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PERK eIF2α Kinase Regulates Neonatal Growth by Controlling the Expression of Circulating Insulin-Like Growth Factor-I Derived from the Liver

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Humans afflicted with the Wolcott-Rallison syndrome and mice deficient for PERK (pancreatic endoplasmic reticulum eIF2α kinase) show severe postnatal growth retardation. In mice, growth retardation in *Perk*−/− mutants is manifested within the first few days of neonatal development. Growth parameters of *Perk*−/− mice, including comparison of body weight to length and organ weights, are consistent with proportional dwarfism. Tibia growth plates exhibited a reduction in proliferative and hypertrophic chondrocytes underlying the longitudinal growth retardation. Neonatal *Perk*−/− deficient mice show a 75% reduction in liver IGF-I mRNA and serum IGF-I within the first week, whereas the expression of IGF-I mRNA in most other tissues is normal. Injections of IGF-I partially reversed the growth retardation of the *Perk*−/− mice, whereas GH had no effect. Transgenic rescue of PERK activity in the insulin- secreting β-cells of the *Perk*−/− mice reversed the juvenile but not the neonatal growth retardation. We provide evidence that circulating IGF-I is derived from neonatal liver but is independent of GH at this stage. We propose that PERK is required to regulate the expression of IGF-I in the liver during the neonatal period, when IGF-I expression is GH-independent, and that the lack of this regulation results in severe neonatal growth retardation. (*Endocrinology* 144: 3505–3513, 2003)

POSTNATAL GROWTH IN humans and other mammals is primarily regulated by the concerted action of IGF-I and GH. Genetic experiments have confirmed the importance of IGF-I and GH in postnatal growth in mice and have delineated the developmental periods in which these factors are individually and jointly required for normal growth (1). *Igf1*−/− knockout mice are 30–40% smaller than normal at birth and continue to lag in postnatal growth (2, 3). In contrast, mice that are deficient for GH, the GH release factor, or GH receptor (GHR) grow normally for the first 2 wk before subsiding to postnatal growth retardation (1, 4–6). Thus, early neonatal growth in mice appears to be independent of GH. Later in postnatal development, a primary function of GH is to induce the expression of IGF-I mRNA in the liver via the transmembrane GHR and a signaling cascade that culminates in the transcriptional activation of the *Igf-1* gene. IGF-I, synthesized in the liver, is secreted into the circulatory system, and most evidence supports the hypothesis that the liver is the predominant if not sole source of circulating IGF-I (4, 7, 8). IGF-I is also expressed in many other tissues, including the skeletal system, where it may have autocrine and/or paracrine functions. Unlike the liver, however, IGF-I expression is completely or partially independent of GH/GHR in most other tissues (4). Because liver-derived IGF-I has been argued to be strictly dependent upon GH (4, 9), it has been assumed that circulating levels of IGF-I are very low and functionally inconsequential during the first 2 wk of neonatal growth when longitudinal growth is not dependent on GH/GHR. Inasmuch as neonatal growth is clearly dependent upon IGF-I, this would imply that neonatal growth is exclusively dependent on the paracrine/autocrine action of IGF-I. The importance of circulating IGF-I to longitudinal growth was also questioned by experiments showing that mice with a liver-specific knockout of IGF-I (LID) did not show postnatal growth retardation, although circulating IGF-I was reduced by approximately 75% at the adult stage (7, 8). However, this interpretation, which arises from studies of the LID mice, is contradicted by investigation of the *Ghr*−/− knockout mice, which exhibit severe postnatal growth retardation and are completely deficient for circulating IGF-I, but have normal levels of IGF-I expressed in most other tissues. To explain the unexpected normal growth in the LID mice, it has been suggested that these mice may have substantial expression of circulating IGF-I during the early postnatal development before the Cre-induced deletion of the *Igf-1* gene in the liver has occurred in most of the hepatocytes (4). The level of circulating IGF-I in the LID or *Ghr*−/− knockout mice during the neonatal period is unknown. Moreover, the expression and functional activity of IGF-I during the neonatal period has not been investigated in either humans or mice.

Genetic analysis of various mouse strains has revealed that polygenic variation for IGF-I expression maps to a small number of loci with major effects on skeletal growth and development (10). Such loci may correspond to genes that regulate IGF-I. PERK (pancreatic endoplasmic reticulum

Abbreviations: GHR, GH receptor; HNF, hepatocyte nuclear factor; LID, liver-specific knockout of IGF-I; PERK, pancreatic endoplasmic reticulum eIF2α kinase; P21, postnatal d 21; WRS, Wolcott-Rallison syndrome.

eIF2α kinase), a type I transmembrane protein kinase spanning the endoplasmic reticulum membrane, maps within a large multigene region on chromosome 6 of mice that bears one of these loci. Recently, we have generated a knockout mutation of the mouse *Perk* gene (11). Loss-of-function mutations in the mouse *Perk* gene result in three major defects: postnatal growth retardation, multiple skeletal dysplasias and osteopenia, and loss of endocrine and exocrine functions in the pancreas. The same array of defects is seen in the Wolcott-Rallison syndrome (WRS), a human genetic disease associated with mutations in the *Perk* gene (12). *Perk*−/− mice are normal at birth but exhibit an immediate lag in growth rate during the neonatal period. Inasmuch as this growth retardation precedes the development of diabetes and exocrine pancreatic insufficiency by more than 3 wk, we speculate that the early growth retardation is not caused by general metabolic defects. Because the early neonatal growth retardation in *Perk*−/− mice is similar than that seen in mice deficient for IGF-I, we investigated the expression of IGF-I in *Perk*−/− mice. We show herein that liver IGF-I mRNAs and serum IGF-I are dramatically reduced in *Perk*−/− mice during the neonatal period, whereas the IGF-I receptor and GH are elevated. Injection of IGF-I, but not GH, reversed the severe growth retardation of the *Perk*−/− mice, thus supporting the hypothesis that PERK is a major regulator of IGF-I-dependent neonatal growth. During the course of investigating the growth retardation of *Perk*−/− mice, we discovered that IGF-I is actively transcribed in the liver during early neonatal development, resulting in significant levels of circulating IGF-I before the period when liver IGF-I expression becomes GH-dependent.

Materials and Methods

Growth plate analysis

All animal experiments were performed in accordance with the rules and regulations of the Institutional and Animal Care Use Committee. Tibias from prediabetic mice were isolated and fixed in 10% formalin overnight, decalcified using Cal-Ex (Fisher Scientific, Pittsburgh, PA) for 3–5 d, embedded in paraffin, cut longitudinally into 5-μm midsagittal sections, and stained with hematoxylin and eosin. Morphometry was performed on light microscopy photographs of the growth plates captured at ×100 magnification. The total height of the epiphyseal growth plate was calculated as the distance between the proximal end of the chondrocyte resting zone to the distal base of the hypertrophic zone. Proliferative chondrocytes typically appear as flattened discs such that their height is much smaller than their width. The width of the hypertrophic chondrocytes is similar to proliferative chondrocytes, but their height is proportionally much greater. The boundary between the proliferative zone and hypertrophic zone was defined as the point where the ratio of cell width to cell height is approximately 2.0, less than 2.0 defined as hypertrophic chondrocytes, and more than 2.0 defined as proliferative chondrocytes (13). All zone height and cell height measurements were calculated in centimeters on photographs printed at ×30 and ×60 magnification, respectively. Averages from five *Perk*−/− mice and three *Perk*+/+ mice were analyzed, with multiple sections of each sample calculated. For cell number calculations, at least four columns per section around the midline were counted by at least three different coauthors. All measurements were performed on coded photographs to mask the genotype information from each observer.

RNA extraction and IGF-I mRNA analysis

Long bones (femur and tibia) were isolated from mice after removal of skin and skeletal muscles. Bone marrow was removed by flushing the bone cavities with PBS. The bones were cut into small pieces in TRIzol reagent (Sigma, St. Louis, MO) and homogenized with a Polytron homogenizer. Liver and pancreas were homogenized in TRIzol reagent with a sonicator, and the RNA was extracted. DNA contaminating the RNA sample was digested with RNase-free DNase (Ambion, Inc., Austin, TX) at 37 C for 60 min. An equal quantity of RNA was primed with random hexamer primers and was reverse transcribed into cDNA using M-MLV (Moloney murine leukemia virus) reverse transcriptase (Promega Corp., Madison, WI). PCR primers for IGF-I amplification are exon 1 forward 5′-GAT GGG GAA AAT CAG CAG CC-3′, exon 2 forward 5′-TGC TGT GTA AAC GAC CCG-3′, and common reverse primer 5′-CAA CAC TCA TCC ACA ATG CC-3′. Primers for tubulin are forward 5′-TCC TTC AAC ACC TTC TTC AGT GAG A-3′ and reverse 5′-GAG GAT GGA ATT GTA GGG CTC AAC-3′. IGF-I and tubulin cDNA was quantified using real-time PCR. Real-time PCRs were carried out in 1× SYBR Green PCR buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.8% glycerol, 0.01% Tween 20, 0.25× SYBR Green (Molecular Probes, Inc., Eugene, OR), and 60 nM ROX I (Synthegen, Houston, TX). Real-time PCR was performed using the GeneAmp 5700 DNA amplification system (PE Applied Biosystems, Foster City, CA). The reaction program included a 94 C denaturation step for 5 min followed by 35 cycles of 94 C denaturation for 60 sec, 60 C annealing for 60 sec, and 72 C extension for 60 sec. Detection of fluorescent product was carried out at the end of the 72 C extension period. PCR products were subjected to a melting curve analysis and agarose gel electrophoresis. Data were analyzed and quantified with the GeneAmp 5700 SDS software. At a specific threshold in the linear amplification stage, the cycle differences between amplified tubulin and IGF-I cDNA were used to determine the relative quantity of IGF-I mRNA.

Calvarial osteoblasts isolation and culture

Calvarial osteoblasts were isolated from mice using a protocol modified from Ecarot-Charrier *et al.* (14). Calvaria were removed aseptically and stripped of the periosteum in culture media (low-glucose DMEM media with 100 U/ml penicillin and 100 μg/ml streptomycin). The calvaria were cut into small pieces and incubated in the same DMEM complete media with 10% fetal calf serum at 37 C, 5% CO_2 for 1 wk, and then the bones were removed. Cells were grown for another week until reaching confluence. Half of the medium was changed with fresh media every 3 d. For IGF-I secretion, cells were first grown to confluence in the complete media and then washed and placed in conditioned media in which the 10% fetal calf serum was substituted with 0.1% BSA. After 24 h, conditioned media and cells were collected separately. Secreted IGF-I was normalized to total protein in the cell lysates.

IGF-I detection

Serum IGF-I was measured in mice using either enzyme immunoassay or RIA-based systems. IGF-I from the serum of neonatal mice and IGF-I secreted into the conditioned culture media were measured with an ultrasensitive RIA kit (022-IGF-R21, ALPCO, Windham, NH) with an assay sensitivity equal to 0.02 ng of IGF-I/ml. This IGF-I assay includes removal of IGF binding proteins by acid extraction and reducing cross-reactivity of IGF-II through the addition of a saturating concentration of exogenous IGF-II. Cross-reactivity to IGF-II is small (0.05%), and the intraassay coefficient of variation was 0.42%. For adult and juvenile mice, a rat IGF-I enzyme immunoassay kit (DSL-10-2900, Diagnostic Systems Laboratories, Webster, TX) was used to detect IGF-I after acid-ethanol extraction to remove IGF binding proteins. The intraassay and interassay coefficients of variation were 10 and 12%, respectively.

Results

Perk−/− *knockout mice exhibit postnatal growth retardation and a reduction in hypertrophic chondrocytes in the tibia growth plate*

The body weight and size of *Perk*−/− mice is indistinguishable from wild-type littermates at birth, but within a few days they begin to noticeably lag in growth. By the end of the neonatal period [postnatal d 21 (P21)], male and female *Perk*−/− mice are on average 50% smaller than normal as a

consequence of a 4-fold reduction in daily growth rate (Fig. 1). The growth rates of *Perk*−/− males are somewhat more negatively impacted than females, but this varies among litters. Examination of the tibia growth plate chondrocytes (Fig. 2) revealed that *Perk*−/− mice show on average a 22% reduction in the width of the growth plate, which is largely due to a 26 and 19% reduction in the width of the hypertrophic and proliferative zones, respectively (Table 1). The width of the resting chondrocyte zone of the *Perk*−/− growth plate is normal, however. The reduction in the hypertrophic zone is due to a 28% reduction of the number of cells in the longitudinal axis (Table 1), consistent with a deficiency in a primary growth factor. We also noted an abrupt transition from the proliferative to the hypertrophic zone in the *Perk*−/− growth plates, as indicated by a deficiency in chondrocytes exhibiting intermediate width/height ratios.

Comparison of body length (rump to nose) to total body weight of *Perk*−/− mice was assessed using the allometric scaling equation $y = ax^b$, where y = body length, x = body weight, and b is the slope of the regression. A regression slope near 0.33 is typically found in normal mice as well as growth-deficient mice that retain allometric proportions (4, 15). The allometric scaling slope of body weight and length for the *Perk*−/− mice was determined to be 0.32, very close to previous estimates of wild-type mice as well as *Ghr*−/− and *Igf1*−/− deficient mice. With the exception of the brain, major organs of *Perk*−/− mice also showed a proportional reduction in size relative to body weight (Table 2). The weight of the brain was only marginally reduced in *Perk*−/− mice and constituted 5.36% of the total body weight, compared with 2.75% for wild-type mice. Others have noted that the brain does not decrease proportionally to total body weight in *Igf-1*- and *Ghr*-deficient mice and speculated that because the brain grows more rapidly in fetal development than other organs (16), it is less impacted by postnatal growth

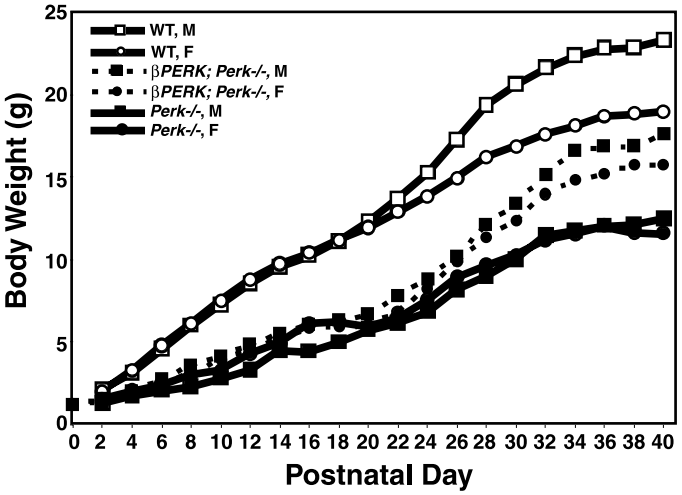

FIG. 1. Growth curves of *Perk*−/− and wild-type mice. Total body weights of wild-type (WT; *open circles and squares*), *Perk*−/− *(filled circles and squares with solid line*), and *βPERK*; *Perk*−/− (*filled circles and squares with dashed lines*) were recorded from birth until P40. *Circles* represent female mice and *squares* represent male mice. Average sample size: WT males, 33; WT females, 25; *Perk*−/− males, 7; *Perk*−/− females, 5; *βPERK*; *Perk*−/− males, 6; and *βPERK*; *Perk*−/− females, 2.

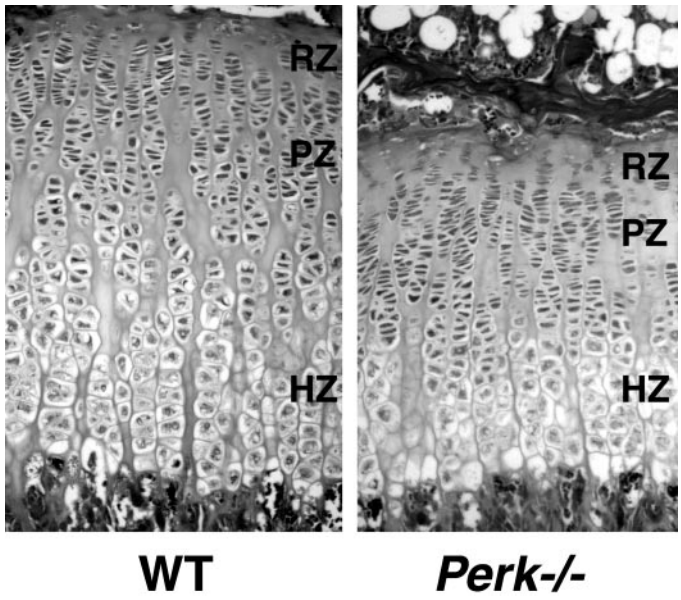

FIG. 2. Tibia growth plates of *Perk*−/− and wild-type (WT) mice. Representative longitudinal sections of WT and *Perk*−/− proximal tibia growth plates showing similar resting zones (RZ) and decreased proliferative zone (PZ) and hypertrophic zone (HZ) in the *Perk*−/− mice. Micrographs are shown at ×100 magnification.

TABLE 1. Analysis of tibia growth plate chondrocyte zones in *Perk*−/− and wild-type (WT) mice

	WT	*Perk*−/−	*P*
GP (cm)	6.97 (±0.14)	5.45 (±0.16)	≪ 0.0001
RZ (cm)	0.65 (±0.05)	0.65 (±0.03)	ns
PZ (cm)	2.18 (±0.10)	1.77 (±0.06)	0.0003
HZ (cm)	4.17 (±0.13)	3.07 (±0.11)	≪ 0.0001
HZ cell number	13.12 (±0.27)	9.40 (±0.25)	≪ 0.0001

Values are the mean (± SEM). The heights of the resting (RZ), proliferative (PZ), and hypertrophic (HZ) zones and the number of hypertrophic cells were measured as described in *Materials and Methods*. WT, n = 3; *Perk*−/−, n = 5. A significant difference between WT and *Perk*−/− is indicated as a *P* value (*t* test). GP, Growth plate; ns, not significant.

factor deficiencies (4). The spleen in *Perk*−/− mice is somewhat proportionally smaller than expected and shows considerable variation independent of total body size.

Transgenic rescue of PERK expression in the insulin-secreting β-cell defect in Perk−/− *mice rescues juvenile, but not neonatal, growth retardation*

Perk−/− knockout mice continue to display a 3- to 4-fold reduction in growth rate during the juvenile period (3–6 wk). Between P22 and P25, these mice develop severe hyperglycemia (11), which may negatively impact their growth. To determine whether the onset of diabetes at the juvenile stage contributes to the continued growth retardation in *Perk*−/− mice, a *Perk* transgene targeted to the insulin-secreting β-cells of the islet of Langerhans (denoted *βPERK*) was introduced into the genome of the *Perk*−/− knockout strain. The resultant mice (*βPERK; Perk*−/−) are deficient in PERK in all tissues except for the insulin-secreting β-cells of the endocrine pancreas. The expression of PERK in the insulin-secreting β-cells of these mice reverses the loss of β-cell mass and the

TABLE 2. Comparison of organ weights between wild-type (WT) and *Perk*−/− mice

	Weight (± SEM)		% Body weight		
	WT	KO	WT	KO	KO/WT (%)
Body weight (g)	15.4 ± 0.8	7.5 ± 0.7			
Brain (mg)	438.2 ± 8.5	356.3 ± 15.6	2.75	5.36	195.0
Heart (mg)	109.0 ± 9.3	51.8 ± 6.9	0.67	0.74	110.4
Lungs (mg)	141.4 ± 2.7	84.8 ± 5.3	0.89	1.27	143.2
Liver (mg)	722.1 ± 65.1	368.6 ± 108.2	4.42	4.89	110.6
Spleen (mg)	97.2 ± 5.8	40.6 ± 11.1	0.64	0.50	78.4
Kidneys (mg)	210.5 ± 15.0	120.6 ± 19.2	1.37	1.55	113.3
Pancreas (mg)	95.6 ± 8.1	43.7 ± 11.0	0.59	0.59	99.6

Values are weight averages (± SEM) of five to nine animals and relative (percentage of body weight) values. The *last column* shows percentage values of *Perk*−/− organ weight relative to wild-type. KO, Knockout.

development of hyperglycemia (Zhang, P., and D. R. Cavener, unpublished data). The *βPERK; Perk*−/− mice still exhibit a 4-fold reduction in growth rates during the neonatal period, indistinguishable from the *Perk*−/− global knockout strain (Fig. 1). However, at the neonatal-juvenile transition (P21) these mice exhibit accelerated growth compared with *Perk*−/− knockout mice. Although *βPERK; Perk*−/− mice do not catch up to the wild-type mice in total size, their growth rates between P20 and P40 are indistinguishable from wild-type mice.

Perk− / − *mice are deficient in serum IGF-I and liver IGF-I mRNA*

Genetic analysis of *Perk*−/− mice showed that PERK is essential for normal growth, particularly during neonatal development. Comparison of the growth rates of *Perk*−/− mice with various mutations affecting GH, IGF-I, and their receptors suggested that the *Perk*−/− mice might be deficient in IGF-I because of the early neonatal manifestation of growth retardation. Analysis of serum IGF-I revealed a dramatic reduction in *Perk*−/− mice during neonatal development (Fig. 3, *top*). The severest reduction (25% of normal) was apparent during the early neonatal period in which IGF-I is clearly the dominant, if not exclusive, growth factor. In contrast, GH serum levels in juvenile mice were somewhat elevated (*Perk*−/−, 7.07 ± 2.64 ng/ml, *vs.* wild-type, 4.04 ± 0.43 ng/ml) as expected due to the reduction in the normal negative feedback regulation of IGF-I on GH (17–19). In late neonatal development, the *βPERK; Perk*−/− rescued mice exhibit a deficiency in circulating IGF-I, which is indistinguishable from the nonrescued *Perk*−/− knockout mice (Fig. 3, *bottom*). However, IGF-I levels in the *βPERK; Perk*−/− mice recover to normal levels during the second postnatal month, whereas in contrast, IGF-I levels in *Perk*−/− knockout mice remain relatively low.

To ascertain the molecular and cellular basis for the reduction in IGF-I in the *Perk*−/− mice, the level of the two major IGF-I mRNA isoforms was determined in liver, because it is the major source of circulating IGF-I (Fig. 4). Both IGF-I mRNA isoforms 1 and 2 were substantially reduced in liver during neonatal development at P5, P7, P10, and P14. The relative abundance of liver IGF-I mRNA is highly correlated with serum IGF-I levels, suggesting that the primary defect in the IGF-I deficiency in *Perk*−/− mice is caused by misregulation of the steady state levels of liver IGF-I mRNA. The expression of IGF-I mRNA was also similarly reduced in

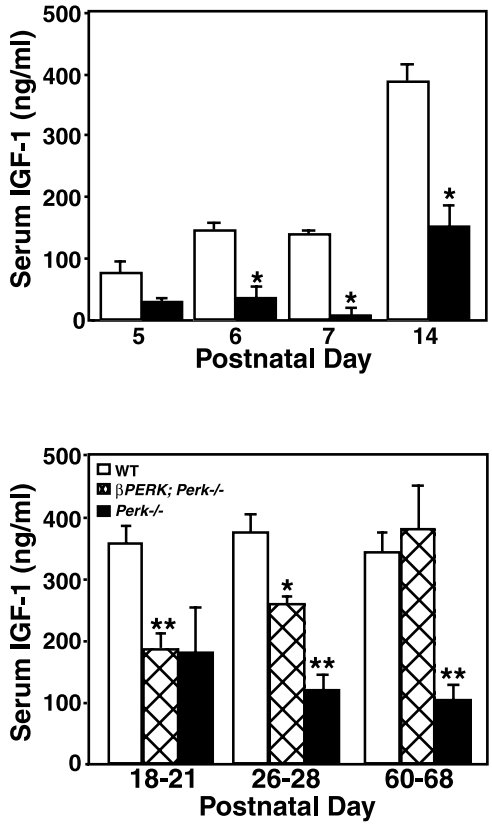

FIG. 3. Serum IGF-I levels in neonatal, juvenile, and adult mice. *Top*, Serum IGF-I levels of neonatal mice from WT (*white bars*) and *Perk*− / − (*black bars*). For each time point, n = 3–7 for WT, and n = 2–4 for *Perk*− / −. *, $P < 0.05$ based upon a one-tailed *t* test. *Bottom*, Serum IGF-I levels of WT, *Perk*− / −, and *βPERK; Perk*− / − (*hatched bars*) mice (n = 3–4). *Error bars* represent SE values of the mean. *, $P < 0.05$. **, $P < 0.01$ based on a two-tailed *t* test comparing *Perk*− / − and *βPERK; Perk*− / − mice to wild-type.

the pancreas of neonatal *Perk*−/− mice (Table 3). However, in contrast, normal levels of IGF-I mRNA were found in long bone, kidney, lung, and skeletal muscle of neonatal *Perk*−/− mice (Table 3).

Although the level of IGF-I mRNA in long-bone tissue is normal, we found that the level of the IGF-I receptor in bone tissue was elevated (Fig. 5). An increased level of the IGF-I receptor has been shown in other cases of IGF-I deficiency in humans (20) and is purported to be due to a relaxation of the normal negative feedback regulation of IGF-IR by circulating

IGF-I. To further assess potential defects in IGF-I expression in bone tissue, IGF-I expression and secretion were assayed in primary cultures of calvarial osteoblasts isolated from *Perk*−/− mice. Calvarial osteoblasts of *Perk*−/− mice exhibit normal levels of IGF-I mRNAs and secrete normal amounts of mature IGF-I (Fig. 6). Together, these results suggest that the neonatal growth retardation is not due to an autocrine/paracrine IGF-I deficiency in bone or other tissues, but rather due to a reduction of circulating IGF-I secreted from the liver. However, we have not been able to quantitatively assess the level of IGF-I expression specifically within the growth plate chondrocytes, and therefore we cannot rule out a possible reduction in locally expressed IGF-I in the growth plate that may negatively impact longitudinal growth of long bones.

Beyond the neonatal period, the expression of IGF-I mRNA is largely controlled by GH as mediated by the GHR (2, 4). To ascertain whether the deficiency of liver IGF-I in the *Perk*−/− mice was caused by a deficiency of GHR, as seen in the Laron syndrome (22), GHR mRNA was quantitatively measured in the liver by real-time RT-PCR and normalized to tubulin mRNA. GHR mRNA was detected in the livers of P14 *Perk*−/− mice and was not substantially different from that seen in wild-type littermates [GHR mRNA *Perk*−/−, 0.17 ± 0.008 (n = 3); wild-type, 0.26 ± 0.026 (n = 3)].

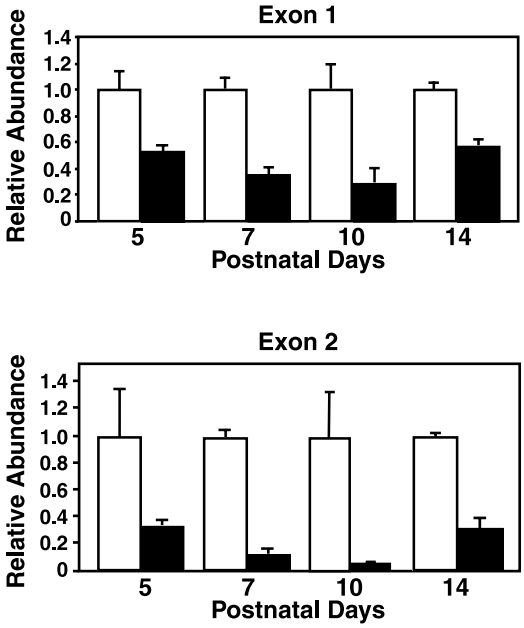

FIG. 4. Relative abundance of IGF-I transcripts in livers of neonatal *Perk*−/− and wild-type (WT) mice. WT (*white bars*) and *Perk*−/− (*black bars*) levels of IGF-I transcripts initiated from exon 1 and exon 2. For each group, n = 3–4. *Error bars* represent SE values of the mean.

TABLE 3. *Igf-1* mRNA expression in major organs and tissues of neonatal wild-type (WT) and *Perk*−/− mice

Organ/tissue	WT	*Perk*−/−	WT *vs.* *Perk*−/−
Pancreas	100 (100) ± 10.9	49 ± 11.5	$P = 0.011$
Bone	100 (10) ± 15.8	110 ± 18.3	ns
Kidney	100 (27) ± 35.5	103 ± 7.4	ns
Lung	100 (21) ± 14.8	116 ± 12.7	ns
Muscle	100 (24) ± 8.3	125 ± 36.2	ns

Values are the mean (± SEM) of three to six animals, normalized to WT. Values in *parentheses* are WT *Igf-1* averages for each organ normalized to *Igf-1* levels in the pancreas to provide relative comparison among organs. A significant difference is indicated by a *P* value (*t* test).

Injection of IGF-I reverses the severe growth retardation of the Perk−/− *mice.*

We reasoned that if the deficiency in circulating IGF-I in the *Perk*−/− mice is the cause of the neonatal growth retardation, injection of IGF-I would lead to a reversal of the growth retardation. *Perk*−/− neonatal mice were injected sc twice daily with 125 ng IGF-I/g total body weight during the entire neonatal period (P2–P21). Within a few days after the onset of IGF-I injection, the injected *Perk*−/− mice began to

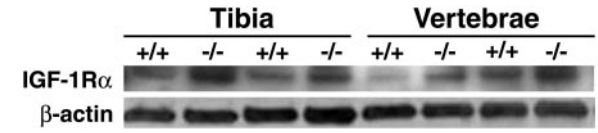

FIG. 5. Expression of IGF-I receptor-α in bone tissue. IGF-IR (α subunit) level of WT (+/+) and *Perk*−/− tibia and thoracic vertebrae. Ten micrograms of total protein were loaded in each lane. β-Actin was used for a loading control.

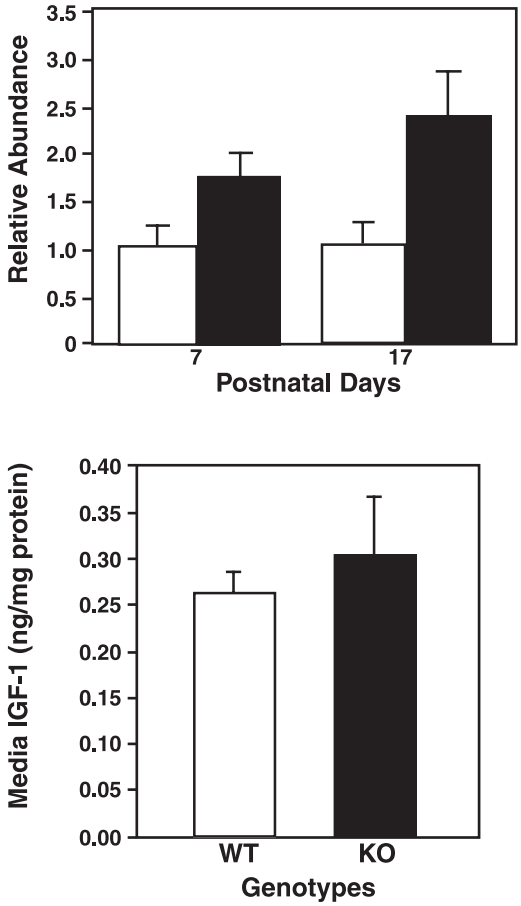

FIG. 6. Expression of IGF-I mRNA and secretion of IGF-I in primary cultured osteoblasts. *Top*, Relative abundance of IGF-I mRNA in cultured osteoblasts from P7 and P17 WT (*white bars*) and *Perk*−/− mice (*black bars*). *Bottom*, Levels of IGF-I secreted into culture media. For WT, n = 7; for *Perk*−/−, n = 5. *Error bars* represent SE values of the mean.

exhibit an accelerated growth rate compared with noninjected *Perk*−/− mice (Fig. 7). Averaged over the neonatal developmental period, the IGF-I injected *Perk*−/− mice exhibited a 2-fold higher daily growth rate than noninjected mutant mice. In contrast, twice-daily injection of GH (3 μg/g body weight) during the late neonatal and juvenile state (P15–P42) had no effect on the growth rate of the *Perk*−/− mice (data not shown).

Serum titers of IGF-I and body weight are highly correlated during neonatal but not juvenile development

IGF-I levels are modulated by a number of environmental and genetic factors, and normal variation of IGF-I may impact postnatal growth. Considerable variation in IGF-I levels was seen for *Perk* mutant and wild-type mice. During neonatal and juvenile development, body weight generally reflects relative growth rates. We determined the correlation of body weight and IGF-I titers at the neonatal (0–3 wk) and juvenile (3–6 wk) stages for *Perk*−/− and wild-type mice (Fig. 8). During the neonatal period, a highly significant correlation (r = 0.93) was observed between body weight and IGF-I titers; this strong positive correlation was observed for both wild-type (r = 0.94) and *Perk*−/− (r = 0.80) mice when analyzed separately as well. In contrast, no significant correlation was observed during the juvenile stage for either wild-type mice or *Perk*−/− mice. These findings suggest that IGF-I levels present in both normal and *Perk*−/− mice are limiting for neonatal growth but permissive for juvenile growth.

Discussion

According to the somatomedin hypothesis of postnatal growth, GH mediates skeletal growth by activating the expression of IGF-I in the liver and secretion into the circulation (17, 18). GH has been shown to regulate the transcription of IGF-I in the liver via signaling through the GHR and downstream effectors such as STAT5b (signal transducer and activator of transcription 5b) and hepatocyte nuclear factor

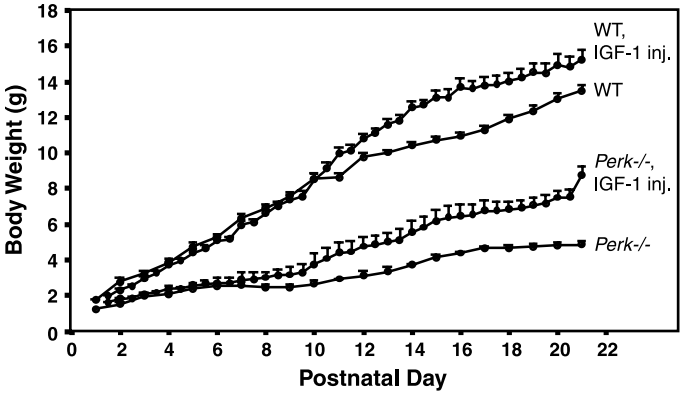

FIG. 7. Growth curves of *Perk*−/− and WT mice injected with IGF-I. Body weights were recorded from mice with and without IGF-I injection (125 ng/g body weight, sc injections, twice daily) from P2 to P21. On average, n = 22 for noninjected WT, n = 3 for noninjected *Perk*−/−, n = 6 for IGF-I-injected WT, and n = 3 for IGF-I-injected *Perk*−/−. *Error bars* represent SE values of the mean.

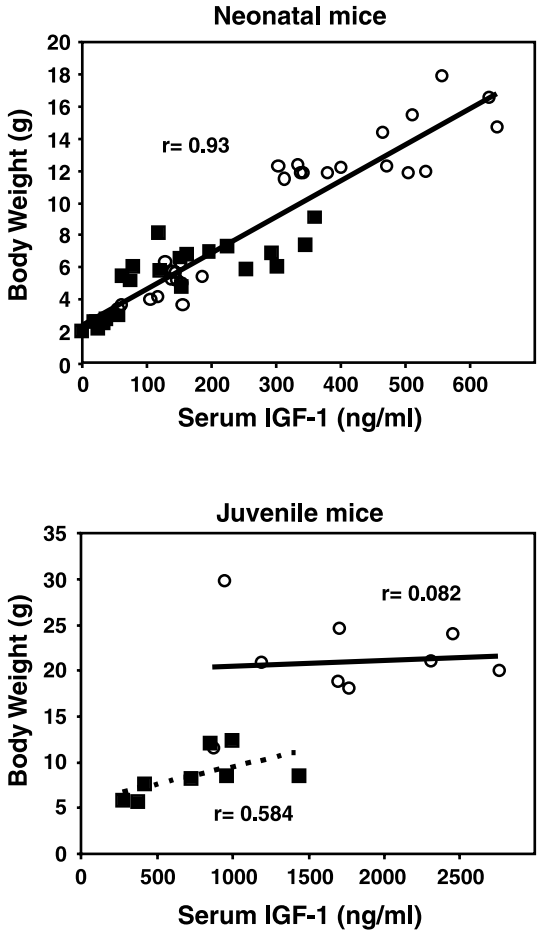

FIG. 8. Correlation of body weight to serum IGF-I in *Perk*−/− and WT mice. *Top*, Correlation of body weight to serum IGF-I in *Perk*−/− (*black squares*) and WT (*white circles*) neonatal mice ranging from age P5–P14. Correlation coefficient (r) for *Perk*−/− mice is 0.80, whereas the correlation coefficient for WT mice is 0.94. *Bottom*, Correlation of body weight to serum IGF-I in *Perk*−/− and WT juvenile mice ranging in age from P23–P42.

(HNF)1α. During the first 2 wk of neonatal development, however, GHR appears to have no effect on growth (4). Knockout mutations of *Igf1* in mice, however, show that the lack of IGF-I leads to perinatal growth retardation (2, 3, 23), and by P14 *Igf1*−/− mice are only half the size of normal. An unresolved issue is the source and regulation of IGF-I during the neonatal period when IGF-I expression is independent of GH. In addition to the liver, IGF-I has been shown to be expressed in a variety of tissues, and in many of these other tissues IGF-I expression is apparently independent of GH (4, 9). Thus, others have speculated that neonatal growth may be under paracrine and/or autocrine control of IGF-I independently of GH (7, 8). The potential contribution of liver-derived serum IGF-I to early neonatal growth has been largely ignored due to the prevalent view that IGF-I expression in the liver is strictly GH-dependent. However, the notion that IGF-I expression in the liver is strictly GH-dependent is based on studies of juvenile and mature adult mice. Genetic ablation of IGF-I in the mouse liver as achieved in the LID mice using the Cre/loxP system reduced serum

IGF-I by 75%, but surprisingly had no impact on postnatal growth (7, 8). However, the ablation of IGF-I in these experiments either was not induced or likely did not occur in the liver of the LID mice until after the early neonatal period and therefore would not have significantly reduced the expression of IGF-I during neonatal development. We show herein that a significant level of IGF-I mRNA is expressed in the liver of 5- to 7-d-old mice as well as a significant level of IGF-I in the serum. Moreover, Rotwein and colleagues (24, 25) have reported significant IGF-I mRNA in the liver in neonatal rats (p7), which they argue is GH independent. In the absence of a functional PERK eIF2α kinase gene, both liver IGF-I mRNA and serum IGF-I are dramatically reduced in neonatal mice, whereas the IGF-I mRNA is normally expressed in most other tissues including bone. However, the tibia growth plates in neonatal *Perk−/−* mice showed a reduction in the proliferative and hypertrophic zone consistent with the longitudinal growth defect. Injection of IGF-I, but not GH, partially reverses the neonatal growth retardation of the *Perk−/−* mice. Together, these data support the hypothesis that liver-derived IGF-I, whose expression is PERK dependent but independent of GH, plays a major role in neonatal longitudinal growth. Our data do not exclude the possibility that IGF-I also plays a paracrine/autocrine role of IGF-I during neonatal growth; however, our finding that IGF-I is normally expressed in major organs and tissues in *Perk−/−* mice, with the exception of the liver and pancreas, suggests that the endocrine role of IGF-I dominates longitudinal growth during the neonatal period.

We showed that although a 2-fold reduction in circulating IGF-I during the neonatal period negatively impacts growth, it does not retard growth during the juvenile stage. This finding is consistent with genetic ablation experiments of the *Igf-1* gene, which show that global and early ablation of IGF-I dramatically impacts growth, whereas a reduction of circulating IGF-I during the juvenile period has no significant impact on growth (2, 3, 7, 8, 23). In addition, we showed that serum IGF-I and body weights are highly correlated during neonatal development, but showed no correlation during juvenile development. Variation in circulating IGF-I among human infants has also been shown to be significantly correlated with growth and the age at which the childhood growth stage is reached (26). The correlation between IGF-I and growth is apparent in infants before the stage at which the effect of GH on linear growth becomes prominent. Similar to mice, IGF-I continues to rise in humans beyond the neonatal/childhood stage, but growth rates are no longer correlated with normal variation in IGF-I levels. We propose that circulating IGF-I is the major limiting factor for neonatal growth but may only limit juvenile growth if its levels are exceedingly low (<10% of normal). Thus, IGF-I is permissive for juvenile growth over a broad range.

Yakar *et al.* (27) have drawn a similar conclusion regarding the importance of a threshold of circulating IGF-I during the juvenile and adult stages. They showed that mice lacking liver-specific expression of IGF-I (LID) and mice globally deficient for the ALS (acid labile subunit) each exhibit a substantial reduction in circulating IGF-I (25 and 35% normal, respectively), but neither exhibited a substantial reduction in postnatal growth rates. However, the double mutant combination LID/ALSKO (LID/acid labile subunit knockout) exhibits a further reduction of circulating IGF-I (10–15% normal) and showed a substantial reduction in postnatal growth rates. Quantitative variation in circulating IGF-I above a minimal threshold at juvenile and adult stages thus appears to have a negligible impact on longitudinal growth; however, variation in IGF-I associated with different mouse strains has been shown to strongly influence bone density and skeletal morphology (10, 28, 29). Moreover, the LID mouse exhibits diminished bone density suggesting that the level of circulating IGF-I derived from the liver has a pronounced effect on bone architecture, although longitudinal growth is normal in these mice. Thus, we conclude that the endocrine function of IGF-I is rate limiting for longitudinal growth during the neonatal period, whereas it is only limiting for bone density and bone modeling during the juvenile and adult stages.

Humans afflicted with the WRS, caused by a loss-of-function mutation in the *Perk* gene, typically develop diabetes within the first 2 months, similar to *Perk−/−* mice. IGF-I deficiency has been reported in one WRS patient (30), but apparently has not been examined in most WRS patients. Because humans do not exhibit as rapid longitudinal growth during the first postnatal month as seen in mice, it has not been possible to determine whether the growth retardation apparent in the first 2 yr in WRS is due to a primary defect in IGF-I or due to an indirect effect of the severe hyperglycemia. For *Perk−/−* mice, however, we show that a dramatic reduction in neonatal growth precedes the development of hyperglycemia, which does not occur until the early juvenile stage (11). Thus, the neonatal growth retardation is not caused by diabetes. We speculate that growth retardation manifested in the WRS during the neonatal period is also caused by a deficiency of IGF-I and may therefore respond to neonatal IGF-I therapy.

In contrast, we have shown that rescuing PERK expression in the insulin-secreting β-cells of the *Perk−/−* mice reverses the juvenile growth retardation thus implicating hyperglycemia as the cause of growth retardation in the *Perk−/−* knockout mice during this later postnatal stage. Hyperglycemia is known to precipitate a reduction in circulating IGF-I, and the longitudinal growth of some individuals suffering from other forms of early onset juvenile diabetes may be delayed (31, 32). The β-cell rescued *Perk−/−* mice (*βPERK; Perk−/−*) recover normal titers of IGF-I during the juvenile period, suggesting that hyperglycemia may be repressing the expression of IGF-I in the *Perk−/−* mice once hyperglycemia is manifested in the early juvenile state (P22–P24). Although the increase in IGF-I titers in the *βPERK; Perk−/−* rescued mice is correlated with an increase in growth rates compared with the global *Perk−/−* mice, we suggest the increase in IGF-I may not be responsible for increasing growth rates based upon studies of conditional knockouts of IGF-I in the liver (LID). The LID mice exhibit IGF-I levels that are comparable to the *Perk−/−* mice in 1-month-old mice, yet do not exhibit growth retardation, suggesting that the reduced level of IGF-I does not limit growth at this stage. Hyperglycemia affects a number of other metabolic processes that directly impact growth, including repression of the GLUT4 transporter and the IGF-I receptor in humeral growth plates (33).

We postulate that the growth retardation seen in the *Perk*−/− mutants, specifically during the juvenile period, may be caused by such hyperglycemic effects.

Liver dysfunctions are known to reduce the expression of IGF-I resulting in postnatal growth retardation (34, 35). However, *Perk*−/− mice appear to express normal levels of liver-enriched genes including albumin, apolipoprotein E, apolipoprotein CI, and serum AST (aspartate aminotransferase) levels (data not shown). In addition, histological examination of the liver of *Perk*−/− mice appears normal. We conclude that the down-regulation of IGF-I in the liver of *Perk*−/− mice has a specific effect on the transcription and/or stability of the IGF-I mRNAs and is not due to a general liver dysfunction. The mechanism underlying the regulation of IGF-I in the liver during the neonatal period by PERK is unknown. We have examined the expression of the key transcriptional regulatory factors that are purported to regulate IGF-I expression in the liver, including HNF1α, HNF3β, HNF4α, CAAT enhancer binding protein (C/EBP)α, and C/EBPβ, but we did not detect any significant differences in their expression in the liver of *Perk*−/− mice (Li, Y., and D. R. Cavener, unpublished data). However, these factors may only be required for GH-dependent regulation of IGF-I later in development. Regulatory factors that specifically control GH-independent expression of liver IGF-I during the neonatal period have been speculated to exist in rats (24, 25) but have not yet been identified.

The translation initiation factor eIF2α is the prime, if not exclusive, substrate of PERK. Phosphorylation of eIF2α (Ser51) may have two diametrically opposed effects on translation initiation, repression or activation, depending on the specific mRNA and the degree to which the eIF2α is phosphorylated. Hyperphosphorylation, particularly under stress conditions, may lead to the repression of global protein synthesis, whereas more modest levels of eIF2α phosphorylation under normal developmental and physiological states can have a very different effect, namely the activation of translation of specific genes, particularly those encoding regulatory functions (36, 37). We speculate that PERK may be required in the liver to regulate the translation initiation of one or more regulatory factors that control neonatal expression of IGF-I mRNA. Although some of these factors that control the neonatal expression of IGF-I in the liver may be identical to those required for juvenile and adult regulation of IGF-I, we propose that one or more neonatal-specific factors are required for IGF-I expression and are under the control of PERK.

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