protein levels were slightly but not statistically significantly decreased in Ames dwarf mice as compared with controls (Fig. 1, C and D).

3.3. Tyrosine phosphorylation and protein levels of IRS-1 and IRS-2

In both groups of animals insulin administration resulted in an increase in IRS-1 tyrosine phosphorylation. (Fig. 2, A and B). However, in Ames dwarf mice the level of IRS-1 tyrosine phosphorylation reached after stimulation with insulin was reduced by 79% ($P < 0.001$) as compared with their control littermates (Fig. 2, A and B).

Basal IRS-2 tyrosine phosphorylation was barely detectable in both control and dwarf mice (Fig. 3, A and B). The level of IRS-2 tyrosine phosphorylation after insulin stimulation was reduced by 51% ($P < 0.01$) in Ames dwarf mice (Fig. 3, A and B) relative to normal animals. As evaluated by scanning densitometry, the levels of both proteins were decreased in dwarf mice. IRS-1 protein levels were decreased by 66% ($P < 0.001$) (Fig. 2, C and D), while IRS-2 was decreased by 43% ($P < 0.001$) relative to normal animals (Fig. 3, C and D).

3.4. p85 association, PI 3-kinase activity and p85 levels

In control mice, insulin stimulation resulted in an important increase in the association of p85 with IRS-1 (Fig. 4, A and B) and in the amount of p85 associated with IRS-2 (Fig. 4, C and D). Compared with control

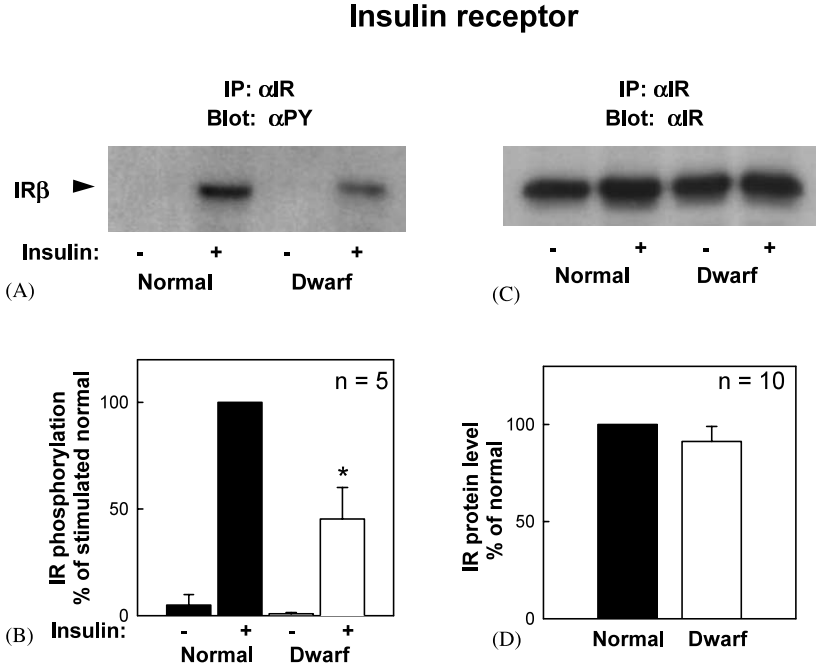

Fig. 1. IR tyrosine phosphorylation and protein levels in skeletal muscle of normal and Ames dwarf mice. A sample of muscle was obtained after injection with insulin (+) or its diluent (−) into the portal vein. Equal amounts of solubilized protein obtained as described in Section 2 were immunoprecipitated (IP) with an anti-insulin receptor antibody (αIR), run on SDS-PAGE, and analyzed by Western blotting with phosphotyrosine antibody (αPY) (A) or αIR (C). (B and D) Data quantification by scanning densitometry: means±S.E.M. of five independent experiments. IR tyrosine phosphorylation is expressed as%, assigning a value of 100% to the mean of insulin-stimulated normal mice (B). The level of IR is expressed as relative to normal values, which were set as 100% (D). * $P < 0.01$ vs. normal mice.

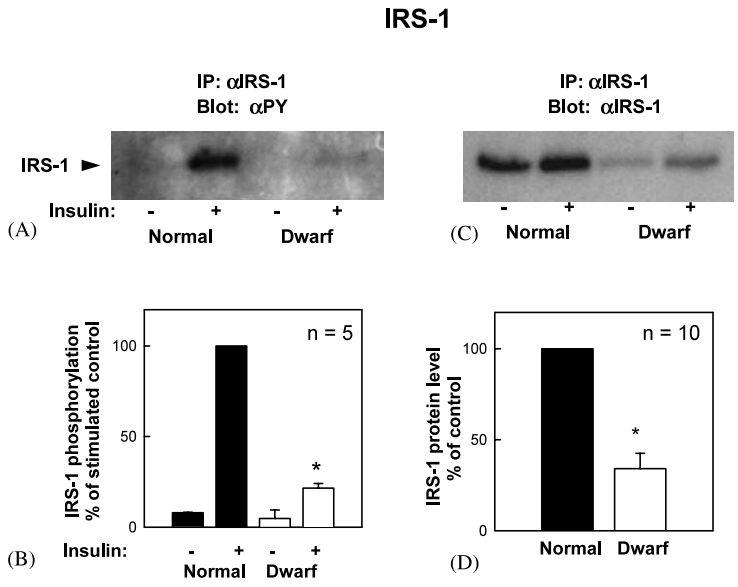

Fig. 2. IRS-1 tyrosine phosphorylation and protein levels in skeletal muscle of normal and Ames dwarf mice. A sample of muscle was obtained after injection with insulin (+) or its diluent (−) into the portal vein. Equal amounts of solubilized muscle protein were immunoprecipitated (IP) with an anti-IRS-1 antibody (αIRS-1), run on SDS-PAGE, and analyzed by Western blotting with αPY (A) and αIRS-1 (C). (B and D) Data quantification by scanning densitometry: mean ± S.E.M. of five independent experiments. IRS-1 tyrosine phosphorylation is expressed as%, assigning a value of 100% to the mean of insulin-stimulated normal mice (B). IRS-1 protein level is expressed as relative to normal values, which were set as 100% (D). * $P < 0.001$ vs. normal mice.

mice, Ames dwarf mice exhibited a reduced response to insulin at this level. After insulin stimulation the amount of p85 associated with IRS-1 was reduced by 80% ($P < 0.01$) compared with control animals (Fig. 4, A and B). As shown if Fig. 4, C and D, the amount of p85 associated to IRS-2 reached after insulin stimulation was also lower than that detected in the control group (41% reduction; $P < 0.05$).

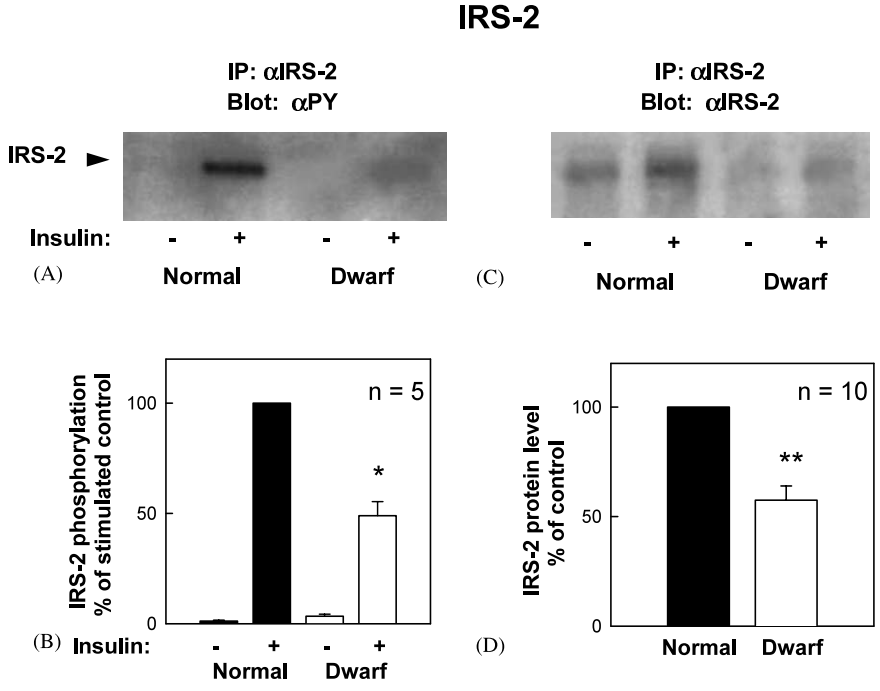

Fig. 3. IRS-2 tyrosine phosphorylation and protein levels in skeletal muscle of normal and Ames dwarf mice. Animals were injected as for Figs. 1 and 2. Equal amounts of solubilized muscle protein were immunoprecipitated (IP) with an anti-IRS-2 antibody (αIRS-2), run on SDS-PAGE, and analyzed by Western blotting with αPY (A) and αIRS-2 (C). (B and D) Data quantification by scanning densitometry: mean ± S.E.M. of five independent experiments. IRS-2 tyrosine phosphorylation is expressed as%, assigning a value of 100% to the mean of insulin-stimulated normal mice (B). IRS-2 protein level is expressed as relative to normal values, which were set as 100% (D). * $P < 0.01$ and ** $P < 0.001$ vs. normal mice.

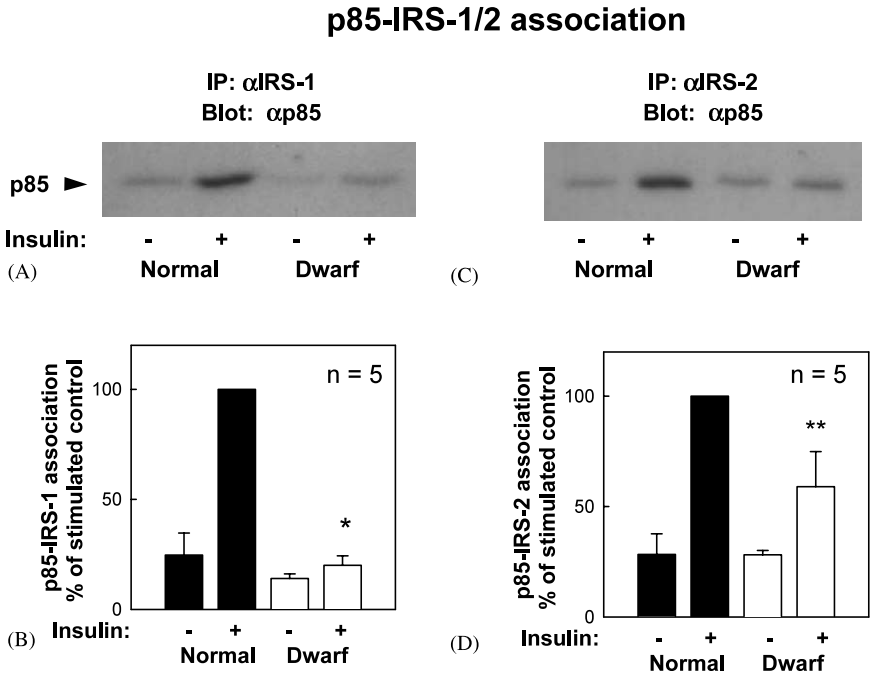

Fig. 4. Association of IRS-1 and IRS-2 with p85 in skeletal muscle of normal and Ames dwarf mice. Animals were injected as described in Figs. 1 and 2. Equal amounts of muscle protein were subjected to immunoprecipitation with αIRS-1 (A) and αIRS-2 (B), run on SDS-PAGE, and analyzed by Western blotting with an anti-p85 antibody (αp85). (B and D) Scanning densitometry on autoradiograms from five independent experiments: mean ± S.E.M. expressed as relative to normal assigning a value of 100% to the mean value in insulin-stimulated normal mice. * $P < 0.01$ and ** $P < 0.05$ vs. normal mice.

In control mice, insulin stimulation resulted in a marked increase in phosphotyrosine-derived PI 3-kinase activity (Fig. 5, A and B). Comparison of the intensity of spots corresponding to phosphatidylinositol 3-phosphate detected after insulin injection revealed that the level of insulin-stimulated PI 3-kinase activity was

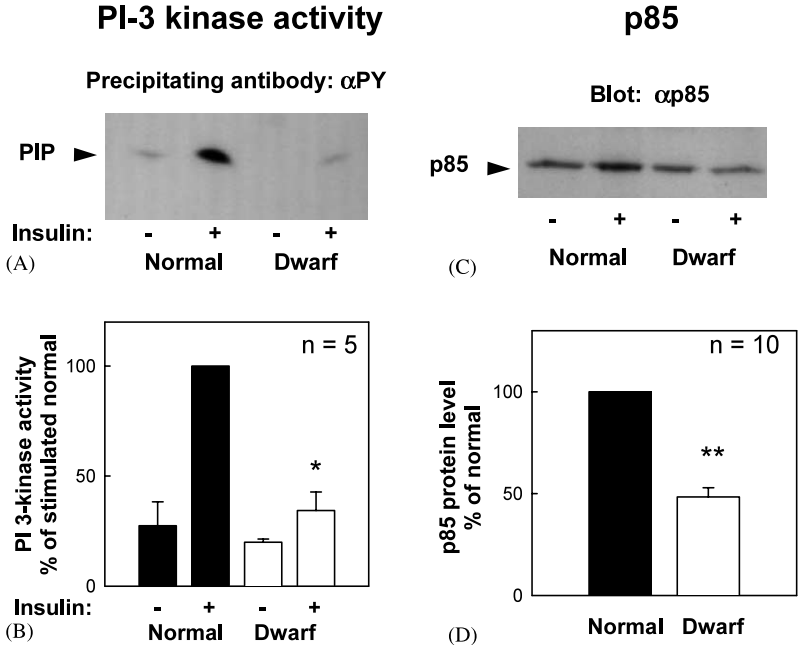

Fig. 5. Phosphotyrosine-associated PI 3-kinase activity and total content of p85 in skeletal muscle of normal and Ames dwarf mice. PI 3-kinase activity was determined as described in Section 2 (A). (B) Values are expressed as relative to normal, assigning a value of 100% to the mean value in insulin-stimulated normal mice. Data shown are means ± S.E.M of five independent experiments. (C) Equal amounts of solubilized muscle protein were separated by SDS-PAGE and immunoblotted with αp85. (D) Quantification of p85 protein levels by scanning densitometry: mean ± S.E.M. of five independent experiments. * $P < 0.002$ and ** $P < 0.005$ vs. normal mice.

reduced by 66% ($P < 0.002$) in skeletal muscle of dwarf mice (Fig. 5, A and B) relative to control animals. To determine whether the reduction in insulin-stimulated PI 3-kinase activity and p85 association with the IRS phosphoproteins in dwarf mice was associated with changes in the content of p85, homogenates of skeletal muscle were immunoblotted with p85 antibodies. There was a 52% reduction in the levels of p85, when Ames dwarf mice were compared with their normal littermates ($P < 0.005$) (Fig. 5, C and D).

3.5. Akt activation

In normal mice, insulin administration resulted in a noticeable increase in the phosphorylation of Akt at Ser473 (Fig. 6, A and B). Ames dwarf exhibited a reduced response to insulin, and the level of phosphorylation of Akt after insulin stimulation was decreased by 21% ($P < 0.02$) compared with values measured in normal mice (Fig. 6, A and B). There was no statistically significant change in expression of Akt, when Ames dwarf mice were compared with their normal littermates (Fig. 6, C and D).

4. Discussion

Several rodent models of GH deficiency or resistance have been shown to display extended longevity (Brown-Borg et al., 1996; Coschigano et al., 2000; Flurkey et al., 2001, 2002). The common factor linking these observations seems to be the reduction in the exposure to growth factors like insulin, GH and IGF-1 (Bartke, 2001; Parr, 1999). Thus, reduced insulin levels and a consequent attenuation of insulin/IGF-1 signaling should favor an extension of life span. In an effort to test this theory, we have previously analyzed the status of insulin signaling and insulin action in Ames dwarf mice and demonstrated a state of increased sensitivity to insulin together with an important upregulation of the first components of insulin signaling in the liver of these animals (Dominici et al., 2002). Skeletal muscle is a pivotal target of insulin action and the disposal of glucose that takes place in this tissue is central to fuel metabolism (Saltiel and Kahn, 2001). Thus, to further characterize this mammalian model of longevity, in the current work we analyzed the status of the proximal steps of the insulin signaling system in the skeletal muscle of Ames dwarf mice.

Remarkably, in skeletal muscle of Ames dwarf mice we found changes in the proximal insulin signaling steps which are the opposite of those we have previously found in the liver of these animals (Dominici et al., 2002). With the exception of the IR levels which were not significantly altered, a significant reduction was found in the expression of IRS-1, IRS-2 and p85 in this tissue. Moreover, a reduced response to a high dose of insulin in terms of IR, IRS-1 and IRS-2 phosphorylation, p85-IRS-1/IRS-2 association, PI 3-kinase activity and Akt activation was detected. The alterations ob-

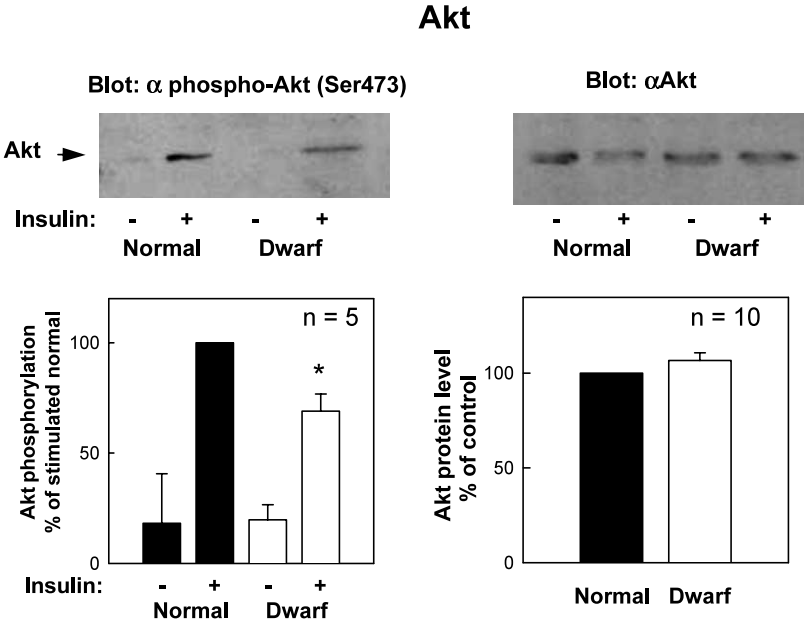

Fig. 6. Akt phosphorylation and protein levels in skeletal muscle of normal and Ames dwarf mice. Animals were injected i.v. with insulin. Equal amounts of muscle protein were run on SDS-PAGE and subjected to Western blotting with specific antibodies against Akt-Ser473 (αphospho-Akt) (A). (B) Data quantification by scanning densitometry: mean ± S.E.M. of five independent experiments. Values are expressed as%, assigning a value of 100% to the mean value in insulin-stimulated normal mice. (C) The same membranes were reprobed with an anti-Akt antibody (αAkt). (D) Band intensities were quantitated by scanning densitometry: means ± S.E.M. of five independent experiments, expressed as relative to normal values, which were set as 100%. * $P < 0.02$ vs. normal mice.

served suggest that p85 may be a major site of attenuation of the insulin signaling pathway in this tissue. Our data showing no change in Akt levels and only a slight decrease in the phosphorylation of Akt suggest that the *Prop1* mutation has no major effect on Akt in skeletal muscle and raises the possibility that other p85 downstream targets might be involved in transducing the reduction of insulin signaling detected. Possibly the effects of this mutation on Akt may become more apparent in older animals.

In the context of the current theory of aging, the alterations found in skeletal muscle of Ames dwarf mice could be related to the substantial extension of life span that has been detected in these animals, as was postulated in Snell dwarf mice (Hsieh et al., 2002a,b). In this sense, our findings would represent the first demonstration of a reduction in both the expression and in the maximal response to insulin at the proximal steps of the insulin signaling in an insulin target tissue of a mammalian model of longevity. However, it is important to point out that the experimental design utilized does not allow us to conclude that there is a difference in the response to insulin between Ames dwarf mice and their control animals under physiological conditions, since the status of the insulin signaling components was analyzed only after administration of a large dose of insulin that attains supraphysiological circulating concentrations of the hormone.

The reasons for the contrast found between liver and skeletal muscle are not clear. A similar differential regulation of insulin signal transduction in liver and muscle has also been detected in dexamethasone-treated rats (Saad et al., 1993). However, the participation of glucocorticoids in our current results appears to be excluded since corticosterone levels were found to be decreased in Ames dwarf mice of the same age and sex as the ones utilized in the present study (Borg et al., 1995). It is important to note that the decreased levels of the insulin signaling components analyzed together with the limited maximal of insulin response in skeletal muscle of Ames dwarf mice reported in the present paper is concomitant with increased insulin sensitivity evidenced by a concomitant reduction in plasma insulin and plasma glucose levels, together with an increased hypoglycemic response to insulin after an insulin tolerance test as was previously reported (Dominici et al., 2002). Although it remains to be determined if the differences observed reflect the status of the insulin signaling system under normal conditions, the alterations detected in skeletal muscle of these relatively hypoglycemic mice could represent a physiological response to avoid severe hypoglycemia. Thus, the ability to down-regulate some of the proximal insulin signaling molecules and to reduce their response to insulin according to changes in the metabolic millieu could be important for glucose homeostasis in these animals and may also be important for longevity.

An extensive evaluation of insulin and IGF-1 signal transduction in the available mammalian models of extended longevity might be useful to elaborate a theory relating insulin signaling and action to longevity in mammals. The status of the proximal steps of the insulin signaling system has been analyzed in liver of Snell dwarf mice at two different ages. Young Snell dwarfs (3–6 months of age) exhibit a large increase in the IRS-1 pool levels and particularly in the IRS-2 pool levels in this tissue, while in comparison to age-matched controls, old (20–23 months of age) Snell dwarfs exhibit a further increase in the IRS-1 pool levels together with a dramatic decrease in the content of IRS-2 (Hsieh et al., 2002a,b). The phosphorylation of the IR, IRS-1 and IRS-2, together with the p85-IRS association and the activity of PI 3-kinase under basal conditions were found to be decreased in Snell dwarf mice (Hsieh et al., 2002a,b). Our previous studies demonstrated an upregulation of insulin signal transduction in the liver of GHR-KO and Ames dwarf mice (Dominici et al., 2000, 2002). Data showing reduced basal insulin signaling in young Snell dwarf mice does not contradict these results since important increases in the hepatic expression of IRS-1 and IRS-2 were found (Hsieh et al., 2002a,b). The impairment of insulin signaling found in skeletal muscle of Ames dwarf mice in the present study, together with our preliminary results indicating a similar alteration in GHR-KO mice (Argentino DP et al., personal communication) suggest that the changes in the insulin signaling system exhibited by these models of longevity might be tissue-specific. From our current results we postulate that enhanced hepatic insulin sensitivity could be responsible for the hypersensitivity to insulin exhibited by Ames dwarf mice, by reducing the need for circulating insulin. The reduced expression of IRS-1, IRS-2 and p85 together with the limited maximal capacity of insulin response in skeletal muscle of these animals could represent a physiological mechanism to avoid severe hypoglycemia and may be important for the control of glucose homeostasis and also for longevity. However, the corroboration of this hypothesis requires further analysis.

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References

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., Hemmings, B.A., 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. 15, 6541–6551.

Andersen, B., Pearse, R.V., Jenne, K., Sornson, M., Lin, S.C., Bartke, A., Rosenfeld, M.G., 1995. The Ames dwarf gene is required for Pit-1 gene activation. Dev. Biol. 172, 495–503.

Araki, E., Lipes, M.A., Patti, M.A., Bruning, J.C., Haag, B., Johnson, R.S., Kahn, C.R., 1994. Alternative pathway of insulin signaling in mice with target disruption of the IRS-1 gene. Nature 372, 186–190.

Bartke, A., 1964. Histology of the anterior hypophysis, thyroid and gonads of two types of dwarf mice. Anat. Rec. 149, 225–235.

Bartke, A., 1979. Genetic models in the study of anterior pituitary hormones. In: Shire, J.G.M. (Ed.), Genetic Variation in Hormone Systems. CRC Press, Boca Raton, FL, pp. 113–126.

Bartke, A., 2001. Mutations prolong life in flies; implications for aging in mammals. Trends Endocrinol. Metab. 12, 233–234.

Borg, K.E., Brown-Borg, H.M., Bartke, A., 1995. Assessment of the primary adrenal cortical and pancreatic hormone basal levels in relation to plasma glucose and age in the unstressed Ames dwarf mouse. Proc. Soc. Exp. Biol. Med. 210, 126–133.

Brown-Borg, H.M., Borg, K.E., Meliska, C.J., Bartke, A., 1996. Dwarf mice and the ageing process. Nature 384, 33.

Chandrashekar, V., Bartke, A., 1993. Induction of endogenous insulin-like growth factor-1 secretion alters the hypothalamic–pituitary–testicular function in growth hormone-deficient adult dwarf mice. Biol. Reprod. 48, 544–551.

Cho, H., Mu, J., Kim, J.K., Thorvaldsen, J.L., Chu, Q., Crenshaw, E.B., III, Kaestner, K.H., Bartolomei, M.S., Shulman, G.I., Birbaum, M.J., 2001. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science 292, 1728–1731.

Clancy, D.J., Gems, D., Harshman, L.G., Oldham, S., Stocker, H., Hafen, E., Leevers, S.J., Partridge, L., 2001. Extension of life-span by loss of CHICO, a *Drosophila* insulin receptor substrate protein. Science 292, 104–106.

Coffer, P.J., Jin, J., Woodget, J.R., 1998. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem. J. 335, 1–13.

Coschigano, K.T., Clemmons, D., Bellush, L.L., Kopchick, J.J., 2000. Assessment of growth parameters and life span of GHR/BP gene-disrupted mice. Endocrinology 141, 2608–2613.

Dominici, F.P., Balbis, A., Bartke, A., Turyn, D., 1998. Role of hyperinsulinemia on hepatic insulin receptor concentration and autophosphorylation in the presence of high growth hormone levels in transgenic mice overexpressing GH gene. J. Endocrinol. 159, 15–25.

Dominici, F.P., Cifone, D., Bartke, A., Turyn, D., 1999. Alterations in early steps of the insulin-signaling system in skeletal muscle of GH-transgenic mice. Am. J. Physiol. 277, E447–E454.

Dominici, F.P., Arostegui Diaz, G., Kopchick, J.J., Bartke, A., Turyn, D., 2000. Compensatory alterations of insulin signal transduction in liver of growth hormone receptor knockout mice. J. Endocrinol. 166, 579–590.

Dominici, F.P., Hauck, S., Argentino, D.P., Bartke, A., Turyn, D., 2002. Increased insulin sensitivity and upregulation of insulin receptor, insulin receptor substrate IRS-1 and IRS-2 in liver of Ames dwarf mice. J. Endocrinol. 173, 81–94.

Fabrizio, P., Pozza, F., Pletcher, S.D., Gendron, C.M., Longo, V.D., 2001. Regulation of longevity and stress resistance by Shc9 in yeast. Science 292, 288–290.

Flurkey, K., Papaconstantinou, J., Miller, R.A., Harrison, D.E., 2001. Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. Proc. Natl. Acad. Sci. USA 98, 6736–6741.

Flurkey, K., Papaconstantinou, J., Harrison, D.E., 2002. The Snell dwarf mutation can increase life span in mice. Mech. Ageing Dev. 123, 121–130.

Hsieh, C.C., DeFord, J.H., Flurkey, K., Harrison, D.E., Papaconstantinou, J., 2002a. Implications for the insulin signaling pathway in Snell dwarf mouse longevity: a similarity with the *C. elegans* longevity paradigm. Mech. Ageing Dev. 123, 1229–1244.

Hsieh, C.C., DeFord, J.H., Flurkey, K., Harrison, D.E., Papaconstantinou, J., 2002b. Effects of the Pit1 mutation on the insulin signaling pathway: implications on the longevity of the long-lived Snell dwarf mouse. Mech. Ageing Dev. 123, 1245–1255.

Kimura, K.D., Tissenbaum, H.A., Liu, Y., Ruvkun, G., 1997. daf-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. Science 277, 942–946.

Parr, T., 1999. Insulin exposure and unifying aging. Gerontology 45, 121–135.

Saad, M.J.A., Folli, F., Kahn, J.A., Kahn, C.R., 1993. Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. J. Clin. Invest. 92, 2065–2072.

Saltiel, A.R., Kahn, C.R., 2001. Insulin signaling and the regulation of glucose and lipid metabolism. Nature 414, 799–806.

Shepherd, P.R., Withers, D.J., Siddle, K., 1998. Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem. J. 333, 471–490.

Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., Sekchara, H., Yoshioka, S., Horikoshi, H., Furuta, Y., Ikawa, Y., Kasuga, M., Yazaki, Y., Aizawa, S., 1994. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature 372, 182–186.

Tatar, M., Kopelman, A., Epstein, D., Tu, M.-P., Yin, C.-M., Garofalo, R.S., 2001. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science 292, 107–110.

Whiters, D.J., Gutierrez, J.S., Towery, H., Burks, D.J., Ren, J.M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G.I., Bonner-Weir, S., White, M.F., 1998. Disruption of IRS-2 causes type 2 diabetes in mice. Nature 391, 900–904.