Hyperammonemia in cirrhosis induces transcriptional regulation of myostatin by an NF- κ B-mediated mechanism

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Loss of muscle mass, or sarcopenia, is nearly universal in cirrhosis and adversely affects patient outcome. The underlying cross-talk between the liver and skeletal muscle mediating sarcopenia is not well understood. Hyperammonemia is a consistent abnormality in cirrhosis due to impaired hepatic detoxification to urea. We observed elevated levels of ammonia in both plasma samples and skeletal muscle biopsies from cirrhotic patients compared with healthy controls. Furthermore, skeletal muscle from cirrhotics had increased expression of myostatin, a known inhibitor of skeletal muscle accretion and growth. In vivo studies in mice showed that hyperammonemia reduced muscle mass and strength and increased myostatin expression in wild-type compared with post-developmental myostatin knockout mice. We postulated that hyperammonemia is an underlying link between hepatic dysfunction in cirrhosis and skeletal muscle loss. Therefore, murine C2C12 myotubes were treated with ammonium acetate resulting in intracellular concentrations similar to those in cirrhotic muscle. In this system, we demonstrate that hyperammonemia stimulated myostatin expression in a NF- κ B-dependent manner. This finding was also observed in primary murine muscle cell cultures. Hyperammonemia triggered activation of I κ B kinase, NF- κ B nuclear translocation, binding of the NF- κ B p65 subunit to specific sites within the myostatin promoter, and stimulation of myostatin gene transcription. Pharmacologic inhibition or gene silencing of NF- κ B abolished myostatin up-regulation under conditions of hyperammonemia. Our work provides unique insights into hyperammonemia-induced myostatin expression and suggests a mechanism by which sarcopenia develops in cirrhotic patients.

signaling | portosystemic shunting

Sarcopenia, or loss of muscle mass, is the most common and clinically significant complication in cirrhosis, but the mechanisms mediating this loss are not known (1). Even though the term sarcopenia has been suggested to define age-related loss of muscle mass (2), it has also been used to describe muscle loss in chronic disorders (3). Hyperammonemia is a consistent biochemical abnormality in cirrhotic patients due to both hepatocellular dysfunction and portosystemic shunting (4). Hyperammonemia has been shown to impair skeletal muscle protein synthesis, but its molecular mechanisms are ill-defined (5).

Recently, elevated plasma concentrations of myostatin were reported in patients with cirrhosis (6). Myostatin is a critical autocrine/paracrine inhibitor of skeletal muscle growth and mass (7). The downstream targets and effects of myostatin in skeletal muscle have been extensively studied (8, 9). Myostatin inhibits skeletal muscle protein synthesis in vivo and in cell culture models using C2C12 murine myoblasts (10, 11). Other effects of myostatin include inhibition of satellite cells (12) as well as increased proteolysis mediated by Akt inhibition (8, 9, 13, 14). The

mechanism that up-regulates skeletal muscle myostatin in cirrhosis is unknown. The goal of the present study was to determine whether hyperammonemia, a consistent metabolic abnormality in cirrhosis, is responsible for increased expression of myostatin.

Hepatocellular dysfunction and portacaval shunting of cirrhosis result in systemic hyperammonemia with increased skeletal muscle uptake of ammonia (15). Studies comparing healthy subjects to cirrhotic patients have shown that the skeletal muscle metabolizes >50% of ammonia present in liver disease (15). These data suggest that the skeletal muscle detoxification of ammonia is a physiological response for metabolic support of the liver with no significant adverse pathological consequences. However, hyperammonemia transcriptionally regulates gene expression in other model systems (16–18). Our studies in the skeletal muscle demonstrate a unique, NF- κ B-dependent up-regulation of myostatin transcription during hyperammonemia. Our findings indicate that hyperammonemia mediates the cross-talk between the liver and skeletal muscle and results in sarcopenia via up-regulation of myostatin expression.

Results

Cirrhotic Patients Have Elevated Plasma and Skeletal Muscle Ammonia Levels, Which Correlate with Decreased Muscle Mass and Increased Myostatin Expression. Because hepatic metabolism of ammonia is impaired in cirrhosis and hyperammonemia impairs

Significance

Loss of skeletal muscle mass, or sarcopenia, is nearly universal in cirrhosis and adversely affects the outcome of these patients. There are no established therapies to prevent or reverse sarcopenia because the mechanisms are not known. We show that the expression of myostatin, a negative regulator of skeletal muscle mass, is increased in the cirrhotic muscle and is mediated by increased ammonia concentration. Skeletal muscle ammonia concentrations are significantly increased in cirrhosis, resulting in activation of the transcription factor NF- κ B, which in turn increases the expression of myostatin. Given the high prevalence of cirrhosis, these studies are of broad general interest because ammonia-lowering strategies, NF- κ B antagonists, and myostatin blocker are potential therapies to reverse sarcopenia of cirrhosis.

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Fig. 1. (A) Skeletal muscle expression of myostatin mRNA showed significantly higher expression in cirrhotics compared with controls ($n = 13$ each). (B) Representative immunoblots and densitometry of myostatin expression in skeletal muscle from patients with cirrhosis and controls. *** $P < 0.001$ (cirrhotics compared with controls). Ctrl, control.

skeletal muscle protein synthesis, we quantified ammonia concentration in the skeletal muscle of cirrhotic patients and controls (13 individuals per group). The patients with cirrhosis had significantly lower core skeletal muscle area ($22.6 \pm 6.3 \text{ cm}^2$) compared with controls ($29.8 \pm 8.4 \text{ cm}^2$; $P < 0.01$). Both the plasma ammonia and skeletal muscle ammonia concentrations were significantly higher ($P < 0.01$) in cirrhotics than in controls (312.8 ± 56.3 vs. $28.6 \pm 18.2 \text{ } \mu\text{mol/L}$ and 4.98 ± 0.41 vs. $0.52 \pm 0.29 \text{ mmol/g protein}$, respectively). This finding was accompanied by significantly elevated myostatin mRNA and protein expression in the skeletal muscle of patients with cirrhosis compared with controls (Fig. 1).

Hyperammonemia in Vivo Induces Myostatin and Reduces Skeletal Muscle Mass. To demonstrate that hyperammonemia in vivo is responsible for muscle loss by a myostatin-mediated mechanism, hyperammonemia was induced in wild-type and myostatin-knockout mice. Muscle mass was reduced by $25.4\% \pm 4.5\%$ (Fig. 2A) and grip strength was decreased by $30.8\% \pm 5.3\%$ (Fig. 2B) in hyperammonemic mice compared with wild-type controls. In contrast, the postdevelopmental myostatin-knockout mice had greater muscle mass and increased grip strength that were not affected by hyperammonemia. Myostatin expression increased significantly in response to ammonium acetate in wild-type, but not in myostatin-knockout mice (Fig. 2C and D). These data, in conjunction with our previous studies in the hyperammonemic portacaval anastomosis (PCA) rat (19–21) and the human studies (above), show that hyperammonemia in vivo induces myostatin expression and reduces muscle mass.

Myotubes Exposed to Hyperammonemia Have a Smaller Diameter. Hyperammonemia could directly affect muscle mass through decreased cellular size. We modeled hyperammonemia in cell culture through the addition of 10 mM ammonium acetate to differentiated murine C2C12 myotubes. This concentration of ammonium acetate was selected because it resulted in intracellular ammonia concentrations similar to that observed in cirrhotic muscle, while maintaining a neutral pH (Fig. S1A and B). Under these conditions, 96% of the cells were viable as assessed by both trypan blue and resazurin dye reduction assays, as we reported earlier (22). Cell size of C2C12 myotubes exposed to ammonium acetate had significantly smaller diameters, suggesting that hyperammonemia reduces muscle size (Fig. 3A and B).

Myostatin Expression Is Transcriptionally Up-Regulated by Hyperammonemia. Exposure of C2C12 myotubes to ammonium acetate induced a time- and concentration-dependent increase in myostatin mRNA (Fig. 4A) and protein expression (Fig. 4B and C). This increase in myostatin expression was induced by hyperammonemia, because exposure of these cells to sodium acetate had no effect on either myostatin mRNA (Fig. S2A) or protein expression (Fig. 4B and C). Additionally, no change in myostatin mRNA expression was observed when cells were exposed to oxidative (H_2O_2) or

Fig. 2. (A and B) Hyperammonemia resulted in reduced muscle mass (A) and grip strength (B) in wild type in contrast to greater muscle mass and increased strength in postdevelopmental myostatin knockout mice. a–b, $P < 0.05$; a, b–c $P < 0.01$. (C) Relative fold change in expression of myostatin mRNA in mice in response to hyperammonemia showed significantly increased expression in the myostatin^{+/+} mice. a–b and a–c, $P < 0.001$; b–c, $P < 0.01$. (D) Representative immunoblots for myostatin protein and the densitometry in response to hyperammonemia in myostatin^{+/+} and myostatin^{−/−} mice. a–b and a–c, $P < 0.001$; b–c, $P < 0.01$. $n = 5$ in each group. AA, ammonium acetate; WT, wild type; KO, postdevelopmental myostatin knockout mice.

osmotic (sorbitol) stress (Fig. S2B). Hyperammonemia does not appear to induce a global up-regulation of muscle regulatory genes, because transcription of insulin-like growth factor 1 (IGF1) and atrogen expression was unaffected under conditions when ammonium acetate enhanced myostatin expression (Fig. S2C). Similar findings were observed in primary mouse and rat muscle cells, with ammonium acetate stimulating transcriptional up-regulation of myostatin with no appreciable changes in IGF1 or atrogen levels (Fig. S2D). Together, these results demonstrate that hyperammonemia specifically induces transcriptional up-regulation of myostatin expression in murine myotubes.

Transcription Factor NF- κ B Is Activated by Hyperammonemia via the Classical Pathway. Previous studies in astrocytes have shown that hyperammonemia causes nuclear translocation of the p65 subunit of the transcription factor NF- κ B (23). Therefore, we tested

Fig. 3. (A) Representative images of phase-contrast microscopy of C2C12 murine myotubes to quantify the myotube diameter. (B) Myotubes treated with 24 h of 10 mM ammonium acetate had significantly lower ($P < 0.01$) mean diameter ($\pm$ SEM) compared with control conditions. A total of at least 85 cells in three different plates were quantified. *** $P < 0.001$ (compared with controls).

Fig. 4. (A) Time course of relative fold change in myostatin mRNA at different concentrations of ammonium acetate in differentiated C2C12 myotubes. Significant increase in myostatin mRNA was observed at all concentrations of ammonium acetate at 6 and 24 h compared with control. (B) Representative immunoblots of the time course of expression of myostatin protein in C2C12 myotubes treated with ammonium acetate (Am. Ac.) and sodium acetate (Na. Ac.) at 10 and 100 mM concentration. C, control. Myostatin expression increased only in response to ammonium acetate. (C) Densitometry of the immunoblots showed increased expression of myostatin protein in a time- and concentration-dependent manner only in response to ammonium acetate, demonstrating that these changes are specific to hyperammonemia. All experiments were performed in triplicate. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared with controls).

whether ammonium acetate stimulates NF- κ B activity in C2C12 myotubes. The p65 subunit of NF- κ B (p65 NF- κ B) is sequestered in the cytosol through interactions with I κ B α and β . In response to an activating signal, the kinases IKK α/β (IKK α/β) phosphorylate I κ B α and β , which leads to the degradation of these inhibitory molecules and the subsequent translocation of p65 NF- κ B to the nucleus. Once in the nucleus, p65 NF- κ B heterodimerizes with other NF- κ B subunits, binds to specific sites in gene promoters, and activates transcription. In C2C12 myotubes, IKK α/β phosphorylation and kinase activity was stimulated by treatment of ammonium acetate (Fig. 5A and B). Subsequently, increased phosphorylation of I κ B α was observed as assessed by phosphospecific immunoblotting leading to degradation of I κ B α and β (Fig. 5C and D). Also in response to ammonium acetate stimulation, p65 NF- κ B translocated to the nucleus as seen in the immunofluorescent confocal micrographs (Fig. 5E). Finally, the activation of NF- κ B transcriptional activity by ammonium acetate was confirmed by using a reporter gene assay that detects NF- κ B-dependent expression of luciferase (Fig. 5F). These results indicate that NF- κ B is activated in myotubes under the condition of hyperammonemia.

Hyperammonemia Stimulates NF- κ B to Bind to the Myostatin Promoter and Activate Transcription. Because the myostatin promoter has two putative NF- κ B binding sites (24), we assessed whether p65 NF- κ B binds to the myostatin promoter to mediate transcriptional up-regulation of myostatin expression in response to ammonium acetate in C2C12 myotubes (Fig. 6A). In response to ammonium acetate, on EMSA, two nuclear protein complexes bound to the labeled oligonucleotide probes containing the putative NF- κ B binding sites (Fig. 6B). Excess unlabeled oligonucleotides comprising both binding sites displaced the DNA-protein complex, demonstrating the specificity of the nuclear protein complex binding to the probes.

To further confirm that p65 NF- κ B binds to myostatin promoter under conditions of hyperammonemia, C2C12 myotubes were stimulated with ammonium acetate or TNF- α (as a positive control) followed by chromatin immunoprecipitation (ChIP). Amplification (ChIP PCR) of specific promoter sequences encompassing the NF- κ B binding sites in the mouse myostatin promoter showed that p65 NF- κ B binds to the myostatin promoter (Fig. 6C). As a positive experimental control, p65 NF- κ B binding to the myostatin gene promoter was increased in response to TNF- α treatment (Fig. 6C).

These data were complemented by a reporter gene assay in which luciferase expression is controlled by the mouse myostatin promoter. In C2C12 myotubes transfected with the myostatin

reporter construct, ammonium acetate enhanced expression of the luciferase reporter gene (Fig. 6D). Ammonium acetate stimulation of luciferase expression was abrogated when the two NF- κ B DNA binding sites were deleted from the construct. To demonstrate that the deletional construct was functional, C2C12 myotubes with the full-length and deletional construct were incubated with dexamethasone (100 μ M) for 6 h, and luciferase assay was performed and normalized to Renilla activity (Fig. 6E). These results demonstrate that conditions of hyperammonemia induce the binding of p65 NF- κ B to specific sites within the myostatin promoter to activate gene transcription in C2C12 myotubes.

NF- κ B Mediates Myostatin Expression in Response to Hyperammonemia.

To determine whether p65 NF- κ B is required for induction of

Fig. 5. (A) Representative immunoblots and densitometry of C2C12 myotubes exposed to 10 mM ammonium acetate and probed for phosphorylation of IKK showed increased phosphorylation of IKK. (B) Time course of percent change in IKK activity in response to 10 mM ammonium acetate in C2C12 myotubes showed significant increase in activity in response to ammonium acetate. (C) Representative immunoblots and densitometry of C2C12 cell myotubes treated with or without (control) 10 mM ammonium acetate for different time points showed increased phosphorylation of I κ B- α . (D) Representative immunoblots and densitometry from C2C12 myotubes treated with or without (control) 10 mM ammonium acetate showed degradation of p65 NF- κ B binding proteins, I κ B- α and - β , during hyperammonemia. (E) Confocal microscopic images of complete and cut sections (yellow V-shaped) of C2C12 myotubes treated with 10 mM ammonium acetate demonstrating nuclear localization of p65 NF- κ B (green stain). TNF- α (50 ng/mL) was used as a positive control. All experiments were performed in triplicate. (F) C2C12 myotubes were nuclear transfected with p65 NF- κ B-luc-6 $\times$ (luciferase reporter gene construct controlled by oligomerized κ B binding sites). The luciferase fluorescence response was significantly higher in response to both 10 mM ammonium acetate and 50 ng/mL TNF- α (positive control) compared with controls. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared with control). All experiments were performed in triplicate.

Fig. 6. (A) Myostatin promoter binding sites for p65 NF- κ B and PCR primer design strategy for the analysis of ChIP of myostatin promoter using anti-NF- κ B p65. MSTN, myostatin; CDS, coding sequence; BS1, binding site 1; BS2, binding site 2. (B) Representative blots of EMSA and densitometry normalized to controls, demonstrating that p65 NF- κ B binds to the myostatin promoter. C2C12 cells were treated with 10 mM ammonium acetate for binding specificity. Arrows indicate the positions of protein-DNA probe complexes and free probes, respectively. C2C12 cells not exposed to ammonium acetate served as negative controls. All experiments were performed in triplicate. BS1, p65 NF- κ B binding site 1 on the myostatin promoter; BS2, p65 NF- κ B binding site 2 downstream of BS1; Ctrl, control. $^{**}P < 0.01$; $^{***}P < 0.001$ (compared with control). (C) ChIP assay and ChIP real-time PCR demonstrate binding of endogenous p65 NF- κ B to the myostatin promoter in C2C12 cells exposed to 10 mM ammonium acetate for 1 h. Cells treated with TNF- α (50 ng/mL) for 30 min served as positive controls, and IgG was used as a negative control. As described in *Materials and Methods*, primers 1 and 2 correspond to a segment covering the two p65 NF- κ B binding sequences within 185-bp myostatin promoter. (Upper) Representative gels showing precipitated chromatin. (Lower) Representative quantitative real-time PCR of the precipitated chromatin. $^{*}P < 0.05$; $^{**}P < 0.01$ (compared with IgG control group). IgG, normal rabbit IgG; anti-NF- κ B p65, rabbit polyclonal IgG anti-p65 NF- κ B. All experiments were performed in triplicate. (D) Luciferase reporter assay demonstrates that in response to ammonium acetate, nuclear p65 NF- κ B binds to the myostatin promoter, and using a deletion construct with the binding sites deleted, the luciferase response with hyperammonemia was not observed. $^{**}P < 0.01$ (compared with control). All experiments were performed in triplicate. (E) Dexamethasone (100 μ M for 6 h) induced a luciferase response in the deletion construct, demonstrating a functional reporter.

myostatin expression, we created a stable p65 NF- κ B knockdown C2C12 line. This stable cell line is characterized ~50% reduction in expression of p65 NF- κ B at baseline and following treatment with ammonium acetate (Fig. 7A). By using these cells, the myostatin mRNA and protein expression was induced by ammonium acetate in the control shRNA line and completely blocked in p65 NF- κ B knockdown cells (Fig. 7B and C). Furthermore, immunoblots of C2C12 myotube nuclear extracts showed a significant increase in nuclear p65 NF- κ B that preceded myostatin up-regulation (Fig. 7D). Importantly, inhibition of NF- κ B activation by the chemical inhibitor pyrrolidine dithiocarbamic acid resulted in blockade of NF- κ B nuclear translocation in response to ammonium acetate and decreased myostatin expression in myotubes. Myotube diameter was significantly greater in p65 NF- κ B knockdown cells compared with controls and did not show a reduction during hyperammonemia, supporting our *in vivo* human and mouse data that hyperammonemia-mediated activation of p65 NF- κ B is directly responsible for sarcopenia (Fig. 7E). These results indicate that p65 NF- κ B is the pivotal player in regulating myostatin expression during hyperammonemia.

Discussion

Hyperammonemia is a consistent abnormality that occurs in cirrhosis due to reduced urea synthesis that occurs almost exclusively in the liver because of hepatocellular dysfunction with or without portosystemic shunting (15, 25). In cirrhotic patients and animal models of hyperammonemia, the skeletal muscle removes large quantities of ammonia from the circulation. Importantly, ammonia is detoxified in the liver through the urea cycle, and the skeletal muscle is an alternate organ for ammonia

detoxification (26, 27). To the best of our knowledge, the levels of intracellular ammonia in the skeletal muscle of cirrhotics and controls have not been previously quantified. Our results demonstrate a significant accumulation of ammonia in the skeletal muscle of cirrhotics. Using this information, we have established unique *in vivo* mouse and cellular systems to reproduce the cellular levels of hyperammonemia present in the skeletal muscle of cirrhotic patients.

Ammonia neurotoxicity has been extensively described in liver disease (15, 28), and we postulate that the uptake of ammonia by the skeletal muscle produces a “buffering effect” to limit the rise of blood ammonia levels and reduce ammonia neurotoxicity. However, our data suggest that this uptake by muscle also results in activation of deleterious signaling pathways that contribute to sarcopenia. Although it has been speculated that a liver–muscle axis exists *in vivo* that is responsible for sarcopenia of cirrhosis (29), the mediator of this axis has not yet been identified. Using a combination of studies in human and mouse muscle, primary muscle cell cultures, and differentiated C2C12 murine myotubes, we demonstrate that hyperammonemia causes transcriptional up-regulation of myostatin in a p65 NF- κ B-dependent manner. Studies in human and animal model systems have suggested that myostatin mediates sarcopenia in a number of disorders (6, 30–33). In the context of cirrhosis, our previous studies in the PCA rat model of sarcopenia of cirrhosis with portosystemic shunting (20, 21) have shown that myostatin is a critical regulator of protein synthesis and muscle loss (20). This finding is complemented by our *in vivo* studies in myostatin^{+/+} and myostatin^{−/−} mice with hyperammonemia. These observations suggest that hyperammonemia is a mediator of a pathogenic liver–muscle axis via increased myostatin expression.

Fig. 7. (A) Representative immunoblots and densitometry of C2C12 myotubes stably transfected with nontargeted sequence (scramble) and knockdown with p65 NF-κB shRNA treated with ammonium acetate for 1 h. Approximately 50% knockdown of p65 NF-κB was achieved at the basal state, and the expression of p65 NF-κB did not increase in the knockdown cells. All experiments were performed four times. $^{**}P < 0.01$ [compared with nontargeted (scramble) shRNA]; $^{***}P < 0.001$ (compared with cells transfected with scrambled shRNA in control medium and cells transfected with p65 NF-κB shRNA treated with ammonium acetate). (B) Relative expression of myostatin mRNA in stable p65 NF-κB knockdown and nontargeted transfected C2C12 myotubes exposed to ammonium acetate for 6 and 24 h. Significant increase in myostatin mRNA in myotubes with nontargeted transfection compared with cells with p65 NF-κB knockdown was found. a–b, $P < 0.001$; b–c, $P < 0.001$; a–c, $P < 0.01$. (C) Representative immunoblots and densitometry for myostatin protein in C2C12 myotubes stably transfected with nontargeted (scramble) plasmid or p65 NF-κB shRNA for knockdown showed significant reduction in myostatin protein expression with knockdown of p65 NF-κB. a–b, $P < 0.001$; b–c, $P < 0.001$; a–c, $P < 0.01$. (D) Representative immunoblots and densitometry of the time course of expression of p65 NF-κB in nuclear lysates and myostatin protein in the cytoplasm of C2C12 murine myotubes exposed to 10 mM ammonium acetate. An increase in p65 NF-κB in the nucleus in response to ammonium acetate precedes the expression of myostatin. Upon blocking p65 NF-κB with 10 μM pyrrolidine dithiocarbamate (PDTC; inhibitor of activation of NF-κB by IκB-α stabilization), nuclear p65 NF-κB expression and myostatin expression did not increase with ammonium acetate. All experiments were performed in triplicate. $^{*}P < 0.001$ (compared to control); $^{§}P < 0.01$ (compared with control). (E) Myotube diameter in C2C12 myotubes stably transfected with p65 NF-κB or scrambled shRNA, differentiated for 48 h, and exposed to ammonium acetate (10 mM) or control. a–b, $P < 0.001$; a–c, $P < 0.001$; b–c, $P < 0.001$.

Even though the mechanisms of regulation of skeletal muscle mass by myostatin has been extensively studied (14, 34), there is very limited data on the upstream modulators of myostatin (35, 36). Human and murine myostatin promoters contain a number of transcriptional response elements, and bioinformatic studies have identified regions homologous to the consensus sequence of the NF-κB binding site (24). Our study demonstrates that hyperammonemia stimulates NF-κB-dependent myostatin transcription through activation of the classical pathway mediated by IKKα/β, IκBα, IκBβ, and p65 NF-κB. Further studies are necessary to elucidate the mechanism(s) and cellular sensors that mediate hyperammonemia-mediated IKKα/β activation. In these studies, we also confirm binding of p65 NF-κB to the myostatin promoter is essential for increasing myostatin expression in response to hyperammonemia.

We postulate that increased myostatin expression induced by activation of NF-κB by hyperammonemia may mediate loss of muscle mass. This interpretation is supported by recent studies in transgenic mice that modulate levels of NF-κB activity. Skeletal muscle atrophy was observed in mice with constitutively active NF-κB, whereas repression of NF-κB led to muscle hypertrophy (37). In this context, our previous studies in the PCA rat model show that inhibition of myostatin ameliorates sarcopenia (20). Correspondingly, the present studies show that myostatin is a critical

regulator of hyperammonemia-induced muscle loss. In contrast, others have reported that p65 NF-κB is a positive regulator of myogenesis (38). Our data are consistent with previous studies that have suggested that p65 NF-κB is a negative regulator of myogenic differentiation (39). This difference in muscle cell response can be

Fig. 8. Mechanism of ammonia-induced transcriptional up-regulation of skeletal muscle myostatin.

explained by the stage of cellular differentiation induced by different culture conditions. In fact, replacing the proliferating medium with serum-deprived differentiation medium has been shown to induce p65 NF- κ B in the first 24 h, whereas replacing the differentiation medium in myotubes did not have the same effect (38). The present studies were performed in myotubes differentiated for 48 h, which we believe is a better model of *in vivo* muscle responses.

In summary, our studies suggest that skeletal muscle functions as a metabolic partner to the liver for ammonia detoxification in cirrhotics. We elucidated a unique role for hyperammonemia in liver disease that mediates elevated expression of myostatin via activation of NF- κ B by the classical pathway (Fig. 8). Hyperammonemia appears to be a critical link in the liver–muscle axis and is significant because it will provide us with the basis for unique intervention strategies targeting this NF- κ B–dependent pathway.

Materials and Methods

Human Studies. Rectus abdominis muscle biopsies were obtained from cirrhotic and control subjects after written informed consent was obtained. All studies were approved by the Institutional Review Board at the Cleveland Clinic. The details of the clinical characteristics are shown in *SI Materials and Methods*.

In Vivo Mouse Studies. Hyperammonemia was induced in postdevelopmental myostatin knockout and wild-type mice as described in *SI Materials and Methods*.

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Cell Cultures. *In vitro* studies in C2C12 myotubes were performed after 48-h differentiation. The details of the protocols followed are discussed in *SI Materials and Methods*.

Analytical Methods. Real-time PCR and immunoblots were performed to quantify gene and protein expression by using methods standardized in our laboratory. The primer sequences, antibodies used, and methodology are described in *SI Materials and Methods*. Assays for IKK activity and ammonia quantification are described in *SI Materials and Methods*. The source of all chemicals and antibodies is also described in *SI Materials and Methods*.

EMSA and ChIP Assay. Binding of p65 NF- κ B to the myostatin promoter was examined by using commercial kits with modifications, the details of which are provided in *SI Materials and Methods*.

Reporter Assays. Luciferase reporter assays for nuclear translocation of p65 NF- κ B and binding to the consensus sequence as well as for myostatin promoter activation are described in *SI Materials and Methods*.

Stable Knockdown of Genes. A lentivirus transduction was used to generate stable knockdown of p65 NF- κ B as described in *SI Materials and Methods*.

Microscopy. Confocal microscopy was performed in C2C12 myotubes, and myotube diameter was quantified on light microscopy by using methods described in *SI Materials and Methods*.

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