Milk-derived ribonuclease 5 preparations induce myogenic differentiation in vitro and muscle growth in vivo

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ABSTRACT

Ribonuclease 5, also known as angiogenin, is a stable and abundant ribonuclease in milk whey protein, which is able to regulate several cellular functions, including capillary formation, neuron survival, and epithelial cell growth. Ribonuclease 5 is important for protein synthesis directly stimulating rRNA synthesis in the nucleolus. Here, we show that biologically active RNase5 can be purified from bovine milk. Furthermore, we show that milk-derived RNase5 directly stimulates muscle cell differentiation in vitro, inducing C2C12 cell differentiation and myogenesis. When supplemented into the diet of healthy adult mice, milk-derived RNase5 preparations promoted muscle weight gain and grip strength. Collectively, these data indicate that milk-derived RNase5 preparations exhibit a novel role in skeletal muscle cell function.

Key words: growth factor, RNase5, myogenic differentiation, muscle growth

INTRODUCTION

Milk provides a rich source of nutrients, immune factors, and bioactive proteins for the growing newborn and is also known to assist in muscle accretion (Burrin et al., 1992; Pennings et al., 2011). Numerous studies have shown that milk whey protein supplementation can promote an increase in the size of muscles subjected to mechanical stimulus, such as physical activity or exercise (Burke et al., 2001; Anderson and Behm, 2005). This anabolic effect has been attributed to either branched-chain amino acids, especially leucine (Phillips et al., 2009), or growth factors. Although the oral consumption of IGF has been implicated, no scientific evidence exists to substantiate its efficacy (Belford et al., 1995). In addition the oral consumption of whey

protein supplementation also has been shown to improve serum protein balance after resistance exercise (Tipton et al., 2007) and improve muscle satellite cell regulation (Olsen et al., 2006).

Bovine milk whey protein comprises several major proteins such as β -LG (rich in branched-chain amino acids), α -LA, and BSA as well as relatively low concentrations of a group of basic proteins. The most abundant of these are lactoferrin and lactoperoxidase. Several growth factors are also present in the basic protein fraction, which, because of their high isoelectric points, can be readily separated from the bulk of the other basic proteins and further purified, using cation-exchange chromatography. Such fractions are frequently referred to as growth factor extract (Dyer et al., 2008) and contain IGF (IGF-1 and IGF-2), platelet-derived growth factor, fibroblast growth factor (FGF; Rogers et al., 1995), and transforming growth factor- β (Rogers et al., 1996). Numerous preparations of milk whey fractions are commercially available and have the potential to prevent and promote muscle growth (Dyer et al., 2008; Pennings et al., 2011).

An initial objective of this study was to characterize a commercially available bioactive milk whey preparation and identify the active component(s) that contribute to the beneficial effects of oral consumption on muscle growth and strength (Dyer et al., 2008). Traditional chromatographic techniques were used as well SDS-PAGE to analyze the protein composition and proteomic profile. Ribonuclease 5 was identified and characterized as an abundant and stable protein. Several preparations of RNase5 estimated to contain 10, 50, and 95% RNase5 were purified from milk whey protein and their biological activity investigated in in vitro and in vivo models of myogenic differentiation and muscle growth.

MATERIALS AND METHODS

Recombinant proteins used in proliferation and myogenesis studies were sourced from R&D Systems

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Inc. (Minneapolis, MN), unless otherwise stated. Milk whey preparations (**RNase5-10**, **RNase5-50**, and **RNase5-95**) were obtained by successive purification from milk whey protein. The -10, -50, and -95 refer to intact RNase5 protein content in the preparation being 10, 50, and 95%, respectively.

Extraction Procedure Used to Enrich for RNase5 from Milk Whey

The bovine RNase5 extract referred to as RNase5-10 was purified by passing skim milk (3,300 L) through a column filled with SP resin (GE Life Sciences, Foster City, CA; Brown et al., 2010), rinsing with 180 L of water, and then eluting with 180 L of 2.5% NaCl. A semi-purified milk RNase5 (RNase5-50) was isolated from RNase5-10 by applying 1 kg of RNase5-10 solution to a column filled with SP resin (GE Life Sciences), rinsing with 360 L of 2.0% NaCl, and then eluting with 180 L of 2.5% NaCl. The RNase5-10 and RNase5-50 were desalted by UF with 10-kDa membranes (Brown et al., 2010). The RNase5-95 was purified from RNase5-50 solution using cation-exchange HPLC with a linear gradient of NaCl in 50 mM phosphate buffer (pH 7.0), followed by size exclusion chromatography using Mono S 10/100 GL and Sephacryl S-100 HR columns (GE Life Sciences). The RNase5-10, RNase5-50, and RNase5-95 were freeze-dried and stored at 4°C.

To quantify the major whey proteins (lactoperoxidase, lactoferrin, and RNase5) present in each of the RNase5-10, -50, and -95 preparations, the respective protein samples were resuspended at 5 mg/mL in 50 mM sodium phosphate containing 5% acetonitrile (pH 7.0). Each sample was then separated on a Mono S 5/50 GL column (GE Life Sciences), pre-equilibrated with buffer A [50 mM sodium phosphate containing 5% (vol/vol) acetonitrile, pH 7.0]. One hundred microliters of the 5 mg/mL solution was loaded onto the column in buffer A and a linear gradient was applied to increase to 100% buffer B (50 mM sodium phosphate and 1 M NaCl, pH 7.0) in 3.5 min after injection and continued at 100% buffer B for the remainder of the Mono S 5/50 GL column chromatography. The lactoferrin and lactoperoxidase concentrations in the eluate were determined using absorbance spectrophotometry at 280 nm for lactoferrin and lactoperoxidase and 220 nm for RNase5. The amount of IgA present in the RNase5-10, -50, and -95 protein preparations was determined using a Bovine IgA ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, TX) according to the manufacturer's instructions. The amount of IgG present in the RNase5-10, -50, and -95 protein preparations was determined by protein G affinity chromatography according to Abernethy et al. (2010). The remaining

protein content was characterized as other whey proteins.

Confirmation of the enrichment procedure used to purify the RNase5-10, -50, and -95 preparations was performed by SDS-PAGE under reducing conditions on NuPAGE precast gradient gel (4–12%; Invitrogen Corp., Grand Island, NY; Laemmli, 1970). The protein gel was stained with SYPRO Ruby (Invitrogen Corp.) and imaged using a cooled scanning charge-coupled device (CCD) camera (ProXPRESS; PerkinElmer Life Sciences, Waltham, MA).

RNase Activity Assay

The RNase activity assay was performed using the RNaseAlert QC system from Ambion (Invitrogen Corp.) according to the manufacturer's instructions. Ribonuclease activity was detected by a cleavable fluorescent-labeled RNase substrate and recorded on a Synergy 2 plate reader (BioTek Instruments Inc., Winooski, VT).

In Vitro Matrigel Angiogenesis Assay

To confirm that the enriched preparations of RNase5 were biologically active, in vitro Matrigel angiogenesis assays were performed as described by Ponce (2001). Briefly, 6-well plates were coated with ice-cold Matrigel (BD Biosciences, San Jose, CA), which was allowed to polymerize at room temperature for about 30 min. Thereafter, human umbilical vein endothelial cells (**HUVEC**; 5×10^4 cells/mL) that had been pretreated with 100 ng/mL of RNase5-10 or recombinant human RNase5, or 5 ng/mL of vascular endothelial growth factor (**VEGF**) 165 (positive control; R&D Systems Inc.) for up to 5 d were seeded onto the Matrigel. Cells were cultured overnight in Iscove's modified Dulbecco's medium, supplemented with 100 IU of penicillin/mL, 100 µg of streptomycin/mL, 1% (vol/vol) fetal calf serum, and 5 ng of basic FGF/mL in the presence or absence of various RNase5-enriched milk preparations or VEGF. Tube formation was assessed every 12 h and bright field images were captured.

C2C12 Myogenesis Assay

The C2C12 cell line is an immortal line of mouse skeletal myoblasts originally derived from satellite cells from the thigh muscle of a 2-mo-old female C3H mouse donor (Yaffe and Saxel, 1977). The C2C12 cells were used between passages 25 and 30 and seeded in 6-well plates at 2.5×10^5 cells/mL, and allowed to attach overnight; C2C12 myogenesis was then induced through changing the medium from growth medium (DMEM

containing 10% fetal bovine serum) to differentiation medium (DMEM containing 2% horse serum), supplemented with one of the following experimental treatments: control (differentiation medium alone), 200 ng of IGF-1/mL (positive control), 10 μ g of RNase5-50/mL, 10 μ g of RNase5-95/mL, or recombinant human RNase5 (1 μ g/mL). The enzyme creatine kinase (**CK**) was used as a marker of C2C12 myogenesis and activity was quantified by CK activity (CK-NAC; Thermo Fisher, Fair Lawn, NJ) in accordance with the manufacturer's instructions. Immunohistochemistry was used to confirm myogenic differentiation by staining with myosin heavy chain (mouse anti-myosin antibody; Invitrogen Corp.). The presence of myosin heavy chain was visualized using confocal fluorescence microscopy (model number FV10i; Olympus America Inc., Center Valley, PA).

Mouse Supplementation Studies

All experiments were carried out on 8-wk-old (adult) male C57Bl/10 mice obtained from the Animal Resource Centre (Murdoch, WA). They were housed on a 12-h light/dark cycle, under standard conditions, with free access to food and drinking water. All animal experiments were conducted in strict accordance with the guidelines of the National Health and Medical Research Council Code of practice for the care and use of animals for scientific purposes (2004) and the Animal Welfare act of Western Australia (2002) and were approved by the Animal Ethics committee at the University of Western Australia (Perth, Australia; RA-3-100-679).

In 3 separate experiments, mice were fed a control diet consisting of the American Institute of Nutrition (AIN)-recommended AIN93G rodent growth diet (Reeves et al., 1993) or AIN93G containing 166 μ g of RNase5-10/g of feed (diet 1), 17 μ g of RNase5-50/g of feed (diet 2), or 166 μ g of RNase5-50/g feed (diet 3). All diets were prepared by Specialty Feeds Pty Ltd. (Glen Forrest, WA, Australia). Experiments were conducted on sedentary mice for 1 or 4 mo duration or mice exercising voluntarily on a wheel for 1 mo duration (28 d). Each treatment group contained 8 animals. For exercising mice, a metal mouse wheel (300 mm in diameter) was placed inside the cage and the total distance voluntarily run by each individual mouse over the experiment was recorded in kilometers using an attached bicycle pedometer (Hodgetts et al., 2006; Radley and Grounds, 2006; Radley et al., 2008). Mice were monitored for BW (g) weekly. Muscle grip strength was measured weekly using a Chatillon Digital Force Gauge (DFE-002; John Chatillon & Sons Inc., Largo, FL). In brief, the mouse was placed on the front of the triangle bar (attached to a force transducer) and pulled gently until release. Each

mouse underwent 5 consecutive grip-strength trials; the grip-strength value for each mouse was recorded as the average of the 3 best efforts and grip force (Hz; Radley-Crabb et al., 2012). At the completion of the each of the 3 individual feeding trials, all mice were anaesthetized with 1.5% (vol/vol) Rodia halothane (Merial Australia, North Ryde, NSW, Australia), N₂O, and O₂, before being euthanized by cervical dislocation. The entire musculus tibialis anterior (**TA**), musculus gastrocnemius (i.e., gastrocnemius), musculus quadriceps femoris (i.e., quadriceps), and epididymal fat pad were dissected and weighed.

Statistical Analysis

The C2C12 myogenesis data were analyzed using an ANOVA for unbalanced designs for treatment by time with a block on experimental replicate. For mouse supplementation studies, all parameters were analyzed by ANOVA (for unbalanced designs, where appropriate) for the effects of treatment. For treatments that were statistically significant, a 2-tailed Student's *t*-test was performed post-hoc to determine the effect of treatment diet versus the nonsupplemented control diet. All ANOVA statistical analyses were performed in GenStat software (13th ed.; VSN International Ltd., Hemel Hempstead, UK) and all Student's *t*-tests were performed in Microsoft Excel software (version 2003; Microsoft Corp., Redmond, WA).

RESULTS

Preparations of RNase5 from Milk Whey

Enrichment of RNase5 protein from whole milk was observed by SDS-PAGE (Figure 1). Densitometric analysis demonstrated that the RNase5 content increased from approximately 10% of the milk whey protein content in the RNase5-10 preparation to approximately 50% of the milk whey protein content in the RNase5-50 preparation to >95% in the RNase5-95 preparation due to the removal of the major contaminating proteins.

Protein Composition of RNase5-10, -50, and -95

The protein composition of RNase5-10, RNase5-50, and RNase5-95 is shown in Table 1. The RNase5-10 preparation contained numerous proteins that were consistent with proteins known to be in milk whey, the most predominant protein species being lactoferrin and lactoperoxidase. The amount of RNase5 protein present in the RNase5-10 protein preparation varied between purification batches and ranged from 8 to 11% (Table 1; Figure 1).

Figure 1. Sodium dodecyl sulfate PAGE of several different milk-enriched preparations of bovine RNase5. Lane M contains BenchMark protein ladder (Invitrogen Corp., Grand Island, NY).

Determination of Milk RNase5 Biological Activity

Ribonuclease activity and in vitro Matrigel angiogenesis assays were performed to confirm whether the RNase5 derived from milk whey was biologically active. It was found that the RNase activity increased as the concentration of RNase increased with 11.8 relative fluorescence units (RFU)/ng in RNase5-10 to 148 RFU/ng in RNase5-50 and 446 RFU/ng for the highly purified RNase5-95 extracts. The increase in RNase activity in the milk whey extracts was consistent with the increases in RNase5 concentration observed in SDS-PAGE (Figure 1).

Figure 2. Endothelial tube formation assays were performed in cultured human umbilical vein endothelial cells (HUVEC) untreated (A) or treated with 100 ng/mL of RNase5 with 10% protein content (RNase5-10; B), 5 ng/mL of vascular endothelial growth factor (VEGF) 165 (C; positive control), or 100 ng/mL of recombinant human RNase5 (D; positive control). Endothelial tube formation was visualized under an inverted light microscope at 4× magnification. Color version available in the online PDF.

Table 1. Protein composition (%) of 3 different protein preparations of RNase5: RNase5 with 10% protein content (RNase5-10), RNase5 with 50% protein content (RNase5-50), and RNase5 with 95% protein content (RNase5-95)

Protein	Whey protein preparation composition (%)			Molecular weight (kDa)	pI ¹	Method of detection	References
	RNase5-10	RNase5-50	RNase5-95				
Lactoperoxidase	40-50	0-10	Negligible	78	9.6	HPLC method developed by A. Brown	Kussendrager and van Hooijdonk (2000); Pouliot and Gauthier (2006)
Lactoferrin	20-30	20-30	Negligible	~78-80.0	8.7	HPLC method developed by A. Brown	(D'Auria et al. (2005); Pouliot and Gauthier (2006)
Immunoglobulins (IgA and IgG)	8-16	Negligible	Negligible	52-56 ²	5.5-6.5 ³	IgA: Bovine ELISA Kit (Bethyl Laboratories Inc., Montgomery, TX)	Abernethy et al. (2010)
				50 and 25 ²	5.0-9.0 ³	IgG: protein G affinity chromatography	
Other whey proteins	10-15	5-10	5	ND ⁴	ND	HPLC method developed by A. Brown; in vitro Matrigel angiogenesis assay; RNase activity assay (Invitrogen Corp., Grand Island, NY).	Fett et al. (1985); Bond and Strydom (1989); Ponce (2001)
	RNase5	8-11	40-50	95	14.5		

¹Isoelectric point.
²Molecular weight determined under reducing conditions.
³IgA and IgG can be posttranslationally glycosylated and thus have a wide range for their pI.
⁴ND = not detected.

Figure 3. Addition of milk whey-enriched RNase5 preparations to differentiating C2C12 myoblasts resulted in increased creatine kinase (CK) activity, an indicator of myotube formation (ANOVA: $P < 0.05$ for all treatment groups compared with control group). The C2C12 myoblasts were differentiated in 2% horse serum (control) and 2% horse serum with one of the following treatments: 10 $\mu\text{g/mL}$ of RNase5 with 50% protein content (RNase5-50), 10 $\mu\text{g/mL}$ of RNase5 with 95% protein content (RNase5-95), 1 $\mu\text{g/mL}$ of recombinant human RNase5 (rhRNase5), or 200 ng/mL of IGF-1 ($n = 3$ –5 per time point). Error bars represent the standard error of the mean of 5 different cell passages, $n = 3$ to 5 per cell passage.

The *in vitro* Matrigel angiogenesis assay replicates the early stages of angiogenesis where endothelial cells migrate, align, and fuse into tubular networks. Representative bright field images are shown in Figure 2. Tube formation was observed when HUVEC were incubated with RNase5-10 (100 ng/mL) along with the positive controls [VEGF 165 (5 ng/mL) and rhRNase5 (100 ng/mL)], whereas no tubes were formed in the untreated controls. These findings demonstrate that RNase5-10 has angiogenic properties, likely due to the presence of biologically active RNase5.

RNase5 Stimulates Myogenesis *In Vitro*

The ability of RNase5 to mediate myogenesis *in vitro* was examined using differentiating C2C12 myoblasts.

Myogenesis was evaluated by measuring CK activity. RNase5-50 and RNase5-95 increased CK activity in differentiating C2C12 myoblasts compared with untreated differentiating C2C12 myoblasts (Figure 3). Differentiating C2C12 myoblasts were also treated with 2 different positive controls - rhRNase5 (1 $\mu\text{g/mL}$) and IGF-1 (200 ng/mL). Both positive controls also showed increases in CK activity when compared with untreated differentiating C2C12 myoblasts. These results demonstrate enhanced myogenesis *in vitro* and indicate a novel activity for RNase5 in skeletal muscle development. Myosin heavy chain staining of differentiating C2C12 myoblasts revealed that cells treated with milk whey preparations containing RNase5 (i.e., RNase5-50) had visibly more myosin heavy chain content than untreated control C2C12 cells (Figure 4). Studies exam-

Figure 4. Treatment of C2C12 myoblasts with RNase5-enriched milk whey isolates promotes myotube formation. The C2C12 myoblasts were treated with 2% horse serum (A) and 2% horse serum treated with either 10 $\mu\text{g/mL}$ of RNase5 with 50% protein content (RNase5-50; B) or 10 $\mu\text{g/mL}$ of RNase5 with 95% protein content (RNase5-95; C). Myoblasts were allowed to differentiate into myotubes for 4 d. After 4 d of differentiation, the C2C12 cells were fixed with 4% paraformaldehyde and the presence of myosin heavy chain was determined by immunohistochemistry and imaged using an Olympus confocal microscope (Olympus America Inc., Center Valley, PA). Color version available in the online PDF.

ining cell proliferation demonstrated that all RNase5 preparations had minor, but significant effects on C2C12 cell proliferation as determined by water-soluble tetrazolium salt (WST)-1 assay (data not shown).

Effect of RNase5-10 and RNase5-50 on Muscle Weight Gain in C57Bl/10 Mice

The influence of RNase5 on adult mouse muscle growth was analyzed within 3 separate experiments. Mice were fed 1 of 4 diets: the control mouse chow (AIN93G) or AIN93G supplemented with (1) 166 μ g of RNase5-10/g feed (diet 1), (2) 17 μ g of RNase5-50/g feed (diet 2), or (3) 166 μ g of RNase5-50/g feed (diet 3). In the first and second experiments, sedentary mice were treated for a period of 1 mo or 4 mo, respectively, and in the third experiment, mice were allowed to exercise voluntarily for a period of 1 mo.

The diets did not significantly affect the quadriceps muscle weights of sedentary mice in the 1-mo feeding cohort ($P = 0.18$; Figure 5A). However, after 4 mo of supplementation, the quadriceps muscle weights of sedentary mice fed diet 2 were significantly greater than those mice fed the control diet ($P < 0.05$; Figure 5B).

When mice were allowed to exercise voluntarily for 1 mo, both diet 1 and diet 2 significantly increased quadriceps muscle weights by over 55% ($P \leq 0.001$) and 35% ($P = 0.044$), respectively, compared with the control diet (Figure 5C). Unlike the control mice cohort, mice on diet 1 did not gain weight in the study period and, as a result, weighed significantly less than the control mice cohort (Supplementary Figure S1; <http://dx.doi.org/10.3168/jds.2014-7901>). As these mice did not have lower feed intakes or lower levels of epididymal fat, it is unlikely that this reduction in BW was due to nutritional factors. Mice fed diet 1 also exercised approximately 20% more than the control treatment group (control: 6.4 km vs. diet 1: 7.8 km average daily distance run; $P = 0.057$), whereas no differences were observed with diets 2 and 3 (Figure 6). Additionally, functional increases in grip strength of approximately 20% were measured in all experimental diets (Figure 7).

Other muscle weights (i.e., TA and gastrocnemius) were not influenced by any experimental diet and, likewise, abdominal fat was largely unaffected by diet, with an exception for a trend for increased fat in sedentary mice supplemented with diet 3 for 1 mo ($P = 0.094$). Supplementation with RNase5-10 (diet 1) did not influence any of the tissue weights.

DISCUSSION

Milk is an important source of nutrients, immune factors, and bioactive proteins for the developing neonate.

Figure 5. Increase in musculus quadriceps femoris weight (mg) from mice supplemented with milk whey-enriched RNase5. The mice were fed for a period of 1 mo (A) or 4 mo (B) without exercise or 1 mo (C) with voluntary exercise. The 4 diets used in this study were the American Institute of Nutrition (AIN)-recommended AIN93G rodent diet (control diet) or AIN93G supplemented with RNase5 with 10% protein content (RNase5-10) at 166 μ g/g of feed (diet 1), RNase5 with 50% protein content (RNase5-50) at 17 μ g/g of feed (diet 2), or RNase5-50 at 166 μ g/g of feed (diet 3). * represents statistically significant differences in mice fed the experimental diets compared with mice fed the control diet (Student's *t*-test; $P < 0.05$; $n = 8$). Error bars represent the standard error of the mean for each diet.

Figure 6. A tendency existed for mice fed diet 1 to run further over the course of the experiment compared with control mice (Student's *t*-test: $P = 0.057$). Mice fed diet 2 and diet 3 containing various amounts of RNase5 with 50% protein content (RNase5-50) were not significantly different from control mice ($P > 0.10$). Error bars represent the standard error of the mean for each diet.

However, the role of the nonnutritive bioactive protein component is the least understood. The mechanism by which milk bioactive proteins mediate their effects is not clear, but nonnutritive factors in milk stimulate protein synthesis in the vital organs, skeletal muscle, and myofibrillar proteins (Burrin et al., 1997; Fiorotto et al., 2000). Whey protein is a nutrient source derived from milk and contains a group of basic proteins, which, when extracted, are commonly referred to as the growth factor extract because of the presence of higher concentrations of growth factors and their ability to

promote muscle growth and repair in vivo (Burke et al., 2001; Anderson and Behm, 2005; Dyer et al., 2008). However, mechanical stimulus in the form of exercise or physical activity is required for muscle growth to occur.

The initial objective of this study was to purify and identify biologically active proteins derived from basic milk whey protein and characterize their effect on muscle growth. Ribonuclease 5 was identified and purified to produce 3 RNase5 preparations designated RNase5-10, -50, and -95 signify the purity of the RNase5 (10, 50, and 95%, respectively). The HUVEC assays confirmed

Figure 7. Exercised mice fed RNase5 with 10% protein content (RNase5-10; i.e., diet 1) and RNase5 with 50% protein content (RNase5-50) diets (i.e., diets 2 and 3) in particular had increased grip strength compared with control mice. The statistically significant effects were observed for both RNase5-50 diets (ANOVA: $P < 0.001$) and the RNase5-10 diet (ANOVA: $P < 0.05$). Error bars represent the standard error of the mean for each diet at wk 1, 2, 3, and 4.

that the RNase5 preparations were biologically active and contained angiogenic activity. The biologically active RNase5 purified from milk was also shown to induce the myogenic differentiation of cultured C2C12 myoblasts, thus identifying novel growth factor response for RNase5 on cultured muscle cells.

The intracellular actions of RNase5 on skeletal muscle are subject to ongoing experimentation; however, in other cell types, RNase5 is important for protein synthesis, directly stimulating ribosomal RNA synthesis in the nucleolus (Moroianu and Riordan, 1994; Belford et al., 1995). In endothelial and epithelial cell growth, RNase5 is required for the activity of growth factors such as FGF and endothelial growth factor (Kishimoto et al., 2005; Gao and Xu, 2008). In endothelial cells, RNase5 binds to cell surface actin (Hu et al., 1993) and is endocytosed by a process involving transient phosphorylation of extracellular signal-related signal kinase 1/2 (ERK1/2; Liu et al., 2001). Ribonuclease 5 is translocated to the nucleolus (Moroianu and Riordan, 1994; Hu, 1998) and upregulates transcription (Xu et al., 2002; Tsuji et al., 2005; Ibaragi et al., 2009) and endothelial nitric oxide synthase activity via phosphatidylinositol 3-kinase and protein kinase B (Akt) signaling (Trouillon et al., 2010).

To ascertain whether milk-derived RNase5 preparations were orally active and had the ability to increase muscle mass in vivo, adult mice were fed known amounts of milk whey protein preparations enriched for RNase5. The principal outcome of these experiments was that all 3 experimental diets increased grip strength in exercised mice, indicating improved neuromuscular function. Once mice reach their maximum size in adulthood the bones have fused and muscles no longer increase in length, but in response to hypertrophic stimuli such as exercise or growth factors, muscles can increase in width. Therefore, we would predict that the exercised cohort of mice would be likely to respond and benefit from the diet supplements and record induced muscle growth. Indeed we observed that mice on diets 1 and 2 had heavier quadriceps muscles than mice fed the control for 1 mo.

Lacking a hypertrophic stimulus, sedentary adult mice were not expected to exhibit muscle hypertrophy. Therefore, it was a surprising result that sedentary adult mice fed diet 2 for 4 mo recorded increases in quadriceps muscle weights. We cannot exclude the possibility that the contaminating whey proteins present in the RNase5 preparations contributed to this effect. It is possible that degradation of the whey protein by the digestive system would produce hydrolyzed whey, which has been shown to have a positive effect on muscle strength and weight gain (Cribb et al., 2006). However, the current study required physical activity

or exercise for this to occur. Thus, it is difficult to conclude whether the response was specifically due to intact RNase5 directly stimulating muscle growth as observed in vitro or due to whey protein being present in the RNase5 preparations.

In this study, we did not observe a universal increase in muscle weight, as one might expect. Nonetheless, this observation was similar to other studies within the literature that report that all muscles do not respond to a stimulus in the same manner. For example, previous studies have shown that quadriceps but not TA and gastrocnemius increased muscle weight after voluntary exercise (Leiter et al., 2012). Conversely, we did observe positive effects of the RNase5 diets on grip strength of the forelimb muscles, indicating that the RNase5 diets influenced more than quadriceps weight.

In summary, these data show that RNase5 can be purified from bovine milk whey in significant concentrations while preserving biological activity. This paper also describes the novel role of RNase5 in muscle development by increasing muscle fiber growth. In adult mice, milk-derived RNase5 preparations increased neuromuscular function and sedentary and exercise-induced muscle growth.

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