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Gelatinases impart susceptibility to high-fat diet induced obesity in mice

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Abstract

Gelatinases play a role in adipose and muscle hypertrophy, and could be involved in tissue remodeling in response to high-fat diet (HFD) intake. This study tested potential roles of gelatinases (matrix metalloproteinases-2 and -9, MMP-2 and -9) in relationship to an antigrowth factor (myostatin, MSTN) known to be dysregulated in relation to HFD induced obesity (HFDIO) propensity. *In vitro* and *ex vivo* analyses demonstrated that MMP-9 increased mature MSTN levels, indicating a potential role of gelatinases in MSTN activation *in vivo*. HFD intake resulted in increased body weight and circulating blood glucose values in C57BL/6J and MMP-9 null mice, with no changes observed in SWR/J mice. HFD intake attenuated *MMP-9* and *MMP-2* mRNA levels in SWR/J mice while elevating *MMP-2* levels in skeletal muscle in C57BL/6J mice. In MMP-9 null mice, the effects of HFD intake were muted. Consistent with changes in mRNA levels, HFD intake increased MMP-9 activity in muscle tissue of C57BL/6J mice, demonstrating a strong relationship between HFDIO susceptibility and local MMP regulation. Overall, resistance to HFDIO appears to correspond to low *MMP-9* and *MSTN* levels, suggesting a role of MMP-9 in MSTN activation in local tissue responses to HFD intake.

Keywords

Gelatinase; high-fat diet induced obesity; HFDIO; myostatin; MMP-9; MMP-2

Introduction

Propensity for high fat diet-induced obesity (HFDIO) varies within populations based upon genetic background, and HFDIO often results in systemic inflammation and subsequent type 2 diabetes. In obesity, a strong relationship exists between increased systemic inflammatory cytokines, like tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), and muscle insulin resistance. This low-grade inflammatory response, coupled with increased intramyocellular lipid deposition, is a direct consequence of metabolic shift due to increased nutrient bioavailability. As skeletal muscle represents the 'metabolic powerhouse' of an organism, considerations of dietary impacts on muscle tissue must be made. Recent evidence suggests that an interaction between muscle growth and inflammation may play a critical role in

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regulating metabolic dysfunction associated with high fat (HFD) intake. A key antigrowth factor, myostatin (MSTN), has been shown to be dysregulated by HFD intake based upon propensity for HFDIO. In response to HFD, susceptible animals exhibited elevated *MSTN* levels, while resistant animals exhibited decreased *MSTN* levels in muscle. Removal of functional MSTN abrogates the susceptibility to HFDIO and the associated insulin resistance. These results suggest a role for MSTN in local tissue responses to changes in dietary lipid intake, especially in skeletal muscle. A recent study demonstrated an interaction between MSTN and matrix metalloproteinases (MMPs) in regulating muscle cell hypertrophy. This work suggested that MMPs in the extracellular matrix (ECM) might play a role in the proteolytic maturation of MSTN *in vivo*. MMPs are known to regulate many developmental events and to activate several growth factors and cytokines, such as tumor necrosis factor- α (TNF- α) and transforming growth factor- β (TGF- β). Latent TGF- β can be activated by either CD44-bound MMP-9 or MMP-2.

Myostatin is a TGF- β superfamily member that is known to negatively regulate muscle cell proliferation, and a hydroxamate-based inhibitor of metalloproteinases stimulated myoblast fusion and myotube growth accompanied by a reduction in mature MSTN, suggesting that MMPs may be involved in MSTN activity regulation.

MMP-2 (Gelatinase A) is highly expressed in adipose tissue of nutritionally-induced obese mice. MMP-2 enhances adipose tissue development by increasing adipocyte hypertrophy in response to HFD. MMP-9 (Gelatinase B) was found to be upregulated in adipose tissue and in circulation of obese humans. Additionally, recent evidence indicates that MMP-9 may play a role in myofiber size and type determination, as MMP-9^{-/-} mice exhibited smaller myofibers and different type II compositions than did mice with normal MMP-9 activity. It is possible that MMPs coordinate tissue remodeling in response to excessive nutrient intake, leading to changes in the metabolic capacity of those tissues. Because our previous work demonstrated changes in a known muscle anti-growth factor (MSTN) in muscle in response to HFD intake, this study was conducted to evaluate potential interactions between MSTN and MMPs in skeletal muscle.

We, therefore, evaluated the interaction between MSTN and MMPs, utilizing both *in vitro* and *ex vivo* approaches. In addition, we examined the effects of HFD intake on MMP-2 and MMP-9 expression and activity in HFDIO-susceptible and -resistant mice. *We hypothesize that MMPs play a regulatory role in HFDIO, and we predict that their activities and expressions will be correlated with diet just as MSTN is.* Following *in vitro* and *ex vivo* analyses, we also examined the effects of HFD intake on MMP-2 and MSTN expression in mice lacking functional MMP-9 (MMP-9^{-/-}). HFD affects local MSTN expression in skeletal muscle, a tissue known to play key roles in metabolic load. *We hypothesize that the regulatory pathway controlling in vivo MSTN activation includes MMP-2 and -9, and that this pathway contributes to HFDIO-susceptibility.*

Materials and Methods

In vitro and *ex vivo* Cleavage Analyses

The ability of MMP-9 to cleave myostatin was analyzed using *in vitro* and *ex vivo* cleavage alongside either silver staining or Western blotting for visualization and quantification. Briefly, recombinant mouse MMP-9 (250 ng; 909-MM, R&D Systems) was activated with APMA (p-aminophenylmercuric acetate; 3.6 μ g) and added to recombinant mouse precursor MSTN (0.5 μ g; 1539-PG/CF, R&D Systems). To demonstrate specificity of cleavage, we inhibited enzyme activity with 1,10 phenanthroline (10 mM). Visualization of recombinant MSTN cleavage was carried out using silver staining. For *ex vivo* verification of biologically relevant cleavage, we treated whole blood (2 μ L) with activated MMP-9 in the

presence and absence of 1,10-phenanthroline. Cleavage of MSTN was detected by Western blotting with anti-mouse GDF-8/myostatin prodomain antibody (0.1 µg/mL; AF-1539, R&D Systems). Putative precursor MSTN (~50 kDa) and processed MSTN (~37 kDa) were detected in whole blood lysates. A FluorChem FC2 Chemiluminescence imager (Alpha Innotech) was utilized to capture luminescence and AlphaEase FC Analysis software was utilized to quantify immunoreactive peptide intensities between treatment groups using arbitrary densitometry units for comparison.

***In vivo* Experimental Design**

The North Dakota State University Institutional Animal Care and Use Committee (Protocol #A11016) approved all animal procedures prior to experimentation. Six week-old male mice, SWR/J, C57BL/6J, and MMP-9^{-/-} (C57BL/6J background), were used for these experiments. SWR/J and C57BL/6J were obtained from Jackson Laboratories (Bar Harbor, ME, USA), while MMP-9 null (MMP-9^{-/-}) mice were kindly obtained from Dr. Farrah Kheradmand (Baylor College of Medicine, Houston TX). In addition, genotype and lack of MMP-9 activity were verified in our laboratory (data not shown). Mice were allowed to acclimate for a period of two weeks to the facilities, which were maintained at constant 20°C and 50% humidity with a 12:12 hour light:dark cycle. Mice were acclimated to the control diets for two weeks before being placed on experiment. Each strain of mouse was divided equally into two experimental groups, which included 1) control: fed a control diet (10% kcal fat, D12450B; Research Diets Inc., New Brunswick, NJ, USA); or 2) high-fat: fed a HFD (60% kcal fat, D12492; Research Diets Inc.). Mice were fed experimental diets for 6 weeks *ad libitum* and given free access to water. Each experiment was repeated (n=3/group, n=6 total). Individual body weights, whole blood glucose, and average daily food intake were measured weekly. All mice were fasted for 12 hours prior to any biological sampling and were euthanized by CO₂ inhalation prior to end-point sampling. Tissue and blood samples were collected at end-point sampling for further analyses. Skeletal muscle (pool of gastrocnemius, soleus, and plantaris) samples was collected and immediately placed on dry ice, then stored at -80°C until further processing. Blood was collected from live, lightly anesthetized animals via the saphenous vein (50 µL) for analysis of blood glucose using an Accu-Chek® Blood Glucose Meter (Roche Diagnostics, Indianapolis, IN, USA).

Quantitative Real-Time PCR

Changes in gene expression of *matrix metalloproteinase-2* and *-9* (*MMP-2*, *MMP-9*) and *MSTN* were evaluated by real-time reverse transcription quantitative PCR (RT-qPCR) in muscle samples. Total RNA samples were reverse transcribed using ImProm-II Reverse Transcription system (Promega, Madison, WI, USA) and oligo-dT₁₈ primers to obtain first-strand cDNA. First-strand cDNAs were diluted (0.50 ng/µL) and used as templates for RT-qPCR analysis. Reactions (10 µL total volume) containing 1 µL diluted template, 150 nM of each primer, and 2X Quanta PerfectaSYBRGreen qPCR SuperMix (Quanta BioSciences, Gaithersburg, MD, USA) were run in duplicate using the Mx3000P real-time PCR system (Stratagene, Santa Clara, CA, USA) and the following cycling parameters: 94°C for 2 min; 40 cycles of 94°C for 20 s, 56°C (*MMP-2*), 52°C (*MMP-9*), and 54°C (*MSTN*) for 15 s and 68°C for 60 s, followed by a dissociation curve (95°C for 60s, 55-95°C). The following primers were used for specific analysis of *MMP* mRNA expression: *MMP-9*, forward primer (5'-GGAACCTCACACGACATCTTCCA-3'), reverse primer (5'-GAAACTCACACGCCAGAAGAATTT-3'); *MMP-2*, forward primer (5'-CGGTTTATTTGGCGGACAGT-3'), reverse primer (5'-GCCTCATACACAGCGTCAATCTT-3'). Primers utilized for specific analysis of *MSTN* were: forward primer (5'-TAGCAGATTCAATAGTGGTC-3'), reverse primer (5'-ATTGAAATTTGACTGGGAGC-3'). No-template controls were run for all primer pairs and PCR efficiencies were calculated for each primer pair. Standards were generated using

serial dilutions of gene-specific targets produced by standard PCR and cloning techniques. Standard curves were generated for each primer pair and used for relative quantification. Primer PCR efficiencies were calculated and used for PCR correction for all primer pairs and normalized data were analyzed using the relative quantification method.

Zymography

Matrix metalloproteinase activity was analyzed by gelatin zymography, as described previously with a few modifications. Briefly, samples were prepared for analysis by dilution in zymogram buffer consisting of 0.4M Tris, pH 6.8, 5% SDS, 20% glycerol, and 0.03% bromophenol blue (Bio-Rad, Hercules, CA, USA). Tissues were diluted in zymogram buffer at concentrations of 500 $\mu\text{g}/\mu\text{L}$ for muscle. Frozen tissue was weighed and minced prior to addition to zymogram buffer. Diluted samples were allowed to sit on ice for 15 minutes and then loaded onto minigels containing 10% polyacrylamide and 0.1% gelatin. Electrophoresis was carried out at constant 105V for 1.5 to 2 hours, at which time the bromophenol blue dye front had reached the bottom of the gel. The gels were then removed and incubated in renaturing buffer (2.5% Triton X-100) for 30 minutes at room temperature under gentle agitation. Gels were then equilibrated in developing buffer (50 mM Tris, 5mM CaCl_2 , 0.02% sodium azide) for 30 minutes at room temperature under gentle agitation. Developing buffer was then replaced with fresh developing buffer and gels were incubated at 37°C for 16 hours under gentle agitation. Gels were then stained with 0.5% Coomassie blue G-250 in 30% methanol/10% acetic acid for 1 hour and then destained with 30% methanol/10% acetic acid.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism version 5.0c for Mac OS X (GraphPad Software, San Diego, CA, USA, www.graphpad.com). All RT-qPCR data were analyzed by the standard curve method where cycle threshold values were compared to standard curves generated and validated for each primer pair set to result in a relative starting copy number of mRNA in nanograms. All comparisons within strains were analyzed by t-tests and variances were tested by F test. Analyses between strains were conducted using two-way analysis of variance (ANOVA) with strain and treatment as factors. All body weight and blood glucose data were analyzed using two-way ANOVA with factors being strain and treatment. Post-hoc comparison tests (Bonferroni) were conducted when overall interactions were significant ($p < 0.05$) to test for differences between treatment groups at each sampling time. Pairwise multiple-comparison tests were conducted when overall interactions were significant ($p < 0.05$) by the Holm-Sidak method to compare between and within strains. Normality of sample distribution was tested by Normal Quantile Plot (Q-Q Plot), and residuals were plotted against predicted values to evaluate dependency between the means and variances and test the assumption of homogeneity of variances. Results were reported as least square means $\pm$ SEM.

Results

Matrix metalloproteinase-9 releases myostatin from sequestration *in vitro* and *ex vivo*

To examine the potential role of the gelatinases in MSTN activation, we utilized *in vitro* and *ex vivo* cleavage analyses to determine if recombinant activated MMP-9 (aMMP-9) could cleave MSTN. Precursor MSTN cleavage to putative mature MSTN was increased by the addition of aMMP-9 (Figure 1a). This increase in mature MSTN fragments was attenuated when inhibited aMMP-9 (iMMP9) was added (Figure 1a). In addition, we tested the role of aMMP-9 in MSTN expression in whole blood from wild type (C57BL/6J) and MMP-9 null (MMP-9^{-/-}) mice. Western blot analysis revealed that aMMP-9 increased mature MSTN levels in both types of mice (Figures 1b and c). Activated MMP-9 increased mature MSTN

expression 12.9% in whole blood from C57BL/6J mice, and increased precursor MSTN 20.0% and mature MSTN 22.6% in whole blood from MMP-9 null mice. In both cases, inhibition of the aMMP-9 attenuated the increase in myostatin expression.

SWR/J mice exhibit no increase in growth or change in blood glucose in response to HFD intake, while MMP-9 null mice exhibit a delayed increase in growth

C57BL/6J mice exhibited a 61.3% increase in body weight in response to 6 weeks of HFD intake (Figure 2a). SWR/J mice fed a HFD exhibited no change in weight gain over the 6-week trial (Figure 2b). In mice lacking functional MMP-9 (MMP-9^{-/-}), HFD intake increased body weight 28.9% after 6 weeks of dietary intake (Figure 2c). An increase in overall body weight was first detected in C57BL/6J mice following 1 week of HFD intake, compared to 5 weeks on the diet in MMP9^{-/-} mice. A greater change in specific growth rate over the course of the experiment was detected in C57BL/6J mice compared to MMP-9 null mice (0.91 vs. 0.71, respectively). Food intake as a function of body weight was not different between any groups (data not shown).

HFD intake resulted in an increase of whole blood glucose levels in C57BL/6J mice, but not SWR/J mice. In C57BL/6J mice, blood glucose levels were elevated following HFD intake at 1, 3, and 6 weeks on the diet (Figures 3a and b; 26.9%, 51.1%, and 27.3% at 1, 3, and 6 weeks, respectively). In MMP9^{-/-} mice, an increase in blood glucose was only detected at 3 weeks on the HFD (Figure 3c), but overall whole blood glucose levels were elevated 47.6% across the entire experimental period.

Matrix metalloproteinase (gelatinase) expression is increased in response to HFD intake in C57BL/6J mice, yet decreased in SWR/J mice

Using quantitative real-time PCR, we evaluated the effects of HFD intake on *matrix metalloproteinase-2* and *-9* mRNA levels in muscle from C57BL/6J, SWR/J, and MMP-9^{-/-} mice. *MMP-2* mRNA levels in C57BL/6J muscle tissue were increased 66% in response to HFD intake (Figures 4a). *MMP-9* mRNA levels, on the other hand, were elevated 2.59-fold in muscle from C57BL/6J mice in response to HFD intake (Figure 4b).

SWR/J mice exhibited an attenuation of *MMP-2* mRNA expression in response to HFD intake (Figure 4a). HFD intake lowered *MMP-2* levels 40.1% in muscle tissue of SWR/J mice. *MMP-9* mRNA was not significantly decreased by HFD, however lower trend is apparent (Figure 4b).

MMP-9^{-/-} mice exhibited no change in *MMP-2* levels in muscle when fed a HFD (Figures 4a). Steady-state control levels of *MMP-2* were significantly ($P < 0.0001$) higher in MMP-9 null mice compared to control levels in both C57BL/6J and SWR/J mice, regardless of tissue measured (data not shown). *MMP-9* mRNA levels were not detectable in MMP-9 null mice.

HFD intake increases MMP-9 activity in muscle tissue of HFDIO-susceptible mice

Using gelatin zymography, we evaluated the effects of HFD intake on local gelatinase activity levels in muscle from both HFDIO-susceptible (C57BL/6J) and -resistant (SWR/J) strains of mice, as well as MMP-9 null mice. In muscle tissue, we detected pro- and active-MMP-2 (~72 and ~62 kDa, respectively) and MMP-9 (~92 and ~82 kDa, respectively) activity (Figure 5a). Active MMP-2 and MMP-9 were detected in muscle tissue from C57BL/6J and SWR/J mice, but only MMP-2 was detectable in MMP-9 null mice (Figure 5b). Quantification was conducted on active enzyme bands (aMMP-2, ~62 kDa; aMMP-9, ~82 kDa). The only difference in gelatinase activity detected was MMP-9 activity in muscle tissue from C57BL/6J mice (Figure 5d). HFD intake increased activity levels 51.1% in C57BL/6J mice. No differences were detected in MMP-9 activity in muscle tissue from

SWR/J mice (Figure 5d). In addition, no differences were detected in MMP-2 activity in muscle tissue from C57BL/6J, SWR/J, or MMP-9^{-/-} mice (Figure 5c).

HFD intake increases myostatin expression in muscle tissue of HFDIO-susceptible mice, but not in MMP-9 null mice

Using quantitative real-time PCR, we evaluated the effects of HFD intake on *MSTN* mRNA levels in muscle tissue from C57BL/6J, SWR/J, and MMP-9^{-/-} mice. HFD intake had no effect on *MSTN* expression in muscle tissue from MMP-9^{-/-} mice (Figures 6). HFD increased *MSTN* expression 1.91-fold in muscle from C57BL/6J mice and decreased *MSTN* levels 0.56-fold in muscle tissue from SWR/J (Figure 6).

Discussion

HFDIO and Myostatin

While it is known that myostatin (MSTN), a potent negative regulator of muscle growth of the TGF- β superfamily, appears to play a role in susceptibility to HFDIO, few studies have examined specific proteins that regulate the actions of myostatin in the context of HFDIO. The classification of MSTN as a TGF- β superfamily led us to hypothesize that MMP-9, a known regulator of TGF β 2, may also play a role in the activation and/or degradation of MSTN. With the demonstration of a possible connection between these two proteins *ex vivo*, we carried out *in vivo* experiments in strains of mice known to exhibit differential responses to HFDIO to establish a potential regulatory mechanism involving MSTN and MMP-9 in HFDIO.

Through *in vitro* cleavage analysis, we demonstrated that recombinant MSTN treated with activated MMP-9 resulted in an increase in peptide fragments similar in size to mature MSTN (Figure 1a). However, there is no evidence that these fragments are active or mature MSTN peptides. These data only suggest that active MMP-9 cleaves the precursor MSTN protein into smaller fragments, which could be 1) indicative of increased activity; or 2) degradation to decrease functionality. We further explored this relationship with an *ex vivo* approach to identify a more biologically relevant interaction between MMP-9 and MSTN. Our finding that active MMP-9 can increase the abundance of potentially active mature MSTN in whole blood, as demonstrated by Western blot analysis, suggests that MMP-9 might play a role in releasing MSTN from sequestration (Figure 1b). In addition, in MMP-9 null mice, aMMP-9 increased the amount of precursor MSTN in whole blood, indicating that aMMP-9 may release MSTN from sequestration. Miura et al. recently demonstrated that decorin, a small leucine-rich proteoglycan, binds and sequesters MSTN to the ECM, regulating its cell proliferation activity in a manner similar to that of TGF- β . MMPs are known to degrade ECM components to aid in cell migration, remodeling, and even growth factor activation, as reviewed in Greenlee et al. These novel results support our hypothesis that MMP-9 plays a role in regulating MSTN and that the delayed onset of body weight gain in MMP-9 null mice could be due to an altered MSTN feedback regulatory system in the absence of MMP-9. Increased MMP-9 activity in C57BL/6J mice following increased lipid intake, together with elevated mRNA levels of *MMP-2* and *MSTN* in MMP-9^{-/-} mice, demonstrate the relevance of enzyme activity values for biological importance and further support the biological relevance of the *in vitro* and *ex vivo* cleavage data.

HFDIO and MMP-2/9

Consistent with recently published data comparing weight gain and glucose levels over a 12-week period on HFD, C57BL/6J mice are highly susceptible to HFDIO, while SWR/J mice are resistant. This is evidenced by an increase in both body weight and whole blood glucose levels in C57BL/6J mice as early as 1 week on a HFD. SWR/J mice fail to exhibit any

change in body weight or circulating blood glucose levels in response to HFD intake. On the C57BL/6J background, MMP-9 null mice exhibit an increase in body weight, although this response appears slightly delayed, as a significant increase in weight gain was not observed until 5 weeks, as compared to 1 week in C57BL/6J mice (Figure 2). At the beginning of the study, MMP-9 null mice were slightly larger than C57BL/6J and SWR/J mice, even though all mice were of the same age. As our and others' data demonstrate, MMP-9 null mice are larger than their age-matched wild-type background C57BL/6J mice, as previously shown. Therefore, the delay in growth response to HFD intake in the MMP-9 null mice could be an artifact of lower growth rates in these larger knockout mice. In addition, in MMP-9^{-/-} mice, blood glucose values were only elevated at 3 weeks on the diet, which was attenuated after 6 weeks. In C57BL/6J mice, the HFDIO-susceptible mice, whole blood glucose levels were elevated consistently while on the experimental diet. The data show an attenuation or delay in the effects of increased lipid intake when functional MMP-9 is absent, supporting the hypothesis that MMP-9 may act to release mature MSTN and promote adiposity.

In mice susceptible to HFDIO, *MMP-9* levels increased in response to HFD intake in muscle. This upregulation was accompanied by an increase in MMP-9 activity in muscle tissue, indicating that MMP-9 may be involved in increasing lipid deposition. In contrast, HFD intake had no effect on MMP-9 levels or activity in muscle from HFDIO-resistant mice (SWR/J). HFD intake in MMP-9^{-/-} mice resulted in a delayed weight gain compared to wild-type counterparts. In contrast to our results, a previous study identified a decrease in MMP-9 activity in response to HFDIO. However, this study involved a 20-week feeding trial, as opposed to the six-week feeding trial in our study. In addition, Kang et al. utilized the gastrocnemius muscle to analyze MMP-9 activity, while we utilized a pool of the gastrocnemius, soleus, and plantaris. Consistent with our data, total plasma MMP-2 and MMP-9 levels were demonstrated to be elevated in obese human patients.

The potential role of MMP-2 in muscle tissue in response to increased dietary lipid intake is unclear. MMP-2^{-/-} mice with a 100% C57BL/6J genetic background gain less weight on a HFD compared to wild type controls, suggesting a role for MMP-2 in metabolic regulation and potentially in adipogenesis. MMP-2 is thought to play a key role in local proteolytic events and lipid deposition. In this study, increased HFD intake increased *MMP-2* mRNA levels in muscle tissue from HFDIO-susceptible mice, but this change was not accompanied by any change in MMP-2 activity, suggesting that MMP-2 might not be as significant as MMP-9 in the metabolic regulation of muscle tissue. In contrast, HFD intake decreased *MMP-2* levels in muscle from HFDIO-resistant mice with no accompanying change in enzyme activity levels. MMP-2 is known to play a pivotal role in tissue remodeling during disuse-induced muscle atrophy, as it is the dominant MMP activated during skeletal muscle atrophy. The changes in message without any changes in mature enzyme activity could suggest non-proteolytic mechanisms of action in muscle remodeling by the MMPs.

Correlation of MSTN with MMP Expression

No effect of HFD intake on *MSTN* expression in skeletal muscle tissue was observed in MMP-9 null mice compared to an increase in C57BL/6J mice. However, steady-state *MSTN* levels in muscle tissue were close to 500-fold greater in MMP-9 null mice compared to C57BL/6J mice. It is likely that this difference in steady-state expression level is due to a feedback mechanism that is not present in the MMP-9 null animals. Further, we hypothesize that increased *MSTN* levels might be related to increased *MMP-2* expression upon inactivation of MMP-9 in transgenic mice. Consistent with data in C57BL/6J mice, increased gelatinase mRNA expression is correlated with increased *MSTN* mRNA expression. In SWR/J mice, decreased gelatinase mRNA expression is accompanied by a similar decrease in *MSTN* mRNA abundance. MMP-2 and -9 have been shown to possess redundant functions and we speculate that the "overcompensation" of increased *MMP-2*

transcription leads to increased *MSTN* expression in skeletal muscle. In spleen, a similar pattern is seen, further corroborating our hypothesis (data not shown). Previous data indicate that ablation of MMP-9 leads to increased levels of MMP-2 enzyme (both pro- and mature forms) in vein grafts and this is likely true in skeletal muscle.

Taken together, the results of this study combined with previous works indicate that lower levels of functional *MSTN* lead to a more HFDIO-resistant phenotype, compared to elevated *MSTN* levels that are present in HFDIO-susceptible animals. In short, we propose that MMP-9 can activate *MSTN* levels to locally regulate cell proliferation or adipogenesis. Figure 7 describes a broad overview of how attenuated MMP-9 activity would result in lower active *MSTN* that could lead to a more HFDIO-resistant phenotype, compared to normal MMP-9 activity resulting in myostatin activation at local sites resulting in a HFDIO-susceptible phenotype as seen in C57BL/6J mice.

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Figure 1. *In vitro* and *ex vivo* cleavage evidence of MMP action on myostatin. a) Recombinant myostatin (rMSTN), rMSTN plus activated MMP-9 (aMMP-9), and rMSTN plus aMMP-9 plus 1,10-phenanthroline (a gelatinase inhibitor). Silver staining reveals two primary myostatin proteins at ~50 kDa and ~37 kDa. b) whole blood from C57BL/6J and MMP9^{-/-} treated with aMMP-9 or inhibited MMP-9. Western blotting detected precursor and processed myostatin immunoreactive peptides at ~50 kDa and ~37 kDa, respectively. c) Quantification of band intensities from Western blotting of precursor (~50 kDa) and mature (~37 kDa) bands. * denotes differences between dietary groups at a given time, $p < 0.05$.

Figure 2.

Overall body changes as measured by mass (g) in C57BL/6J (a), SWR/J (b), and MMP-9^{-/-} (c) mice fed experimental diets for 6 weeks. Open bars = control diet, 10% kcal fat; gray bars = high-fat diet, 60% kcal fat. * denotes differences between dietary groups at a given time within strains, $p < 0.05$.

Figure 3. Whole blood glucose levels from C57BL/6J (a), SWR/J (b), and MMP9^{-/-} (c) mice fed either a control (open bars) or high-fat diet (gray bars) for 6 weeks. * denotes differences between dietary groups at a given time within strains, p<0.05.

Figure 4. Quantitative PCR analysis of *MMP-2* (a) and *MMP-9* (b) gene expression from C57BL/6J, SWR/J, and MMP-9^{-/-} mice. Gene expression was evaluated in muscle tissue from mice fed either a control (open bars) or high-fat (gray bars) diet for 6 weeks. * denotes differences between dietary groups at a given time within strains, $p < 0.05$.

Figure 5. Matrix metalloproteinase activity in muscle tissue from C57BL/6J, SWR/J, and MMP-9^{-/-} mice. a) Representative MMP-2 and -9 activity from muscle: 1-2. SWR/J, high-fat diet; 3-4. C57BL/6J, high-fat diet; 5-6. C57BL/6J, control diet; 7-8. SWR/J, control diet. Active MMP-2 (aMMP-2) was detected at ~ 62 kDa. Active MMP-9 (aMMP-9) was detected at ~82 kDa. b) Representative MMP-2 and -9 activity in muscle: 1. SWR/J, control; 2. MMP-9^{-/-}, control; 3. C57BL/6J, control. c) Quantification of active MMP-2 (~62 kDa). d) Quantification of active MMP-9 (~82 kDa). * denotes differences between dietary groups at a given time within strains, $p < 0.05$.

Figure 6. Quantitative PCR analysis of myostatin mRNA expression in muscle tissue from C57BL/6J, SWR/J, and MMP9^{-/-} mice fed either a control (open bars) or HFD (gray bars) for 6 weeks.* denotes differences between dietary groups at a given time within strains, $p < 0.05$.

Figure 7.

Proposed model demonstrating the role of MMP-9 activating myostatin (MSTN) in regards to susceptibility or resistance to HFDIO. Results from this study, and others, indicate that lower levels of myostatin are related to HFDIO-resistant phenotype and it is possible that attenuating various feedback regulation steps (in this case MMP-9) for active myostatin might have similar phenotype consequences.