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

Peggy R. Biga[a,b,*], Jacob M. Froehlich[a,b], Kendra J. Greenlee[a], Nicholas J. Galt[a,b], Ben M. Meyer[a], Delci J. Christensen[a]

[a]Department of Biological Sciences, North Dakota State University, Fargo, ND, USA
[b]Department of Biology, University of Alabama at Birmingham, Birmingham, AL, USA

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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 metalloproteinses-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.
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1. 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, and muscle insulin resistance [1]. 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 regulating metabolic dysfunction associated with high-fat diet (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 [2]. Removal of functional *MSTN* abrogates the susceptibility to HFDIO and the associated insulin resistance [3–5]. 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 [6]. 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 [7] and to activate several growth factors and cytokines, such as TNF-α and transforming growth factor-β (TGF-β) [8–10]. Latent TGF-β can be activated by either CD44-bound *MMP-9* or *MMP-2* [11]. *MSTN* is a TGF-β super family 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* [6], 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 [12,13]. *MMP-2* enhances adipose tissue development by increasing adipocyte hypertrophy in response to HFD [14]. *MMP-9* (Gelatinase B) was found to be up-regulated in adipose tissue and in circulation of obese humans [15,16]. In addition, recent evidence indicates that *MMP-9* may play a role in myofiber size

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* Corresponding author. Department of Biology, University of Alabama at Birmingham, Birmingham, AL, USA. Tel.: +1 205 934 9684.
E-mail address: pegbiga@uab.edu (P.R. Biga).

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and type determination, as *MMP-9*$^{-/-}$ mice exhibited smaller myofibers and different Type 2 compositions than did mice with normal *MMP-9* activity [17]. It is possible that *MMP*s 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 antigrowth factor (*MSTN*) in muscle in response to HFD intake, this study was conducted to evaluate potential interactions between *MSTN* and *MMP*s in skeletal muscle.

We, therefore, evaluated the interaction between *MSTN* and *MMP*s, 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 *MMP*s 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.

2. Materials and methods

2.1. In vitro and ex vivo cleavage analyses

The ability of *MMP-9* to cleave *MSTN* 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 *p*-aminophenylmercuric acetate (3.6 μg) and added to recombinant mouse precursor *MSTN* (0.5 μg; 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 μL) with activated *MMP-9* in the presence and absence of 1,10-phenanthroline. Cleavage of *MSTN* was detected by Western blotting with antimouse GDF-8/*MSTN* 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.

2.2. 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, USA). 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 2 weeks to the facilities, which were maintained at constant 20°C and 50% humidity with a 12:12-h light:dark cycle. Mice were acclimated to the control diets for 2 weeks before being placed on experiment. Each strain of mouse was divided equally into two experimental groups, which included (a) control: fed a control diet (10% kcal fat, D12450B; Research Diets Inc., New Brunswick, NJ, USA); or (b) 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 h prior to any biological sampling and were euthanized by CO_2 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 (50μL) for analysis of blood glucose using an Accu-Chek Blood Glucose Meter (Roche Diagnostics, Indianapolis, IN, USA).

2.3. Quantitative Real-Time PCR

Changes in gene expression of *MMP-2* and *−9* (*MMP-2*, *MMP-9*) and *MSTN* were evaluated by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) in muscle samples. Total RNA samples were reverse transcribed using ImProm-II Reverse Transcription system (Promega, Madison, WI, USA) and oligo-dT_{18} primers to obtain first-strand cDNA. First-strand cDNAs were diluted (0.50 ng/μL) and used as templates for RT-qPCR analysis. Reactions (10μL total volume) containing 1 μL diluted template, 150 nMof each primer and 2X Quanta PerfeCTaSYBRGreen qPCRSuperMix (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 60 s, 55–95°C). The following primers were used for specific analysis of *MMP* mRNA expression: *MMP-9*, forward primer (5′-GGAACTCACACGACATCTTCCA-3′), reverse primer (5′-GAAACTCACACGCCAGAAGAATTT-3′); *MMP-2*, forward primer (5′-CGGTTTATTTGGCGGACAGT-3′), reverse primer (5′-GCCTCATACACAGCGT-CAATCTT-3′) [18]. 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 [19–21]. 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 [22].

2.4. Zymography

MMP activity was analyzed by gelatin zymography, as described previously [23] with a few modifications. Briefly, samples were prepared for analysis by dilution in zymogram buffer consisting of 0.4MTris, pH 6.8, 5% sodium dodecyl sulfate, 20% glycerol and 0.03% bromophenol blue (Bio-Rad, Hercules, CA, USA). Tissues were diluted in zymogram buffer at concentrations of 500 μg/μL 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 h, 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 min at room temperature under gentle agitation. Gels were then equilibrated in developing buffer (50 mMTris, 5mM $CaCl_2$, 0.02% sodium azide) for 30 min at room temperate under gentle agitation. Developing buffer was then replaced with fresh developing buffer and gels were incubated at 37°C for 16 h under gentle agitation. Gels were then stained with 0.5% Coomassie blue G-250 in 30% methanol/10% acetic acid for 1 h and then destained with 30% methanol/10% acetic acid.

2.5. 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<.05) to test for differences between treatment groups at each sampling time. Pairwise multiple-comparison tests were conducted when overall interactions were significant (P<.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±S.E.M.

3. Results

3.1. MMP-9 releases MSTN 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* (a*MMP-9*) could cleave *MSTN*. Precursor *MSTN* cleavage to putative mature *MSTN* was increased by the addition of a*MMP-9* (Fig. 1A). This increase in mature *MSTN* fragments was attenuated when inhibited a*MMP-9* (i*MMP9*) was added (Fig. 1A). In addition, we tested the role of a*MMP-9* in *MSTN* expression in whole blood from wild type (C57BL/6J) and *MMP-9* null (*MMP-9*$^{-/-}$) mice. Western blot analysis revealed that a*MMP-9* increased mature *MSTN* levels in both types of mice (Fig. 1B

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