Endocrine, *vol. 19, no. 3, 249–256, December 2002* 0969–711X/02/19:249–256/$20.00

A Model for Tissue-Specific Inducible Insulin-like Growth Factor-I (IGF-I) Inactivation to Determine the Physiological Role of Liver-Derived IGF-I

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Insulin-like growth factor-I (IGF-I) has important growth-promoting and metabolic effects and is expressed in virtually every tissue of the body. The highest expression is found in the liver, but the physiological role of liver-derived IGF-I is unknown. It has been difficult to separate the endocrine effects of liver-derived IGF-I from the autocrine/paracrine effects of locally produced IGF-I in peripheral tissues. Therefore, we have developed a mouse model with a liver-specific inducible deletion of the IGF-I gene (LI-IGF-I–/– mouse). The LI-IGF-I–/– mouse has dramatically reduced (>80%) serum IGF-I levels, demonstrating that the major part of serum IGF-I is liver-derived. Surprisingly, LI-IGF-I–/– mice demonstrate a normal appendicular skeletal growth up to at least 12 mo of age despite the dramatic decrease in circulating IGF-I levels, indicating that liver-derived IGF-I is not required for appendicular skeletal growth. However, the adult axial skeletal growth is reduced in the LI-IGF-I–/– mice. Furthermore, the amount of cortical bone is reduced due to decreased radial growth of the cortical bone, while the trabecular bone mineral density is unchanged in the LI-IGF-I–/– mice. The decreased levels of circulating IGF-I are associated with increased serum levels of growth hormone (GH), indicating a role for liver-derived IGF-I in the negative-feedback regulation of GH secretion. Measurements of factors regulating GH secretion in the pituitary and in the hypothalamus revealed an increased expression of GH-releasing-hormone (GHRH) and GH-secretagogue (GHS) receptors in the pituitary of LI-IGF-I–/– mice. This in turn results in an increased sensitivity to systemically administered GHRH and GHS, demonstrating that the regulatory action of liver-derived IGF-I on GH secretion is at the pituitary rather than at the hypothalamic level. The liver is an important metabolic organ and LI-IGF-I–/– mice are markedly hyperinsulinemic and yet normoglycemic, consistent with an adequately compensated insulin resistance. Interestingly, LI-IGF-I–/– mice display a reduced age-dependent fat mass accumulation compared with control mice. Furthermore, LI-IGF-I–/– mice have increased blood pressure attributable to increased peripheral resistance indicating a role for liver-derived IGF-I in the regulation of blood pressure. In conclusion, liver-derived IGF-I is important for carbohydrate and lipid metabolism and for the regulation of GH secretion at the pituitary level. Furthermore, it regulates adult axial skeletal growth and cortical radial growth while it is not required for appendicular skeletal growth.

Key Words: IGF-I; liver; inducible; bone; fat.

Introduction

The physiological role of liver-derived circulating IGF-I vs IGF-I produced locally in peripheral tissues has been difficult to investigate. To address this issue and to study the importance of liver-derived IGF-I for growth and metabolism, we and others developed a liver-IGF-I deficient mouse model.

Liver-Specific Inducible IGF-I Inactivation

The Cre/*loxP* system is used to achieve cell type and/or time-specific gene targeting *(1)*. Cre/*loxP* is a site-specific recombination system of bacteriophage P1. Cre (causes recombination) has been shown to perform efficient recombination at *loxP* sites (locus of X-ing over) in bacteria as well as in eukaryotic cells *(2,3)*. Cre-mediated excision can be used to remove DNA segments in the genome: Two genetically modified mouse lines are crossed, one that carries the *Cre* gene coupled to a tissue-specific promoter and one where the *loxP* sites have been integrated into the genome by genetic targeting. Cre protein will be expressed in the desired tissue, where it will excise DNA sequences flanked by *loxP* sites. This system was used to establish a transgenic mouse model with a liver-specific, inducible inactivation of the *IGF-I* gene (Fig. 1).

Received October 31, 2002; Accepted November 15, 2002.

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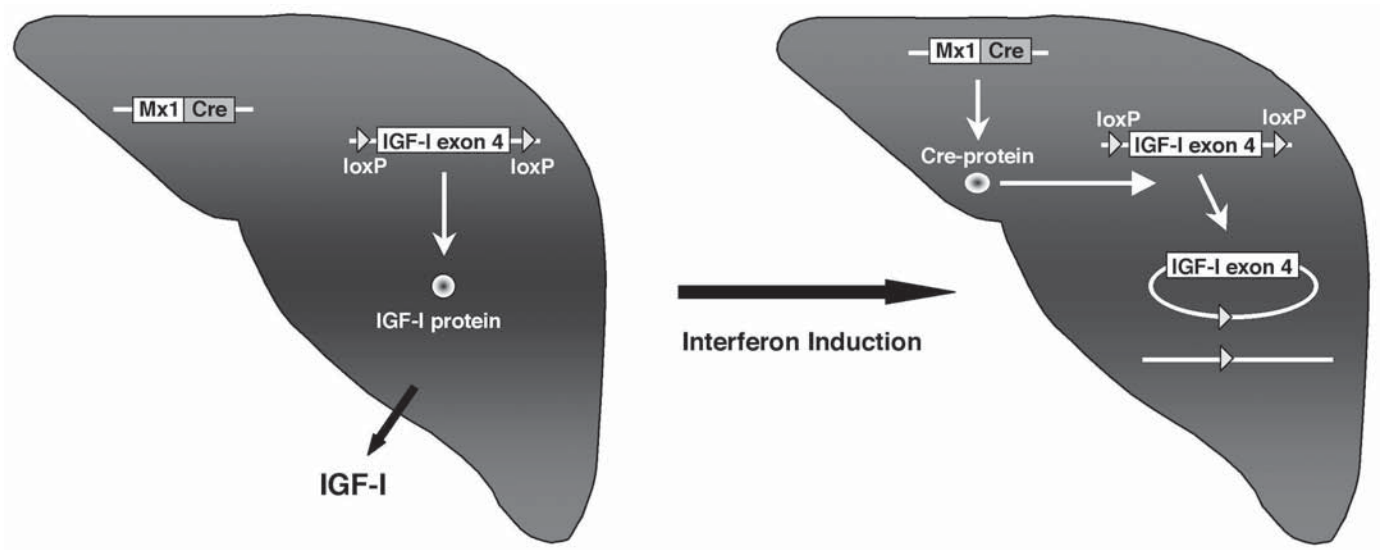

Fig. 1. Liver-specific inducible IGF-I inactivation. At 3 wk of age, mice homozygous for IGF-I exon 4 flanked with *loxP* sites and heterozygous for the Mx1-Cre construction were given INF to induce Cre expression. Cre causes recombination of the *loxP* sites and subsequent excision of IGF-I exon 4 from genomic DNA. No functional IGF-I protein will be expressed in Cre-expressing cells, while in other cells where the Mx1 promoter is silent IGF-I expression is normal. Cre-mediated excision in LI-IGF-I–/– mice led to a 95% decrease in IGF-I mRNA levels in liver, whereas no significant effect was seen in other extrahepatic tissues including fat, muscle, bone, kidney, brain, and heart.

The inducible promoter of the *Mx1* gene was used to drive the expression of Cre *(4)*. The *Mx1* gene is part of the defense against viral infections, and it renders mice resistant to infections with influenza A and B viruses *(5)*. *Mx* gene homologs have been found in organisms ranging from yeast to humans *(6,7)*. The expression of Mx1 can be induced by interferon α (IFN-α) or interferon β (IFN-β) *(8)*. It is also induced by polyinosinic–polycytidylic acid (pI-pC), which is a synthetic, double-stranded RNA that activates the Mx1 promoter directly as well as via induction of endogenous IFN *(9)*. The time course of induction is transient and declines after 6 h in mice *(9)*. The activity of the promoter after induction is largely dependent on the integration site and, in the transgenic mouse line we used, the expression of Cre was high in liver, medium in spleen, and very low or absent in other tissues *(4)*.

The Mx1-Cre mice were crossed with mice that have had their normal exon 4 IGF-I allele replaced with an allele where IGF-I exon 4 is flanked by two *loxP* sites *(10)*. Exon 4 encodes several critical residues for the ligand–receptor interaction, and Cre-mediated excision will result in a truncated IGF-I protein that has no functional capability *(11)*. The crossing resulted in mice homozygous for IGF-I exon 4 flanked by *loxP* sites and either heterozygous for or lacking the Mx1-Cre construction. We waited until weaning to induce Cre expression by giving either human IFNα_2/α_1 or pI-pC. An important advantage of this inducible model is that the mice do not carry the knockout during embryogenesis or early neonatal life, when there can be a risk of disturbed development and/or induction of compensatory mechanisms. The efficiency of recombination was studied by Southern blot. There was an approx 90% recombination in the liver, which consists of both the hepatocytes (with a high proportion of binuclear and polyploid cells) and nonparenchymal cells *(12)*. In purified hepatocytes from LI-IGF-I–/– mice, the IGF-I gene was completely recombined. In all other tissues studied the recombination were less than 20%, except for the spleen where the recombination was 65%. No recombination was found in control mice lacking the Mx1-Cre construction but homozygous for IGF-I exon 4 flanked by *loxP* sites. IGF-I mRNA levels in LI-IGF-I–/– mice were decreased by more than 95% in liver and by approx 60% in spleen, whereas no significant effect was seen in other extrahepatic tissues including fat, muscle, bone, kidney, brain, and heart *(13)*. At 4 wk of age, the LI-IGF-I–/– mice had a >80% decrease in serum IGF-I levels. This decrease was still present when the mice were 13 mo old, indicating a complete and sustained inactivation of the IGF-I gene in hepatocytes *(14)*.

Liver-Derived IGF-I Is Not Required for Appendicular Skeletal Growth but Is of Some Importance for Adult Axial Skeletal Growth

Body growth (weight gain) and skeletal growth during and shortly after sexual maturation was normal in the LI-IGF-I–/– mice, demonstrating that liver-derived IGF-I is not required for postnatal growth. Up to 10 wk of age, mice maintained normal growth rate despite having dramatically decreased serum IGF- I levels (Fig. 2) *(13,15)*. In contrast to humans, mice do not close their growth plates in the long

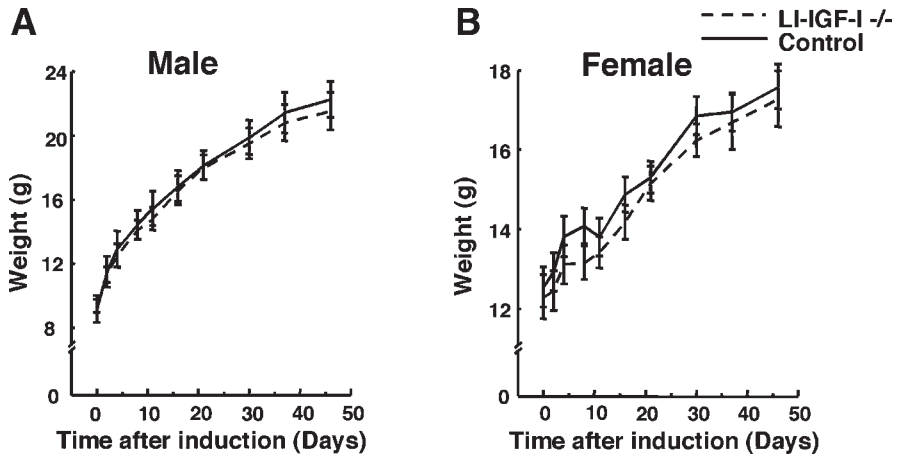

Fig. 2. Body growth in liver-inducible IGF-I–/– (LI-IGF-I–/–) mice. Mice were given IFN at 3 wk of age to induce Cre expression. The weights of male (**A**) and female (**B**) mice at various times after induction are indicated. Number of observations (n) = 4–5. Figure is reproduced from Sjögren et al. *(13)*.

bones completely after puberty, but continue to grow until at least 1 yr of age. To study the long-term effects of liver-specific IGF-I deletion on skeletal growth, mice were investigated at 13 mo of age. Interestingly, the adult axial skeletal growth was decreased by 24% in the LI-IGF-I–/– mice, while no major reduction of the adult appendicular skeletal growth was seen *(16)*. Taken together, these data demonstrate that body growth and skeletal growth during sexual maturation is independent of liver-derived IGF-I. However, liver-derived IGF-I is clearly of some importance for adult axial- but not appendicular-skeletal growth.

In serum, the majority of the IGFs exist in a 150 kDa complex consisting of an IGF molecule, IGF binding protein 3, and acid labile subunit (ALS) *(17)*. This complex prolongs the half-lives of IGFs and is assumed to facilitate their endocrine action. Recently, four different transgenic mouse models with decreased serum IGF-I levels have been reported: (i) the *liver-specific inducible* IGF-I gene inactivation (LI-IGF-I–/–, *13*), (ii) the *liver-specific* IGF-I gene inactivation using the albumin promoter (LID) *(15)*, (iii) the ALS gene inactivation *(18)*, and (iv) the double knockout of the liver IGF-I gene and the ALS gene (LID/ALS) *(19)*. By optimizing the inducible IGF-I inactivation in the liver of the LI-IGF-I–/– mice, we have now been able to reproducibly and with a high efficiency inactivate the IGF-I gene in liver, resulting in serum IGF-I levels of 10–15% of wild-type levels *(16)*. The LID/ALS double-knockout mice have serum IGF-I levels decreased to a similar extent as seen in the LI-IGF-I–/– mice *(19)*. The early postnatal GH-independent longitudinal bone growth of femur was clearly decreased in the LID/ALS double-knockout mice *(19)*. We have not yet studied this early growth period in the inducible LI-IGF-I–/– mouse model. The decreased early postnatal GH-independent longitudinal bone growth in the LID/ALS double-knockout mice might be because this growth is dependent on serum IGF-I or, alternatively, ALS may exert other important functions besides being a passive carrier of IGF in serum. Autocrine/paracrine effects of ALS in peripheral tissues are possible, as expression of ALS has been found in several tissues besides liver including kidney, developing bone, lactating mammary gland, thymus, and lung and in the theca and granulosa cells of the ovary *(20–22)*. In contrast, to the disturbed GH-independent growth during early life, longitudinal bone growth during the GH-dependent growth period is almost unchanged in the LI-IGF-I–/– and the LID/ALS double knockout *(16,19)*. Thus, results from several different mouse models indicate that the GH-dependent longitudinal bone growth is largely independent on serum IGF-I.

One may speculate that IGF-II might compensate for the decreased IGF-I stimulation, but the serum IGF-II levels, IGF-II mRNA levels in bone, and bone content of IGF-II were unchanged in LI-IGF-I–/– mice *(16)*. Insulin-like growth factor binding protein (IGFBP)-2 and -3 in serum were decreased in mice with liver-specific inactivation of the IGF-I gene, and it could be argued that the decrease in serum IGF-I is not within the fraction of free and dissociable IGF-I that would be consistent with their normal appendicular skeletal growth. This is a plausible explanation considering that in one study measurements of free IGF-I showed no significant difference between liver-IGF-I deficient mice and controls *(23)*. However, it is unlikely that the method used to estimate dissociable IGF-I gives a true value of biologically active IGF-I as seen by the cells in vivo *(24)*. Furthermore, several physiological parameters, including increased GH secretion, insulin resistance, decreased fat mass, increased leptin levels, increased blood pressure, decreased periosteal bone growth, and reduced axial skeletal growth clearly suggest that the levels of biologically active free IGF-I are altered in LI-IGF-I–/– mice *(13–16,23,25,26)*.

There have been a few studies addressing the relative importance of local versus systemic effects of IGF-I on body growth. Treatment of patients with GH insensitivity syndrome have shown that IGF-I is not as effective as GH at restoring growth when compared with similarly aged infants with GH deficiency (GHD) treated with GH *(27,28)*. On the other hand, the growth phenotype of GH receptor null mice was almost completely reversed by IGF-I treatment *(29)*. Together these studies show that systemically administered IGF-I may have the capacity to compensate for autocrine/paracrine-acting IGF-I, although the LI-IGF-I–/– mice demonstrate that it is not required for postnatal body growth.

Effects on Adult Bone Metabolism in Liver-IGF-I Knockout Mice

GH and IGF-I are important for normal adult bone metabolism. GHD results in a decreased bone mass in both humans and experimental animals *(30)*. Long-term treatment of GHD patients with GH increases bone mass and accumulation of IGF-I in cortical and trabecular bone *(31,32)*.

Although it is clear that IGF-I affects adult bone metabolism, it is difficult to separate systemic effects of liver-derived IGF-I from the local effects of autocrine/paracrine IGF-I in bone. Mice with a liver-specific inactivation of the IGF-I gene have decreased serum IGF-I levels by >80% but normal expression of IGF-I and -II transcript in bone, which make them a unique model to investigate the effects of liver-derived, endocrine, IGF-I on adult bone metabolism. Cortical and trabecular bone parameters were measured in IGF-I-deficient mice at different ages. LI-IGF-I–/– mice had a clear reduction in the amount of cortical bone. The decreased cortical bone mineral content (BMC) in these mice was mainly due to a decreased periosteal circumference associated with decreased cortical thickness *(16)*. Several studies have shown that GH treatment of normal as well as ovariectomized rats results in an increased cortical bone formation at the periosteal surface *(33–35)*. Results from LI-IGF-I–/– mice indicate that at least some of the stimulatory effect of GH on cortical radial bone growth is dependent on liver-derived IGF-I. In contrast, the trabecular bone mineral density (BMD) was unchanged in the LI-IGF-I–/– mice *(16)*. In summary, liver-derived IGF-I exerts a small but significant effect on cortical periosteal bone growth, while it is not required for the maintenance of the trabecular bone mineral density in adult mice.

Liver-Derived IGF-I Regulates GH Secretion

Absence of liver-derived IGF-I caused a compensatory increase in serum GH levels in mice *(13,15)*. Both male and female LI-IGF-I–/– mice have increased relative liver weights *(25)*. It seems reasonable to hypothesize that the increased liver weight in the LI-IGF-I–/– mice is due to increased GH-levels. Hepatocytes have very high levels of GH receptors, and results from GH-overexpressing mice indicate that enhanced GH levels cause a disproportional increase in liver growth *(36)*, while GH-receptor-knockout mice have a decreased relative liver weight *(37)*.

Two major hypothalamic hormones regulate GH secretion from the pituitary: GH-releasing hormone (GHRH), which induces GH-secretion, and somatostatin, which inhibits GH secretion *(38,39)*. The low basal GH levels between pulses in male rats are probably due to suppression of GH by surges of hypothalamic somatostatin, which may inhibit release of hypothalamic GHRH as well as its action on the pituitary. Neuropeptide Y (NPY) is another hypothalamic peptide that has been reported to mediate negative-feedback inhibition of GH secretion, possibly by increasing somatostatin expression *(40,41)*. Activation of the GH-secretagogue (GHS) receptor induces GH-secretion, involving direct stimulation at the pituitary level as well as stimulation of GHRH release and possibly also inhibition of somatostatin release at the hypothalamic level *(42,43)*. Until recently, the only known ligands for the GHS receptor were synthetic peptides and nonpeptides such as GH-releasing peptide-6 (GHRP-6), MK677, and ipamorelin *(44)*. The first endogenous GH secretagogue, ghrelin, was cloned from rat stomach *(45)*. To investigate the mechanism behind the increased GH levels in LI-IGF-I–/– mice, the mRNA expression of several of these GH secretion regulatory factors were measured in the hypothalamus and the pituitary. LI-IGF-I–/– mice had increased expression of the receptors for GHRH and GHS in the pituitary, while no effect was seen on expression of pituitary somatostatin receptors or hypothalamic GHRH, somatostatin, and NPY. Consistent with an increased number of bioactive pituitary receptors, mice with liver-specific IGF-1 depletion had an increased sensitivity to GHRH and GHS when injected systemically *(25)*.

A GHRH antagonistic effect of IGF-I has been demonstrated earlier in rat pituitary cells in vitro *(46,47)*. An inhibitory effect of IGF-I on GHRH receptor mRNA levels has also been reported in vivo by Kamegai et al. when giving IGF-I replacement to the GH-deficient spontaneous dwarf rat *(48)*. Similarly to LI-IGF-I–/– mice, GH-receptor-knockout mice have markedly decreased levels of IGF-I in the circulation, and they were also found to have increased GHRH receptor and GHS receptor levels in the pituitary *(49)*.

Clinical studies on the effects of pharmacological IGF-I treatment on GHRH responsiveness have not been completely conclusive. IGF-I infusion has been reported to decrease the GH response to GHRH treatment in men but not in women, while this treatment inhibited the spontaneous, pulsatile GH secretion in both men and women *(50)*. In another study, however, IGF-I treatment did suppress GHRH-induced GH-secretion in young women *(51)*. In contrast to the study in LI-IGF-I–/– mice, IGF-I has earlier been shown to have effects on GH secretion at the hypothalamic level, e.g., via enhanced somatostatin release *(46,52,53)*. However, in these studies

IGF-I was administered either intracerebroventricularly *(52,53)* or in an in vitro system *(46)*. Perhaps those studies demonstrate that local IGF-I expression in the hypothalamus can have effects on GH secretion, while the LI-IGF-I–/– mouse model demonstrates the effects of endogenous liver-derived IGF-I.

In summary, loss of liver-derived IGF-I feedback on the hypothalamic–pituitary system increases GH secretion and results in increased expression and sensitivity of pituitary GHRH and GHS receptors. Therefore, the major site of action of liver-derived IGF-I in the regulation of GH secretion seems to be at the pituitary rather than at the hypothalamic level.

Changes in Glucose Metabolism and Decreased Fat Mass in LI-IGF-I–/– Mice

It is well known that IGF-I causes an acute decrease in glucose and insulin concentrations in the blood, and IGF-I has even been suggested as a potential therapy for diabetes because it reduces insulin resistance *(54,55)*. In line with these studies, LI-IGF-I–/– mice have elevated basal as well as glucose-induced insulin levels but normal glucose levels, which could be interpreted as adequately compensated insulin resistance (Fig. 3) *(14,23)*. A similar phenotype is seen in mice overexpressing IGFBP-1, which results in a partial IGF-I deficiency *(56)*. Furthermore, IGF-I overexpressing mice have reduced basal insulin levels *(57)*. In a recent study in humans with normal glucose tolerance at baseline, high circulating levels of IGF-I were associated with reduced risk of development of impaired glucose tolerance and type-2 diabetes *(58)*. Together, these studies may indicate an important role of liver-derived endocrine IGF-I for normal glucose homeostasis.

Insulin resistance is usually associated with obesity, but LI-IGF-I–/– mice had decreased fat content at 13 mo of age and thus seem to be at least partially protected against the obesity that normally develops in older mice (Fig. 4) *(14)*. One possibility is that the decreased fat mass might be secondary to the elevated GH levels in LI-IGF-I–/– mice. GH is known to promote lipolysis directly in adipose tissue by enhancing the reactivity of hormone-sensitive triglyceride lipase to lipolytic hormones *(59)*. In addition, GH renders adipose tissue less sensitive to the antilipolytic effects of insulin *(60)*. Furthermore, GHD humans have increased fat mass, a condition that can be reversed by GH treatment *(61)*. Some studies have shown that the effects of IGF-I on fat mass and lipid metabolism are similar to those of GH, arguing that the low levels of circulating IGF-I in LI-IGF-I–/– mice are not directly involved in the decreased fat mass in these mice. Treatment with IGF-I in normal adults was accompanied by elevated energy expenditure and lipid oxidation and reduced protein oxidation *(62)*. Several studies have also reported that IGF-I treatment decreases body

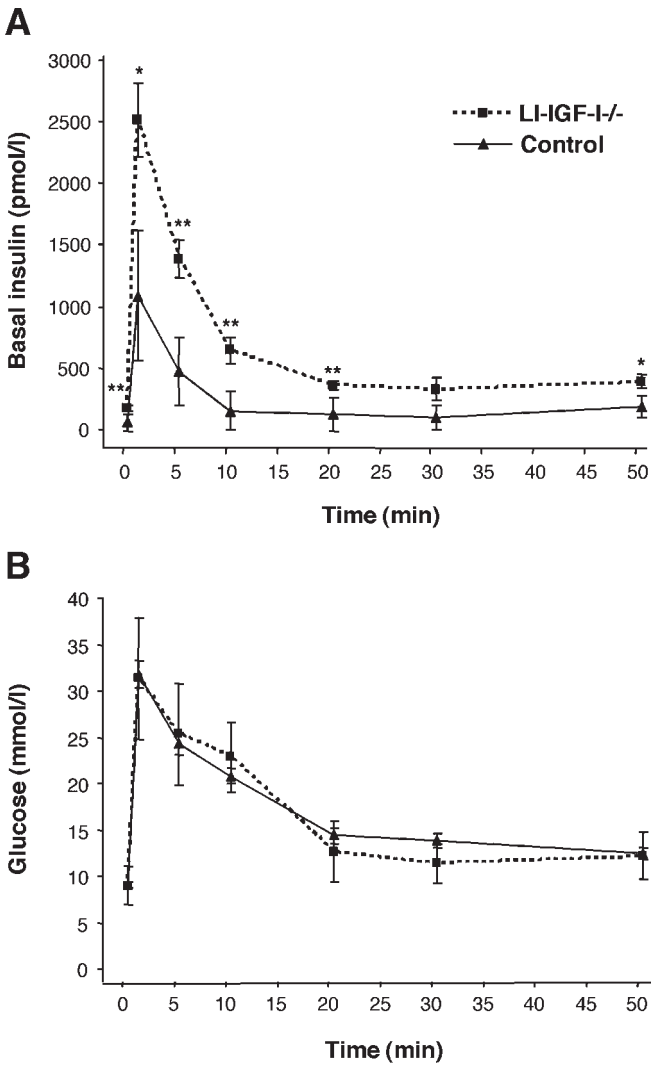

Fig. 3. Intravenous glucose tolerance test. Plasma insulin (**A**) and glucose (**B**) was measured immediately before and 1, 5, 10, 20, 30, and 50 min after intravenous injection of glucose (1 g/kg) in 4-mo-old anaesthetized female mice. Values are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$, LI-IGF-I–/– versus control, Student's t test, $n = 4$–7. Similar results were obtained for male mice. Figure is reproduced from Sjögren et al. *(14)*. (Copyright © 2001 American Diabetes Association. Reprinted with permission from *The American Diabetes Association.*)

fat mass in patients with Laron (also called GH-resistance or -insensitivity) syndrome or GHD *(63–65)* and in Hx rats *(66)*. The mechanism behind these effects is probably not direct because mature adipocytes have low levels of functional type-I IGF receptors. Instead, it has been suggested that IGF-I has an indirect effect by inhibiting insulin secretion and thereby decreasing lipogenesis in adipose tissue *(67)*. However, LI-IGF-I–/– mice have decreased IGF-I levels and increased insulin levels associated with decreased fat mass, which do not support this mechanism of action. Furthermore, in a study by Underwood et al., IGF-I was

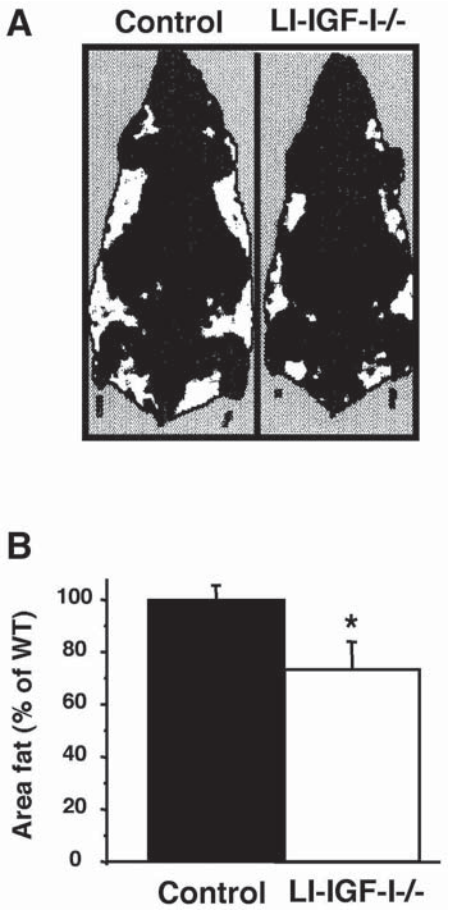

Fig. 4. Total body fat, as measured using dual-energy X-ray absorptiometry, in 13-mo-old pooled male and female LI-IGF-I–/– and control mice. (**A**) DXA/image analysis of fat content in representative LI-IGF-I–/– and control mice. Areas with more than 50% fat are shown as white areas, while areas with lean mass and bone are shown as black areas. (**B**) Fat area. Values are expressed as percentage of control and given as means ± SEM. *$p < 0.05$, LI-IGF-I–/– versus control, Student's t test, $n = 11$. Figure is reproduced from Sjögren et al. *(14)*. (Copyright © 2001 American Diabetes Association. Reprinted with permission from *The American Diabetes Association.*)

shown to decrease fat mass in children with GH-insensitivity syndrome during the first 2 yr of treatment. However, the authors claimed that with prolonged IGF-I treatment, several patients tended to gain excessive amounts of fat *(28)*. A similar observation, of increased fat mass after prolonged IGF-I treatment, was done in a patient with IGF-I gene deletion *(68)*. Together these studies point to a complex and probably indirect relation between IGF-I and fat mass.

Several experimental studies indicate a role for IGF-I in preadipocyte proliferation and differentiation. Mice overexpressing IGFBP-1 have, similar to LI-IGF-I–/– mice, a lean phenotype. As mentioned earlier, the IGFBPs limit the bioavailability of IGF-I, and IGFBP-1 overexpressing mice have a phenotype consistent with partial inhibition of IGF-I action in all tissues. IGFBP-1 overexpressing mice exhibited impaired preadipocyte proliferation and differentiation *(69)*. These results are consistent with a study in mice with partial IGF-I receptor deficiency in all tissues. These mice had decreased fat mass consisting of fewer but larger adipocytes indicating decreased preadipocyte proliferation and stimulation of differentiation into mature adipocytes *(70)*. In comparison, LI-IGF-I–/– mice have decreased fat mass but the IGF-I gene inactivation is limited to the liver with normal IGF-I expression in peripheral tissues, including adipose tissue *(13)*. Further studies are required to investigate whether adipocyte formation is impaired in LI-IGF-I–/– mice.

Leptin is produced in adipose tissue and is known to reduce fat mass by decreasing food intake *(71)*. Interestingly, leptin levels were clearly increased in LI-IGF-I–/– mice *(14)*. One of the most important regulators of leptin is insulin *(72)*. Thus, the increased leptin levels in LI-IGF-I–/– mice might be secondary to the elevated insulin levels. Food intake was normal in LI-IGF-I–/– mice when measured over a 2-wk period, although a small, long-term effect of slightly reduced food intake cannot be excluded (unpublished observation). Further studies are required for the determination of the exact mechanism behind the decreased fat mass in LI-IGF-I–/– mice.

Liver-Derived IGF-I Is Involved in Blood Pressure Regulation

IGF-I is a potent vasodilator that decreases blood pressure and peripheral resistance when administrated in vivo *(73, 74)*. This suggests a role of IGF-I in the regulation of vascular tone. Indeed, we could show that LI-IGF-I–/– mice had increased blood pressure attributable to increased peripheral resistance. Vessels from LI-IGF-I–/– mice showed impaired endothelium-dependent vasodilation in vitro while endothelium-independent vasodilation was normal. LI-IGF-I–/– mice also had increased vascular mRNA expression of the vasoconstrictor endothelin-1 *(26)*. These results imply a role of liver-derived IGF-I in physiological blood pressure regulation.

In conclusion, liver-derived IGF-I is important for carbohydrate and lipid metabolism and for the regulation of GH secretion at the pituitary level as well as physiological blood pressure regulation. Furthermore, it regulates adult axial skeletal growth and cortical radial growth while it is not required for appendicular skeletal growth.

Acknowledgments

This study was supported by the Swedish Medical Research Council, the Swedish Foundation for Strategic Research, the Lundberg Foundation, the Swedish Medical Society, the Novo Nordisk Foundation, the Torsten and Ragnar Söderbergs Foundation, the Emil and Vera Cornell Foundation, the Petrus and Augusta Hedlunds Foundation, and the Wennergren Foundation. We also thank SWEGENE Center for Bio-Imaging (CBI), Gothenburg University, for technical support regarding image analysis.

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