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Liver Regeneration is Impaired in Lipodystrophic *fld* Mice

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Abstract

We previously reported that mice subjected to partial hepatectomy exhibit rapid development of hypoglycemia followed by transient accumulation of fat in the early regenerating liver. We also showed that disrupting these metabolic alterations results in impaired liver regeneration. The studies reported here were undertaken to further characterize and investigate the functional importance of changes in systemic adipose metabolism during normal liver regeneration. The results showed that a systemic catabolic response is induced in each of two distinct, commonly used experimental models of liver regeneration (partial hepatectomy and carbon tetrachloride treatment), and that this response occurs in proportion to the degree of induced hepatic insufficiency. Together, these observations suggest that catabolism of systemic adipose stores may be essential for normal liver regeneration. To test this possibility, we investigated the hepatic regenerative response in fatty liver dystrophy (*fld*) mice, which exhibit partial lipodystrophy and have diminished peripheral adipose stores. The results showed that the development of hypoglycemia and hepatic accumulation of fat was attenuated and liver regeneration was impaired following partial hepatectomy in these animals. *fld* mice also exhibited increased hepatic p21 expression and diminished plasma levels of the adipose-derived hormones adiponectin and leptin, which have each been implicated as regulators of liver regeneration.

Conclusion—These data suggest that the hypoglycemia that develops after partial hepatectomy induces systemic lipolysis followed by accumulation of fat derived from peripheral stores in the early regenerating liver, and that these events may be essential for initiation of normal liver regeneration.

The liver has remarkable regenerative potential, which permits recovery from functional deficits induced following hepatic injury. The rodent partial hepatectomy model has been the most extensively used experimental system for investigating the mechanisms that control this highly regulated response (1). Analyses using this paradigm have identified many signals that are regulated during and necessary for normal liver regeneration (2–6). Nevertheless, an integrated understanding of the mechanisms that regulate liver regeneration does not yet exist, and the signals that initiate and terminate hepatic regeneration remain incompletely defined.

Liver mass is maintained or recovered after injury in proportion to body mass (3;6). This observation, taken together with the central role of the liver as the principle intermediary between dietary nutrient uptake and extrahepatic energy consumption (7), has led us to

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investigate the regulation and functional role of systemic metabolic changes in response to partial hepatectomy during the hepatic regenerative response. We previously reported that genetic and pharmacological interventions that suppress the transient hepatic steatosis characteristic of the early regenerative response result in impaired liver regeneration (8). We also characterized the hypoglycemic response to partial hepatic resection and the inhibitory effect of glucose supplementation on liver regeneration (9). Together, these data suggest a model in which the hypoglycemia that follows partial hepatectomy induces systemic lipolysis and accumulation of fat derived from peripheral stores in the early regenerating liver. The studies reported here were undertaken to further characterize the significance of changes in peripheral adipose metabolism during liver regeneration.

Experimental Procedures

Animal Husbandry, Partial Hepatectomy, and CCl₄ Treatment

Wildtype C57Bl6/J were obtained from The Jackson Laboratory (Bar Harbor, Me). *Lpin1* null (*fld/fld*, BALB/cByJ-*Lpin1*^{fld/J}; Jackson Laboratory), heterozygous (*fld/+*), and wildtype (+/+) mice were obtained by maintaining *fld/fld* × *fld/+* and *fld/+* × *fld/+* mating pairs. Eight-twelve week old C57Bl6/J and *Lpin1* null-, heterozygous-, and wildtype-mice were maintained on 12 h dark-light cycles with *ad libitum* access to standard rodent chow and water throughout the analysis, and subjected to two-thirds partial hepatectomy or sham surgery using standard methodology (see Supplementary Materials and (8;10–12)). Some animals were treated with recombinant leptin using a regimen shown to rescue impaired regeneration in *ob/ob* mice (see Supplementary Materials and (13)); some were subjected to one-third partial hepatectomy, in which only the median lobes of the liver were resected; and some were treated with carbon tetrachloride (CCl₄) (see Supplementary Materials). At serial times after surgery or CCl₄ administration, animals were sacrificed and plasma and liver tissue were harvested. Very little morbidity or mortality occurred in experimental animals (summarized in Supplementary Materials). Three or more animals were examined at each time point for each genotype, surgical, and treatment group. All experiments were approved by the Animal Studies Committee of Washington University and conducted in accordance with institutional guidelines and the criteria outlined in the “Guide for Care and Use of Laboratory Animals” (NIH publication 86-23).

Histology, Immunohistochemistry, mRNA and Protein Expression, Body Composition, Hepatic Triglyceride, and Plasma Leptin and Insulin Analyses

See Supplementary Materials for detailed methods.

Statistical Analysis

Data were analyzed using SigmaPlot and SigmaStat software (SPSS, Chicago, IL). Unpaired Student's t-test for pair-wise comparisons and ANOVA for multiple groups were used with significance (alpha) set at 0.05. Data are reported as mean ± standard error.

Results

Partial Hepatectomy Induces Systemic Catabolism Prior to Onset of Hepatocellular Proliferation

To begin to investigate the systemic metabolic response to partial hepatectomy, total, lean and fat mass were measured at serial times after surgery in wildtype C57Bl/6J mice. The results showed a stereotypical pattern of loss and recovery in each of these parameters after hepatic resection but not sham surgery (Figure 1A–C). Maximum loss of body weight occurred 24 hours after surgery, with subsequent recovery and return to baseline by ~2 weeks (Figure 1A). The amount of weight lost, ~10% of the initial body mass, was greater

than that which could be explained by removal of two-thirds of the liver (~3% of the initial body weight). Next, changes in lean and fat mass during liver regeneration were determined using magnetic resonance (MR) spectroscopy. The results showed that both lean and fat tissue stores declined and reached their respective nadirs 24 hours after partial hepatectomy, with significantly smaller changes seen after sham surgery (Figure 1B–C). At 24 hours, lean mass had declined by ~10% and fat mass by ~20% of the initial values. These catabolic changes followed the onset of hypoglycemia, detectable 3 hours after partial hepatectomy (9), and preceded the initiation of hepatocellular proliferation, which remains almost undetectable at 24 hours and does not peak until 36 hours after surgery (Figure 4, (9;10;12;14)). Recovery of tissue mass followed specific and distinct patterns (Figure 1B–C), with lean mass increasing more rapidly than fat stores. Individual muscles showed changes in mass comparable to those seen by MR spectroscopy, with quadriceps and gastrocnemius mass decreasing by 5% and 10%, respectively, 24 hours after partial hepatectomy. Mice subjected to partial hepatectomy ate ~two-thirds as much food over the first 24 hours after surgery as did controls (Supplementary Figure 1A). Thus, the decline in body mass observed after partial hepatectomy (Figure 1) exceeds that predicted based on decreased caloric intake alone (6–7% after 24 hour fast; D.A.R. unpublished observations). These results demonstrate the induction of a reproducible systemic catabolic response after partial hepatectomy in mice.

Carbon Tetrachloride (CCl₄) Induces Systemic Catabolism

To investigate whether the systemic metabolic response described above is specific for partial hepatectomy or a more common response to hepatic insufficiency, changes in body mass were determined in another model of hepatic regeneration, that induced by administration of CCl₄ (15;16). As seen after partial hepatectomy, CCl₄-treatment induced specific catabolic changes in total, lean, and fat mass prior to onset of hepatocellular proliferation, with earlier recovery of lean versus fat tissue stores (Figure 2A–F). Mice treated with CCl₄ took in ~one-fourth as much food over the initial 24 hours (Supplementary Figure 1B). Together, these data show that systemic catabolism prior to the onset of hepatocellular proliferation occurs in two different models of liver regeneration.

The Systemic Catabolic Response to Partial Hepatectomy is Proportional to the Amount of Liver Resected

The data described above raise the possibility that the systemic catabolic response to a hepatic regenerative stimulus (*e.g.* partial hepatectomy or CCl₄ exposure) might contribute to regulation of liver regeneration. If so, then the extent of this response – like regeneration itself – should occur in proportion to the magnitude of the regenerative stimulus. One-third partial hepatectomy induces significantly less hepatocellular proliferation compared to removal of two-thirds of the liver (17;18). Therefore, the systemic response to two-thirds partial hepatic resection was compared to that seen after one-third hepatectomy. The development of hypoglycemia and accumulation of hepatic triglyceride, which we previously reported as regulated during and important for normal liver regeneration (8;9), was examined first. The results showed that the degree of hypoglycemia was significantly less severe (Figure 3B) and the magnitude of hepatic triglyceride accumulation much lower (Figure 3C) after one-third versus two-thirds hepatic resection. Further analysis showed that removal of one-third of the liver was also associated with significantly less decline in total and fat mass (Figure 3A). The decline in lean mass after one-third hepatectomy was not significantly different than that seen after two-thirds hepatectomy (*p*=0.3). These data show that catabolism of total body and fat mass after partial hepatectomy occurs in proportion to the degree of induced hepatic insufficiency.

Liver Regeneration is Impaired in Lipodystrophic Fatty Liver Dystrophy (*fld*) Mice

Many interventions that suppress hepatic fat accumulation after partial hepatectomy also result in impairment of liver regeneration (8;19;20); however, neither *de novo*- nor dietary-fat dependent hepatic lipogenesis appears to be required for such regeneration (21). Together, these observations suggest that catabolism of existing adipose stores may be essential for normal hepatic regeneration. To address this possibility, regeneration was examined in fatty liver dystrophic (*fld*) mice (22). *fld* mice are homozygous for a mutation in *Lpin1*, which results in markedly diminished adipose tissue depot size throughout the body (22). Strain-matched wildtype and heterozygous mice appear identical to each other and exhibit comparable amounts of total body fat (18 ± 2 and $18\pm 1\%$, respectively, versus $13\pm 1\%$ in *fld* mice; assessed by MR spectroscopy). Wild-type and heterozygous mice also demonstrate equivalent hepatocellular proliferation 36 hours after two-thirds partial hepatectomy, the time of peak proliferation in this model (Figure 4C). Therefore, heterozygous mice were used as controls for analyses of liver regeneration in *fld* animals. These experiments showed that the regenerative response to partial hepatectomy was significantly impaired in *fld* mice, with reduced hepatocellular BrdU incorporation (Figure 4A–C) and cyclin D1 mRNA and protein expression (Figure 5A–C) compared to controls. *fld* mice also exhibited diminished hepatocellular mitotic frequency (Figure 4D–F; * $p=0.11$ at 48 hrs; $p=0.06$ at 72 hours) and delayed recovery of liver mass ($57\pm 5\%$ versus $62\pm 1\%$ in controls at 72 hours after partial hepatectomy, $p=0.2$) however, these differences were not significant. Post-operative mortality was modestly increased in *fld* mice with 3/42 animals dying within 24 hours after partial hepatectomy compared to 0/44 heterozygous controls ($p=0.08$). There was no increase in hepatic tissue necrosis in surviving null mice compared to controls (Figures 4D–E). The hepatic regenerative response to CCl_4 administration was also investigated, with administration of CCl_4 at a dose sufficient to induce robust regeneration in wildtype mice (Figure 2) resulting in lethality in 4 of 4 *fld* mice versus 1 of 4 controls. Together, these data show that liver regeneration is impaired in lipodystrophic *fld* mice.

fld Mice Contain Less Hepatic Triglyceride after Partial Hepatectomy Compared to Controls

Next, changes in systemic metabolism after partial hepatectomy were examined in *fld* mice. The results showed that regenerating liver from *fld* mice contained significantly less triglyceride than controls (Figure 6A). Triglyceride content was also reduced in quiescent *fld* liver, which likely reflects both the systemic adipose deficiency of *fld* mice and the increased hepatic triglyceride content at baseline in the BALBc genetic background (23). *fld* mice exhibited less severe hypoglycemia 12–24 hours after partial hepatectomy (Figure 6B) and higher plasma insulin levels 48–72 hours after surgery (Figure 6C) compared to controls. These findings are consistent with prior characterization of *fld* mice as insulin resistant (24), and show that systemic metabolic changes characteristic of liver regeneration in wild-type animals are deranged in *fld* mice.

fld Mice Exhibit Dysregulated Hepatic p21 Expression after Partial Hepatectomy

The data described above, together with our previous characterization of the inhibitory effect of supplemental glucose on liver regeneration (9), suggest that perturbations in systemic glucose metabolism may contribute to suppressed regeneration in *fld* mice. The impaired regenerative response associated with dextrose supplementation was characterized by augmented expression of C/EBP α , p21, and p27 (9). Therefore hepatic expression of these factors was compared between *fld* and control mice. The results showed that C/EBP α and C/EBP β mRNA and p27 protein expression were comparable in *fld* and controls (Supplementary Figure 2 and Figure 5D–F); however, p21 protein was increased in *fld* liver

(Figure 5D–F). These data raise the possibility that dysregulated p21 expression contributes to impaired regeneration in *fld* mice.

Plasma Adiponectin and Leptin Levels are Decreased in *fld* Mice

The adipose-derived hormones adiponectin and leptin have each been identified as regulators of liver regeneration (13;25–29). To investigate whether deficiency of either hormone might contribute to impaired regeneration in *fld* mice, plasma levels of each were determined before and after partial hepatectomy in *fld* and control mice. This analysis showed that circulating adiponectin and leptin levels were significantly lower in *fld* animals at baseline (Figure 7A–C). Following partial hepatectomy, leptin levels declined in controls and remained low in *fld* mice (Figure 7C). Because leptin deficiency is associated with impaired liver regeneration (13;28;30), the effect of leptin supplementation on regeneration in *fld* mice was investigated. This analysis showed that a regimen of leptin supplementation sufficient to rescue impaired regeneration in CCl₄-treated *ob/ob* mice (13) did not augment and, in fact, suppressed hepatocellular proliferation 36 hours after partial hepatectomy in *fld* mice compared to untreated *fld* mice and leptin-treated controls (Supplementary Figure 3). Adiponectin levels increased after partial hepatectomy in controls (Figure 7B), but remained almost undetectable in *fld* mice (Figure 7A–B). These data suggest that impaired liver regeneration in *fld* mice may be mechanistically related to that recently described in adiponectin-null mice (26). Diminished activation of STAT3 and augmented induction of expression of suppressor of cytokine signaling-3 (SOCS3) were observed in liver after partial hepatectomy in those animals (27). Therefore STAT3 activation and SOCS3 expression, each of which modulate liver regeneration (31;32), were quantified after partial hepatectomy in *fld* and control animals. The results showed comparable STAT3 phosphorylation in both groups; however, the ratio of phosphorylated:total STAT3 was reduced. Moreover, in contrast to adiponectin-null mice (27), hepatic SOCS3 expression after partial hepatectomy was significantly lower in *fld* mice than in controls. (Figure 8A–C) These data suggest that decreased circulating levels of adiponectin and disruption of STAT3 activation (which induces SOCS3 expression) may contribute to impaired liver regeneration in *fld* mice.

Discussion

The studies reported here were undertaken to further characterize the regulation and functional significance of changes in systemic metabolism during normal liver regeneration. The results show that a systemic catabolic response is induced in each of two distinct models of liver regeneration. These experiments also show that catabolism of total and systemic fat mass – like regeneration itself – occurs in proportion to the degree of induced hepatic insufficiency. Surprisingly, catabolism of lean mass was not significantly different after one-third versus two-thirds partial hepatectomy. These data raise an intriguing question about liver:body mass regulation: To what body mass compartment is liver mass proportionately regulated? The answer to this question may offer insight into mechanisms of liver regeneration, and will be the subject of future investigations. Our data provide the first detailed characterization of systemic metabolic changes in these classic models of liver regeneration, and the findings reported here also offer insight into previously published analyses of liver regeneration. For example, TNF α and IL-6 have each been identified as essential regulators of normal liver regeneration after partial hepatectomy and CCl₄ administration (15;16;33–36), and also induce cachexia (37). Thus, the catabolic response to liver injury in these models of liver regeneration may be induced by signals that are required for normal regeneration. Together, these considerations raise the possibility that the requirement of such signals for recovery of liver mass following hepatic injury may be mediated by the catabolic response they induce.

We and others have previously reported that interventions associated with disruption of transient hepatic accumulation of fat during early liver regeneration, including genetic disruption of caveolin-1 or hepatic glucocorticoid receptor expression, and leptin or propranolol supplementation, are associated with impaired regeneration (8;19;20). Subsequent analyses by Newberry *et al.* showed that regeneration is not impaired in mice in which dietary uptake of fat or *de novo* hepatic synthesis of fatty acid is disrupted ((intestine-specific microsomal triglyceride transfer protein- and liver-specific fatty acid synthase-null mice, respectively) (21)). That study also showed that regeneration is unaffected in liver fatty acid binding protein-null mice. In each case, the regenerating livers accumulated triglyceride fat but to a lesser extent than controls (21), leading those investigators to speculate on the existence of a critical “threshold of adaptive lipogenesis” which was not crossed in those animal models. Whether the genetic interventions evaluated in the Newberry study affected systemic adipose stores was not reported. Together with our previously published data, these considerations suggest that catabolism of existing peripheral adipose stores followed by hepatic accumulation of systemically derived fat may promote liver regeneration. To test this possibility, we investigated liver regeneration in *fld* mice, which have diminished peripheral adipose stores (22). The results showed that early hepatic fat content was reduced and liver regeneration impaired following partial hepatectomy in these animals. The increased insulin levels in *fld* mice 48–72 hours after partial hepatectomy is consistent with prior characterization of insulin resistance in these animals (24). Furthermore, the increased blood glucose levels 12–24 hours after surgery in *fld* mice, together with our previous characterization of the hypoglycemic response to partial hepatectomy and the inhibitory effect of glucose supplementation on early hepatic fat accumulation and liver regeneration in wildtype mice (9), suggest that perturbations in systemic glucose metabolism may contribute to impaired regeneration in *fld* mice. Indeed, hepatic p21 expression, which is increased by dextrose supplementation (9), was also augmented in regenerating *fld* mouse liver. Collectively, these data suggest a model in which the hypoglycemia that follows partial hepatectomy induces systemic lipolysis and accumulation of fat derived from peripheral stores in the early regenerating liver, and that these events provide or regulate essential signals for normal liver regeneration.

The specific mechanisms responsible for impaired liver regeneration in lipodystrophic *fld* mice require further elucidation. Future analyses should address whether the requirement for systemic adipose stores during normal liver regeneration is based on adipose as a source of metabolic fuel to support regeneration (38), lipid precursor for new membrane synthesis, a specific signal that initiates the regenerative response itself, or, perhaps, all of these. Our data showing that circulating levels of adiponectin are markedly reduced in *fld* mice together with published data demonstrating that adiponectin-null mice exhibit impaired liver regeneration (26;27) raise the possibility that this hormone may be such an essential adipose-derived signal. Because the gene that is mutated in *fld* mice, *Lpin1*, is also expressed in liver (22), another important consideration is that absence of hepatic *Lpin1* expression contributes to impaired regeneration in *fld* mice. In this regard, it is intriguing to consider that the *Lpin1* gene product (lipin 1) is bi-functional in liver: It catalyzes an essential step in glycerolipid biosynthesis (39), which may be critical for synthesis of new cell membranes, and also co-activates PPAR α activity, which is required for normal liver regeneration (40;41) and may be regulated by binding phospholipid (42). Nevertheless, the *Lpin1* homolog *Lpin2*, whose protein product exhibits similar enzymatic and transcriptional coactivator activity, is also highly expressed in liver, and hepatic *Lpin2* expression is increased in *fld* mice (39;43). Ultimately, analyses of liver regeneration in other adipose-deficient lipodystrophic models and in adipose- and liver-specific *Lpin1*-null mice will be necessary to define the relative importance of each of these activities of Lipin1 during normal regeneration and the precise mechanisms responsible for deranged regeneration in *fld* mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

<i>fld</i>	fatty liver dystrophy
CCl_4	carbon tetrachloride
BrdU	bromo-deoxyuridine

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Figure 1. Systemic Catabolism after Partial Hepatectomy

(A) Total, (B) lean, and (C) fat mass (percentage of corresponding initial mass) after partial hepatectomy or sham surgery (* $p < 0.005$, ** $p < 0.05$ vs. sham).

Figure 2. Systemic Catabolism after CCl₄

(A) Total, (B) lean, and (C) fat mass after CCl₄ or vehicle (Veh; * $p < 0.005$, ** $p < 0.05$ vs. vehicle). Immunohistochemical analysis of hepatocellular BrdU incorporation 48 hours after (D) vehicle and (E) CCl₄ (100 micron bar shown). (F) Summary of hepatocellular proliferation 24–72 hours after CCl₄ or vehicle (# $p < 0.001$ vs. vehicle).

Figure 3. Metabolic Changes after One-Third versus Two-Thirds Partial Hepatectomy (A) Total, lean, and fat mass (* $p=0.03$, ** $p=0.02$ vs. two-thirds partial hepatectomy); (B) blood glucose (* $p=0.03$ vs. two-thirds partial hepatectomy, $p=0.5$ vs. sham; ** $p=0.002$ vs. sham); (C) hepatic triglyceride content (* $p=0.04$ vs. two-thirds partial hepatectomy, $p=0.3$ vs. sham; ** $p<0.001$ vs. sham) 24 hours after sham, one-third, or two-thirds partial hepatectomy.

Figure 4. Liver Regeneration in *fld* Mice

Immunohistochemical analysis of hepatocellular BrdU incorporation 36 hours after partial hepatectomy (A, B) and H&E staining of liver 48 hours after partial hepatectomy (D, E) in control (*fld/+*) and fatty liver dystrophy (*fld/fld*) Lpin1-null mice (100 micron bar shown; arrowheads identify mitoses). (C) Summary of hepatocellular proliferation 0–72 hours after partial hepatectomy in control and *fld* mice (* $p=0.003$); hepatocellular proliferation in strain-matched wildtype (+/+) mice 36 hours after partial hepatectomy is also illustrated. (F) Summary of mitotic frequency 48–72 hours after partial hepatectomy in liver from control and *fld* mice.

Figure 5. Hepatic Cyclin D1, p21, and p27 Expression after Partial Hepatectomy in *fld* Mice
 Expression of Cyclin D1 mRNA (A, * $p=0.02$ vs. *fld*) and protein (B: representative immunoblot; C: summary of densitometric analysis of replicates; * $p=0.04$ vs. *fld*).
 Expression of p21 and p27 protein (D: representative immunoblots; E–F: summary of densitometric analysis of replicates; * $p\leq 0.04$ vs. *fld*).

Figure 6. Hepatic and Systemic Metabolic Changes after Partial Hepatectomy in *fld* Mice
 (A) Hepatic triglyceride content (* $p < 0.02$ vs. *fld*; $p = 0.08$ vs. 12 hr control, 0.49 vs. 24 hr control; ** $p < 0.002$ vs. *fld*). (B) Blood glucose (* $p = 0.05$, ** $p = 0.002$). (C) Plasma insulin (* $p = 0.008$, ** $p < 0.001$).

Figure 7. Plasma Adiponectin and Leptin after Partial Hepatectomy in *fld* Mice

(A) Representative protein immunoblot and quantitative summary of (B) adiponectin and (C) leptin levels in plasma from control and *fld* mice after partial hepatectomy (B: *p<0.001 vs. corresponding control; **p=0.04 and ***p<0.001 vs. 0 hour control; C: *p=0.02 and **p=0.05 vs. 0 hour control, ***p<0.001 vs. 0 hour control and p=0.03 vs. *fld*).

Figure 8. STAT3 Activation and SOCS3 Expression after Partial Hepatectomy in *fld* Mice
 (A) Representative protein immunoblot of hepatic phosphorylated (pSTAT3) and total STAT3, (B) quantitative summary of hepatic phosphorylated:total STAT3 (*p=0.04), and (C) hepatic mRNA expression of SOCS3 (*p≤0.02, **p=0.002 vs. *fld*) in control and *fld* mice after partial hepatectomy.