DISSERTATION

TREATMENT WITH RAPAMYCIN OR RAPAMYCIN IN COMBINATION WITH METFORMIN CONTRIBUTES TO MECHANISMS OF MITOCHONDRIAL PROTEOSTASIS IN VIVO AND IN VITRO

Submitted by

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ABSTRACT

TREATMENT WITH RAPAMYCIN OR RAPAMYCIN IN COMBINATION WITH METFORMIN CONTRIBUTES TO MECHANISMS OF MITOCHONDRIAL PROTEOSTASIS IN VIVO AND IN VITRO

This dissertation describes three sets of experiments with an overall objective of understanding how lifespan-extending treatments influence mechanisms of mitochondrial protein homeostasis (proteostasis). The specific aims of the three experiments were to 1) determine how the mTORC1 inhibitor rapamycin (Rap), or the anti-diabetes medication metformin in combination with rapamycin (Met+Rap) influence mitochondrial protein synthesis in young mice, and to determine if there are sex-specific differences in protein synthesis following treatment with Rap or Met+Rap; 2) examine the influence of Met+Rap treatment on protein synthesis in the heart, liver, and skeletal muscle of older mice and to determine if there are sex-specific differences in protein synthesis following Met+Rap treatment in old mice; 3) and, finally, to investigate the regulation of how protein turnover contributes to maintaining proteostasis during Rap and Met+Rap treatment, and the contribution of autophagic and mitophagic flux to protein turnover in cultured skeletal myotubes. In the first experiment, both Rap and Met+Rap treatments lowered mitochondrial protein synthesis in male mice compared to control in skeletal muscle. However, in female mice, only Met+Rap treatment significantly lowered skeletal muscle mitochondrial protein synthesis compared to control. Additionally, both Rap and Met+Rap treatments significantly elevated skeletal muscle mitochondrial protein synthesis in

female animals compared to males. However, in the heart and liver tissue, there were no differences in mitochondrial protein synthesis between treatments or sexes. In the second experiment, Met+Rap treatment lowered protein synthesis in all three tissues, but in a fraction-specific manner. independent of sex-differences in old mice. For the third experiment, we measured protein synthesis and protein breakdown in cultured skeletal myotubes treated with Rap and Met+Rap. Rap treatment significantly increased mitochondrial protein:DNA compared to control, while Met+Rap did not. We demonstrate that autophagic flux is a large (29%) contributing process to degradation of mitochondrial proteins. Additionally, mitochondrial fission is not essential for mitochondrial protein degradation. The data from these experiments demonstrate that despite sexually dimorphic effects on lifespan, Rap and Met+Rap treatments both enhance the contribution of protein synthesis to maintaining proteostasis in vivo. Further, we demonstrate that both Rap and Met+Rap treatment increased protein:DNA in cultured skeletal myotubes. In summary, these data demonstrate that Rap and Met+Rap treatments increase proteostatic mechanisms, and further research is required to improve the understanding of how Met+Rap treatment influences lifespan.

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TABLE OF CONTENTS

CHAPTER 1 – INTRODUCTION AND EXPERIMENTAL AIMS

Aging is the time-related decline in physiological function that is, in part, related to the accumulation of intracellular damage over the course of the lifespan. Reduced rates of protein synthesis and breakdown, or protein turnover, directly contribute to the age-related loss of cellular function (157, 158). Interventions that increase protein turnover during aging may improve, or maintain cellular function by attenuating the accumulation of protein damage (136, 164). Furthermore, increased mitochondrial protein turnover can maintain cell function through reduced damage accumulation and potentially energy production potential (25, 68, 106). Maintaining cellular function over time should reduce the age-related onset of chronic diseases such as cardiovascular disease and diabetes, thereby increasing the so-called 'healthspan' (142, 179).

Protein homeostasis, or proteostasis is a complex process involving protein turnover as well as a diverse network of regulatory proteins (66, 76, 87, 115, 159, 165). Protein turnover declines during the aging process, directly contributing to the age-related accumulation of damaged proteins and therefore loss of proteostasis (59, 76, 84, 115). Our laboratory measures mechanisms that contribute to proteostasis through assessing protein synthesis and DNA synthesis rates to identify if changes in protein synthesis rates are due to cellular proliferation, or proteome remodeling in response to treatments. During cellular proliferation, both the proteome and the genome must be duplicated to be split into the daughter cells (51). In the case of proteostatic processes, an increase in the protein:DNA ratio will be observed as protein synthesis rates and cellular proliferation rates are coupled (106). Specifically, when protein synthesis is maintained (relative to

control) in response to a treatment that reduces cellular proliferation, any “excess” protein synthesis can be assumed to be for proteostatic processes (51, 106).

Slowed-aging models, specifically chronically calorie restricted (CR) and rapamycin (Rap) treated animals, exhibit reduced signaling through the mTOR complex 1 (mTORC1) signaling pathway (17, 38). Rapamycin is a macrolide antibiotic that has been shown to increase lifespan as well as mitochondrial proteostatic mechanisms (38, 55). Rapamycin treatment started in young or old mice extends maximal lifespan (55, 113). Short term treatment with rapamycin inhibits mTORC1, thereby reducing cap-dependent protein translation as well as cell cycle progression (30, 169). Despite inhibition of cap-dependent translation, some transcripts have been shown to be selectively translated, including nuclear-encoded mitochondrial genes (152, 201). We have previously demonstrated that slowed-aging models, including Rap treated mice, exhibit increased mitochondrial proteostatic mechanisms (38, 106). Thus, Rap treatment may exert its lifespan-extending effects by increasing mitochondrial proteostasis.

Despite the lifespan-extension associated with long-term Rap treatment, prolonged Rap treatment has a negative consequence on glucose handling (90). The use of the anti-hyperglycemia drug metformin in combination with rapamycin (Met+Rap) can combat the negative glucose handling effects associated with chronic rapamycin treatment. Increased AMPK signaling accompanied by reduced mTORC1 signaling extends median and maximal lifespan in multiple organisms (69, 104). Metformin activates AMPK (160), while rapamycin inhibits mTORC1. Accordingly, Met+Rap increased median and maximal lifespan to a greater extent than Rap alone (38, 55, 113,

161). It is currently unknown if there are divergent effects of Rap or Met+Rap treatment on mitochondrial proteostatic mechanisms.

In each investigation of chronic rapamycin treatment in vivo, female mice have lived longer than their male counterparts (55, 112, 113, 189). While the specific mechanisms are still unclear, one investigation revealed that Rap treatment leads to a gene expression pattern similar to long-term calorie restriction in female mice, but not male mice (191). Additionally, whole body S6 kinase 1 ($p70^{S6K1}$) knockout animals exhibit a gene expression profile similar to calorie restriction in both sexes, but only the female animals exhibited an increase in lifespan (144). In humans, differences in protein metabolism are reportedly only detected during changes in hormonal status, such as menopause (101). To date, limited data are available regarding potential sex differences in proteostatic mechanisms during the aging process. Furthermore, it is unknown if there is a sexually dimorphic response of mitochondrial proteostatic mechanisms to Met+Rap treatment in vivo.

In summary, it is currently unclear if there are different effects of Rap and Met+Rap treatments on mitochondrial proteostatic mechanisms in vivo, if these treatments have a sexually dimorphic effect on proteostatic mechanisms, and the mechanisms underlying how the treatments influence mitochondrial protein turnover.

Specific Aim for Experiment 1: Examine the impact of Rap or Met+Rap treatments on protein synthesis in the heart, liver, and skeletal muscle of young male and female mice, and to determine if there are sex-specific changes in protein synthesis.

Specific Aim for Experiment 2: Examine the impact of Met+Rap treatment on protein synthesis in the heart, liver, and skeletal muscle of old male and female mice, and to determine if there are sex-specific changes in protein synthesis.

Specific Aim for Experiment 3: Examine the impact of Rap or Met+Rap treatments on protein turnover and cellular proliferation in cultured skeletal myotubes, and investigate the mechanisms that contribute to mitochondrial protein turnover and proteostasis.

Overall Hypotheses: Rap and Met+Rap treatments will enhance mitochondrial protein synthesis in young and old mice compared to controls. Rap will have a greater effect on mitochondrial protein synthesis in females compared to males, while Met+Rap will enhance protein synthesis to the same extent in both sexes. Both Rap as well as Met+Rap treatments will enhance protein synthesis in the young animals compared to control. Met+Rap treatment will enhance protein synthesis in old animals compared to control. We also hypothesize that Rap and Met+Rap treatments will improve mitochondrial protein turnover, in vitro, by increasing both mitochondrial protein synthesis and degradation.

CHAPTER 2 – THE INFLUENCE OF RAPAMYCIN AND METFORMIN+RAPAMYCIN ON PROTEIN SYNTHESIS IS SEXUALLY DIMORPHIC AND TISSUE SPECIFIC IN YOUNG GENETICALLY HETEROGENEOUS MICE

Maintenance of protein homeostasis (proteostasis) is essential for proper cellular function. Proteostasis refers to protein localization, concentration, folding, and turnover. Because enzymatic capacity for protein repair is limited (158), protein synthesis and degradation (i.e., turnover) are essential for proteome maintenance. However, both protein synthesis (133), and breakdown (126) are energetically costly processes. As such, protein turnover requires efficient energy production to maintain cellular function (116). Data from our laboratory demonstrate that in murine models of slowed aging, mitochondrial protein synthesis as a mechanism of maintaining proteostasis is increased compared to control animals (36-38, 51, 106, 108). Increased mitochondrial proteostasis contributes to maintained mitochondrial function over time by preventing damage accumulation (83).

Methods for dynamic assessments of proteostasis are limited by the complexity of the proteostasis network. However, measuring changes in rates of protein synthesis relative to the rate of cellular proliferation may provide insight into one cellular proteostatic mechanisms. During cellular proliferation, both the proteome and the genome must be duplicated to be split into the daughter cells (51). Thus, measured protein and DNA synthesis rates will both increase, resulting in no change in the ratio of protein:DNA synthesis. Additionally, if proliferation decreases, protein synthesis may be reduced, but newly synthesized proteins will be incorporated into the existing proteome. On the other hand, when an increase in the protein:DNA ratio is observed (e.g., following rapamycin

treatment) despite no difference in protein synthesis between treated and control animals, the apparent “excess” protein synthesis revealed by the ratio is dedicated to proteostatic mechanisms (51, 106).

Rapamycin (Rap) is a macrolide antibiotic that has been shown to increase lifespan as well as mitochondrial proteostatic mechanisms (38, 55). Short term treatment with Rap inhibits mTOR complex 1 (mTORC1) thereby reducing cap-dependent protein translation as well as cell cycle progression (30, 169). Despite inhibition of cap-dependent translation, some transcripts have been shown to be selectively translated, including nuclear-encoded mitochondrial genes (152, 201). Selective translation of certain transcripts is one mechanism through which cells respond and adapt to stressors. Long-term Rap treatment has an inhibitory effect of mTORC2 and this inhibition has a negative consequence on glucose handling (90). Thus, long-term treatment with Rap may not be an ideal intervention for human translation.

The use of the anti-hyperglycemia drug metformin (Met) in combination with Rap (Met+Rap) can combat the negative glucose handling effects associated with chronic Rap treatment. Metformin alone has been shown to inhibit mTORC1 (71), and activate 5’ AMP-activated protein kinase (AMPK) (160). Together, inhibition of mTORC1 and activation of AMPK with Met appear to mimic the intracellular signaling associated with calorie restriction (102, 122, 151). Additionally, Met treatment has been demonstrated to have numerous positive health benefits, resulting in a call for the implementation of Met as an anti-aging intervention (15). However, in a longevity trial at the National Institute on Aging’s Interventions Testing Program (ITP), Met had minimal influence on lifespan

extension while Met+Rap increased median lifespan of both males and females 23% (161).

It is currently unclear what causes the sexually dimorphic lifespan responses to chronic Rap treatment. Rapamycin treatment leads to a gene expression pattern similar to long-term calorie restriction in the liver of female mice, but not male mice (191). We have also demonstrated that twelve-weeks of rapamycin treatment reduced hepatocyte proliferation in male mice, but not female mice (38). Whole body S6 kinase 1 ($p70^{S6K1}$) knockout mice exhibit a gene expression profile similar to calorie restriction in both sexes, but only the female animals exhibited an increase in lifespan (144). In humans, sex differences in protein metabolism have been reported in both young and older individuals (101, 141). Limited data are available regarding potential sex differences in protein synthesis during the aging process, especially following Rap or Met+Rap treatments. Furthermore, it is unknown if Met+Rap treatment elicits a sexually dimorphic response of mitochondrial protein synthesis in vivo.

Investigations from the ITP do not examine the mechanisms that impart changes in longevity. To date, no investigations have examined the influence of Met+Rap on protein synthesis *in vivo*. Furthermore, it is unknown if there are divergent effects of Rap or Met+Rap treatments on protein synthesis. The purpose of this investigation was to compare the effects of control (Con), Rap, and Met+Rap treatments on protein synthesis in the heart, liver, and skeletal muscle of young male and female HET3 mice compared to control. We hypothesized, that because Met+Rap increased median and maximal lifespan, the combined Met+Rap treatment would increase protein synthesis compared

to Rap alone as well as control. We further hypothesized that Met+Rap treatment would increase protein synthesis to the same extent in both sexes.

METHODS

Animals & Experimental Design

All procedures and conditions for the housing and care of the animals was approved by the Animal Care and Use Committee at the University of Michigan. Ninety, 4-month UM-HET3 mice were housed 3-per cage on a 12h light-dark cycle. The UM-HET3 model are genetically heterogeneous mice that were crossbred from two different F1 strains: (BALB/cByJ x C57BL/6J) F1 females (JAX stock 10009) and (C3H/HeJ x DBA/2J) F1 males (JAX stock 10004), as previously described (35). Thirty mice were randomized into control diet (Purina 5LG6, CON), Rapamycin encapsulated into control diet (14 parts per million (ppm)) or Metformin (Met)+Rap encapsulated into control diet (1000 ppm Met and 14 ppm Rap), as in the Interventions Testing Program (55, 161). Rap and Met+Rap groups were treated for 8 weeks (56 days) (Figure 2.1). To measure the increase in protein synthesis over time, and to reduce the bias of highly abundant, rapidly synthesized proteins (110), mice received up to 32 days of isotope labeling. Briefly, after twenty-four days of treatment, the first subset of mice received an intraperitoneal injection of 99% deuterium oxide (D_2O) and subsequently had access to drinking water enriched with 8% D_2O. Those mice continued treatment for another 32 days (32d) at which point they were sacrificed (Figure 2.1). At 41 days of treatment, a second subset of mice received a 99% D_2O injection with subsequent access to drinking water enriched with 8% D_2O, and those mice were sacrificed after fifteen days of labeling (15d). At 48 days of treatment, a third subset of mice received a 99% D_2O injection with subsequent access

to drinking water enriched with 8% D_2O, and those mice were sacrificed after eight days of labeling (8d). At 52 days of treatment, a fourth subset of mice received a 99% D_2O injection with subsequent access to drinking water enriched with 8% D_2O, and those mice were sacrificed after 4 days of labeling (4d). At 55 days of treatment, a final subset of mice received a 99% D_2O injection with subsequent access to drinking water enriched with 8% D_2O, and those mice were sacrificed after one day of labeling (1d). All mice received 8 full weeks of treatment. We aimed to have all animals reach a total body water enrichment of ~5%, as previously described (36, 38, 127). Animals were euthanized using carbon dioxide following a 12h fast. At each time point, 6 mice (3 male and 3 female) were sacrificed from each treatment (CON, Rap, Met+Rap) group. We collected the liver, heart, and gastrocnemius from each animal to assess isotope incorporation into proteins and DNA, and plasma and bone marrow to assess precursor enrichment (described in detail below).

Subcellular Fractionation of Tissues

Excised skeletal muscle, heart, and liver samples were snap-frozen in liquid nitrogen and subsequently powdered in liquid nitrogen. Powdered samples were homogenized using zirconium oxide beads in a bullet blender with mitochondrial isolation buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH=7.5) as previously described (38, 109, 111). Homogenized samples were centrifuged at 800 x *g* for 10 minutes at 4°C. The pellet from this spin was washed and resuspended as the mixed-protein fraction (MIX). The supernatant was transferred to a new tube and centrifuged at 10,000 x *g* for 30 minutes at 4°C. The pellet from the 10,000 x *g* spin was washed and resuspended as the mitochondrial-enriched protein fraction

(MITO). The supernatant from the 10,000 x *g* spin was split into two volumes with one volume being precipitated in 4% sulfosalicylic acid for one hour on ice and one volume being frozen for use in western blotting applications. Following the precipitation, the solution was centrifuged at 16,000 x *g* for 10 minutes at 4°C. The resultant pellet was washed and resuspended as the cytoplasmic-enriched fraction (CYTO). All pellets were solubilized in 1M NaOH at 50°C, and hydrolyzed in 6M HCl for 24h at 120°C.

Preparation of Analytes for GC-MS Analysis

After each protein fraction was hydrolyzed, cation exchange chromatography was used to isolate positively charged amino acids. Amino acids were eluted from the chromatography resin using 4M NH_4OH. Purified amino acids were the. dried on a vacuum centrifuge (SpeedVac Savant 110, Thermo, Rockford, IL). Dried amino acid pellets were resuspended in 1mL of molecular biology grade water, and 500µl of the resuspended amino acids were subjected to our standard derivation procedure (36, 38, 110). Specifically, acetonitrile, 1M potassium phosphate, and pentafluorobenzyl bromide were added to the amino acid solution and incubated at 100°C for one hour. Ethyl acetate was used to extract the pentafluorobenzyl derivatives which were dried under N_2. Dried derivatives were resuspended in 700µl of ethyl acetate and analyzed using a 7890A gas chromatograph coupled to 5975C mass spectrometer with a DB-5MS GC column (30 m x 0.25 mm x 0.25 µm; all from Agilent), as previously described in detail by our laboratory (36, 38, 109).

Deuterium enrichment of the total body water pool was used to estimate the alanine precursor enrichment based upon mass isotopomer distribution analysis (MIDA (38, 109, 111). Body water enrichment was measured in 125µl of evaporated plasma

samples from each animal as previously described (38). Briefly, plasma samples were placed onto inverted plastic microcentrifuge caps with a rubber o-ring. Tubes were then sealed and placed onto a heat block at 80°C overnight. Evaporated water from the plasma was captured in the top of the tubes. The evaporated water then underwent proton exchange with 10M NaOH and acetone overnight. Finally, proton exchanged samples were extracted in hexanes into anhydrous sodium sulfate before being transferred into chromatography vials for analysis on a 7890A gas chromatograph coupled to 5975C mass spectrometer using a DB-17 Column (Agilent).

Protein Synthesis Calculations

Protein synthesis was calculated based on the precursor-product relationship. Briefly, protein synthesis was calculated from the precursor enrichment that was determined from the deuterium enrichment in the body water pool and then adjusted using Mass Isotopomer Distribution Analysis (56, 125). The fraction of new proteins for each time point (1, 4, 8, 15, or 32 days) was plotted as a function of time, as described in detail below.

Analysis and Statistics

Graphpad Prism 7 was used to complete statistical analyses for this investigation. The fraction of new proteins plotted as a function of time create a non-linear plot. To assess the increase in the fraction of new proteins over time, we applied a one-phase association non-linear regression model to the data. The slope of the lines represent the protein synthesis rate constant (k; $1 \cdot day^{-1}$). We compared linear and non-linear analysis to determine which model had the best fit for each tissue and each fraction. For the heart and liver, the data were fit with the one-phase association non-linear regression model

using data from all 5 time points (1d, 4d, 8d, 15d, and 32d). However, for the skeletal muscle, the 32 day samples from female mice were accidentally thawed overnight and could not be used for stable isotope analysis. Therefore, skeletal muscle k values were calculated by a linear regression with the data from the first four time points (1d, 4d, 8d, and 15d) for each group.

To examine the difference in protein synthesis (k values), two-way analysis of variance (ANOVA) tests were used to identify main effects of sex and treatments. Tukey post-hoc tests were used to identify differences between specific groups, where appropriate. For the liver, additional two-way ANOVAs were used to compare protein synthesis (fraction new) at each time point for the 1d, 4d, and 8d animals, as the protein pool in all three fractions was fully turned over by day 15, and there were no significant differences in the k values between treatments or sex. Significance was accepted as $P<0.05$ for these experiments. All data are presented as (Mean±SEM).

RESULTS

Skeletal Muscle Protein Synthesis

There was a significant effect of sex ($P<0.001$), and of treatment ($P<0.001$) on MITO protein synthesis rate (Figure 2.2). Post-hoc analysis revealed that Rap and Met+Rap treatments significantly lowered ($P<0.01$) MITO protein synthesis compared to control in the male mice (Figure 2.2). However, Met+Rap treatment significantly lowered ($P<0.01$) MITO protein synthesis compared to control in the female mice (Figure 2.2). Additionally, following Rap ($P<0.05$) and Met+Rap ($P<0.001$) treatments, MITO protein synthesis values were significantly higher in females compared to male mice. There was a main effect of treatment in the CYTO fraction ($P<0.01$; Figure 2.2). CYTO protein

synthesis values were significantly higher in the female Rap group compared to female Met+Rap ($P<0.05$; Figure 2.2). Finally, there was a significant interaction ($P<0.05$), a main effect of sex ($P<0.001$), and a main effect of treatment in the MIX fraction ($P<0.001$; Figure 2.2). MIX protein synthesis was significantly higher ($P<0.01$) in female control animals compared to male control animals (Figure 2.2). Met+Rap significantly lowered MIX fraction protein synthesis compared to Rap in the male animals only ($P<0.001$; Figure 2.2). Additionally, Met+Rap significantly lowered ($P<0.05$) MIX fraction protein synthesis in males compared to the females (Figure 2.2).

Heart Protein Synthesis

There was a significant interaction of sex and treatment ($P<0.05$) for MITO protein synthesis in the cardiac tissue (Figure 2.3). Post-hoc analyses revealed that Met+Rap significantly lowered ($P<0.05$) MITO protein synthesis compared to control in female mice only (Figure 2.3). There were no significant differences in CYTO or MIX protein synthesis in response to any treatment in male or female mice (Figure 2.3).

Liver Protein Synthesis

For all three fractions of liver protein synthesis, the protein pool was fully turned over by day 15. There were no significant differences in k for any fraction. At 1d, 4d, and 8d, there were no significant differences in MITO or MIX protein synthesis between treatments, or sexes (Figure 2.4 and 2.5).

Rap significantly increased ($P<0.05$) liver CYTO protein synthesis (fraction new) at 1d in males compared to control (Figure 2.6). Additionally, 1d CYTO protein synthesis was significantly higher in Rap treated males compared to Rap treated females ($P<0.05$;

Figure 2.7 and 2.8). There were no significant differences in CYTO protein synthesis between treatment or sex at 4d or 8d (Figure 2.9).

DISCUSSION

Rapamycin and Metformin+Rapamycin treatments are known to inhibit mTORC1 signaling, a primary regulator of numerous cellular processes, including protein translation and cellular proliferation (14, 140, 151). Protein translation is energetically costly, but is also a key contributing process to proteostasis (10, 133). Previous data from our laboratory revealed that 12 weeks of Rap treatment increased the contribution of protein synthesis to maintaining the proteome (i.e., protein:DNA synthesis ratio) (38). To date, Met+Rap treatment has been described to extend mouse lifespan (161), and delay the onset of certain cancers (26, 150, 193). This is the first investigation to examine and compare the effects of 8 weeks of Rap or Met+Rap treatment on protein synthesis in young, genetically heterogeneous mice. We found that Rap and Met+Rap treatments significantly lowered mitochondrial protein synthesis in male and female skeletal muscle compared to Con animals. Additionally, we found that Rap treatment, and contrary to our hypothesis, Met+Rap treatment exhibit tissue-specific and sexually dimorphic effects on protein synthesis in young, genetically heterogeneous mice. These are the first data to identify tissue- and sex- specific effect of the lifespan-extending treatment Met+Rap, while contributing to the understanding of how Rap treatment influences protein synthesis in multiple tissues.

Rapamycin and Metformin+Rapamycin Lower Mitochondrial Protein Synthesis in Skeletal Muscle

Proteostasis requires a coordinated network of processes to maintain a functional proteome. Protein turnover is the balance between protein synthesis and protein degradation, and is a key contributor to overall proteostasis (13, 106). Additionally, enzymatic repair processes to maintain protein quality are limited (158), and thus damaged proteins must be degraded and then resynthesized to maintain proteostasis. In the current study, we measured protein synthesis following 8 weeks of Rap and Met+Rap treatments. There were no effects of Rap or Met+Rap treatment on protein synthesis in the heart or liver tissues compared to control.

Previous investigations have revealed that both acute and chronic interventions with Rap treatment alter protein synthesis in liver and heart tissue. Specifically, Rap treatment in young animals significantly lowered the content of oxidative modifications to proteins relative to control in both liver (73), and heart tissue (32). In both investigations, Rap treatment increased protein half-life (i.e., decreased the protein synthesis) in heart and liver. Those two investigations utilized a dynamic assessment of individual protein half-lives (32, 74), while we measured protein synthesis in more bulk fractions. We have previously reported a mathematical model to explain the bias of highly abundant, rapidly synthesized proteins in measurements of bulk protein synthesis (110). Furthermore, Rap treatment increased ribosome content on transcripts, suggesting more efficient translation without changes in transcription (32, 74). Thus, although in the current investigation Rap and Met+Rap treatment did not influence protein synthesis in the heart or liver of young, genetically heterogeneous mice, the synthesis of specific proteins may reveal different findings.

In the skeletal muscle, we found that Rap and Met+Rap treatments lowered mitochondrial protein synthesis. Our findings are, in part, supported by a previous investigation that reported Rap treatment decreased protein synthesis signaling in the skeletal muscle, but not the liver (162). Additionally, Rap treatment has been shown to elicit tissue specific changes in gene expression across the lifespan of UM-HET3 mice (12). Skeletal muscle is post-mitotic and has limited proliferative potential, making it more sensitive to the accumulation of damage than the liver or heart (85). Additionally, reduced protein synthesis rates contribute to proteostasis by allowing more ribosome-associated chaperones to fold proteins during translation (79). Furthermore, decreased protein synthesis rates reduce the activation of the unfolded protein response, contributing to increased protein quality and therefore maintenance of proteostasis (58). Thus, our data suggest that Rap and Met+Rap treatment in young mice may increase proteostasis in skeletal muscle through decreased protein synthesis rates.

Sex-Specific Effects of Rap and Met+Rap Treatments on Muscle Protein Synthesis

In the current investigation, Rap and Met+Rap treatments elicited sex-specific changes in protein synthesis in the skeletal muscle (Figure 2.2). Specifically, Rap and Met+Rap treatment lowered mitochondrial protein synthesis to a greater extent in the muscle of male mice compared to female mice, and Met+Rap alone lowered mixed protein synthesis in male mice compared to female mice. We hypothesized that Met+Rap treatment may not have sex-specific effects on protein synthesis, as Met+Rap treatment extends median lifespan of male and female mice equally (161). However, Met+Rap treatment, Met treatment alone, and Rap treatment alone elicit sexually-dimorphic changes in maximal longevity (6, 113, 161). Although the effect of Met treatment on

lifespan in both sexes is limited, it exhibits sexually-dimorphic changes in maximal longevity. Specifically, in the inbred 129/Sv mouse strain, Met treatment extends female lifespan, but not male lifespan (5). However, in the UM-HET3 mice, Met treatment alone did not extend male or female maximal lifespan (161), although the authors speculate different statistical approaches may have revealed differences in maximal longevity. Furthermore, Met treatment elicited sexually dimorphic changes in cardiac metabolism, decreasing fatty acid oxidation in hearts from males, but not females (98). To date, however, no investigations have reported the effect of Met alone on protein turnover.

Rapamycin treatment also exhibits sex-dependent changes in protein synthesis and longevity. Specifically, following 12 weeks of Rap treatment in UM-HET3 mice, protein synthesis in heart tissue of female mice was lowered compared to males (38). Additionally, the sex-specific effect of Rap treatment on murine lifespan (female > male) is well documented (7, 55, 113, 120).

One potential explanation for the sex-specific effect of Rap treatment is differences in plasma Rap concentration between sexes. In male and female mice treated with the same dose of Rap, the resulting plasma Rap concentration was 16 ng/ml in female mice and 9 ng/ml in male mice (113). Although no data were collected to identify the mechanism through which circulating Rap concentration was nearly double in the female mice, it may be due to differences in drug detoxification. Specifically, Rap is detoxified by the cytochrome P450, with a greater effect in males than females (182). The higher circulating Rap concentration in female mice likely directly contributed to the difference in male and female lifespan, as lifespan extension by Rap treatment is dose dependent, with higher doses increasing lifespan more than lower doses (113). However, protein

synthesis was higher in females compared to males (Figure 2.2) that received Rap or Met+Rap treatment, suggesting potentially lower Rap concentrations in the female mice, or less sensitivity of female skeletal muscle to Rap treatment. Previous data from our laboratory suggest that skeletal muscle from female mice is less sensitive to mTOR inhibition during Rap treatment (38)

Another potential mechanism for the sex-specific effect of Rap treatment is that female Rap treated mice differentially express ~4500 mRNA compared to control, while male Rap treated mice differentially express only ~300 mRNA compared to control (46). Additionally, contained within the 4500 differentially expressed genes in female mice, numerous cellular processes that contribute to homeostasis, such as mitochondrial function, protein degradation, and oxidative stress responses, were upregulated compare to control (46). These pathways were not changed in Rap treated male mice (46). Skeletal muscle protein synthesis rates have also been demonstrated to have sexually dimorphic responses to interventions such as exercise training (141). Sex-differences in basal levels of protein synthesis are not commonly reported (170). However, small differences in protein metabolism between male and female animals have been purported to result from the effects of sex hormones (27). Specifically, estrogen increases protein synthesis in utero (156), while testosterone increases protein synthesis across the lifespan (134). Despite sexually dimorphic effects of Met+Rap treatment on protein synthesis, Met+Rap treatment may exert sex-independent effects on another aging-related process, such as reducing inflammation, or maintaining the stem cell pool (77).

Perspectives and Conclusions

Maintaining an intact, damage-free proteome is required for proper cellular function. Despite the complex cellular network devoted to maintaining proteostasis, damaged proteins accumulate over time (157). Although we tested Rap and Met+Rap treatments in younger mice (4mo), our findings that Rap and Met+Rap treatment did not decrease protein synthesis in heart and liver, with a reduction in skeletal muscle protein synthesis compared to control-treated mice suggest that these treatments contribute to maintaining a functional proteome. These data emphasize the need to assess the influence of lifespan-extending drugs in multiple tissues to better understand the mechanism of action. Furthermore, our findings that Rap and Met+Rap treatments decrease skeletal muscle protein synthesis add to the growing body of evidence that reductions in protein synthesis contribute to lifespan-extension, potentially by increasing proteome quality.

FIGURES

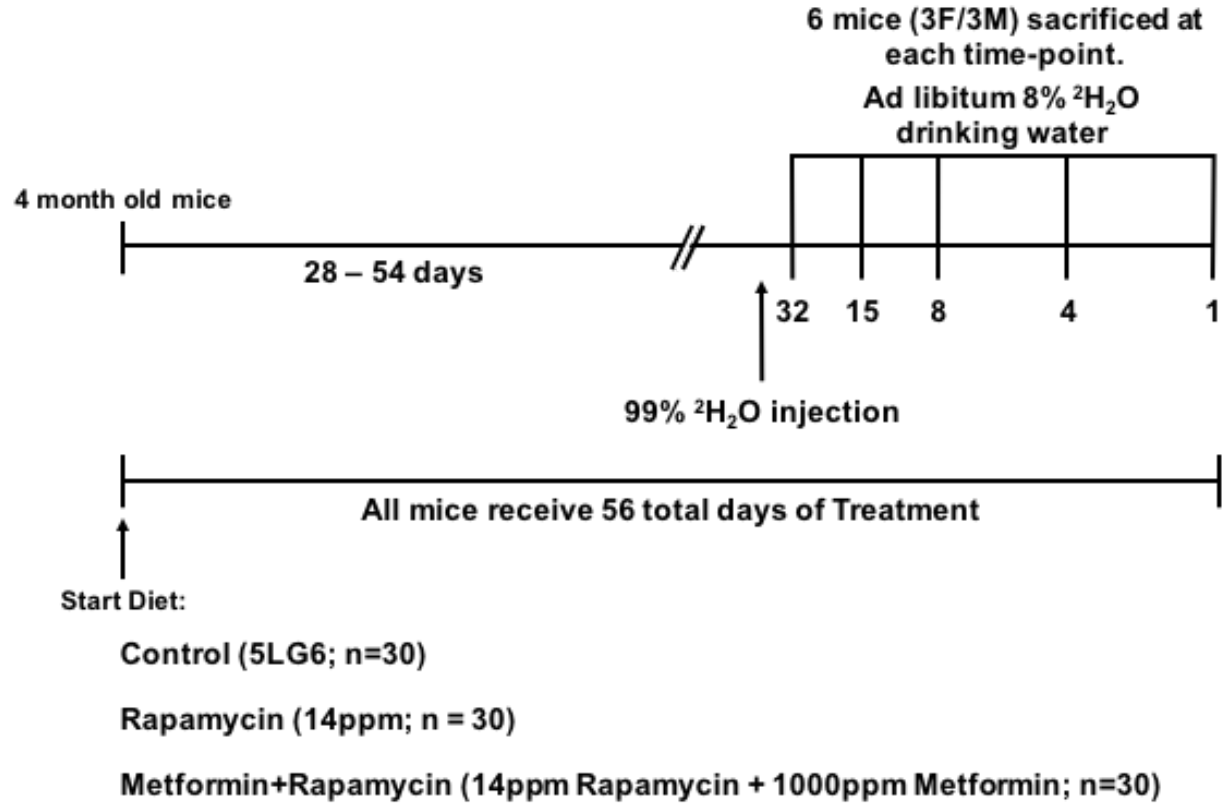

Figure 2.1 Study Timeline and Experimental Conditions. Ninety UM-HET3 mice were randomized into three two groups. One group received Rap encapsulated into the diet, one group received Met+Rap encapsulated into the diet, and the other consumed Con (5LG6) Chow. Animals began treatment at ~4 mo of age, and received 8 full weeks of treatment. All animals received an intraperitoneal injection with 99% D2O the day before receiving ad libitum access to drinking water enriched to 8% with D2O. Animals were then sacrificed one day later (1d), four days later (4d), eight days later (8d), fifteen days later (15d), or thirty-two days later (32d).

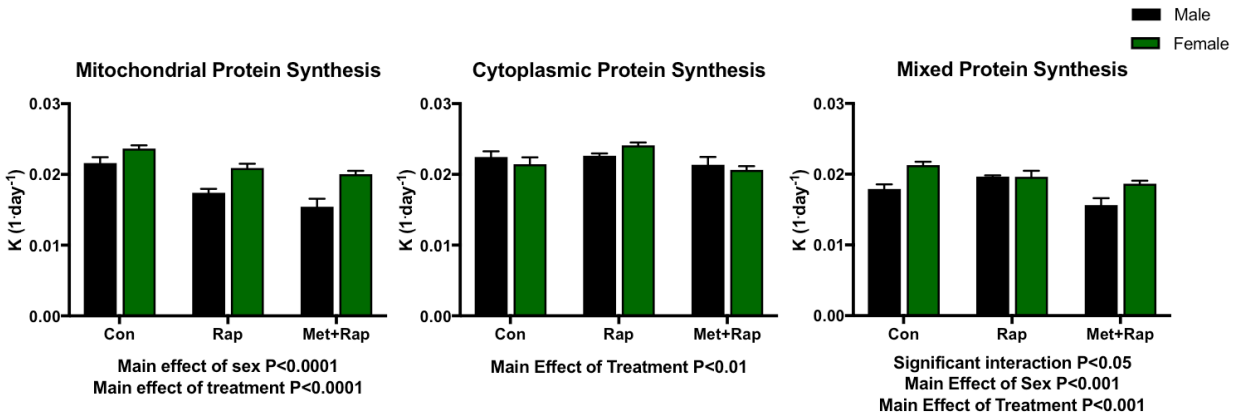

Figure 2.2 Skeletal Muscle Protein Synthesis in MITO, CYTO, and MIX Fractions. Protein synthesis rate constant values are presented from each protein fraction in the skeletal muscle. Data were analyzed by a 2-way ANOVA. There was a main effect of treatment in each fraction. There was a main effect of sex for the MITO and MIX fractions, and there was a significant interaction of treatment and sex in the CYTO and MIX fractions.

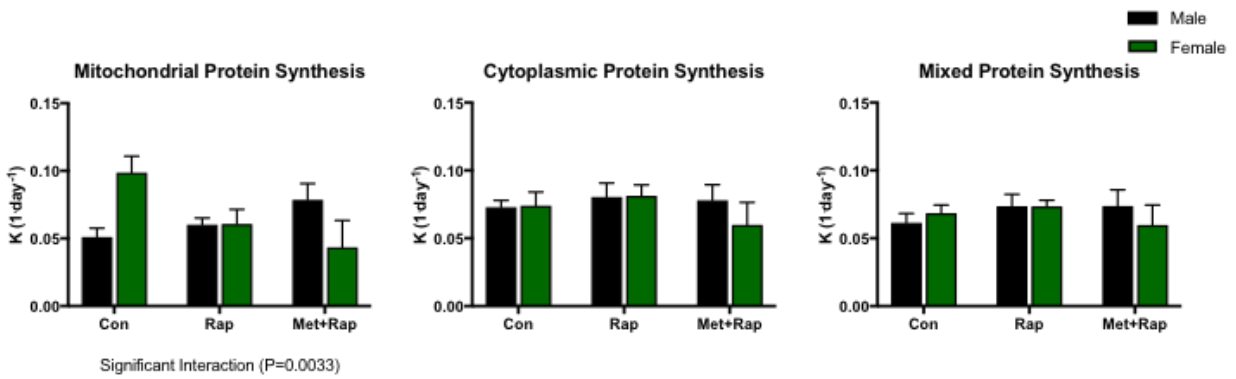

Figure 2.3 Heart Protein Synthesis in MITO, CYTO, and MIX Fractions. Protein synthesis rate constant values are presented from each protein fraction in the heart tissue. Data were analyzed using a 2-way ANOVA. There was a significant interaction between treatment and sex in the MITO fraction. Additionally, Met+Rap treatment lowered MITO protein synthesis compared to Con in female mice only.

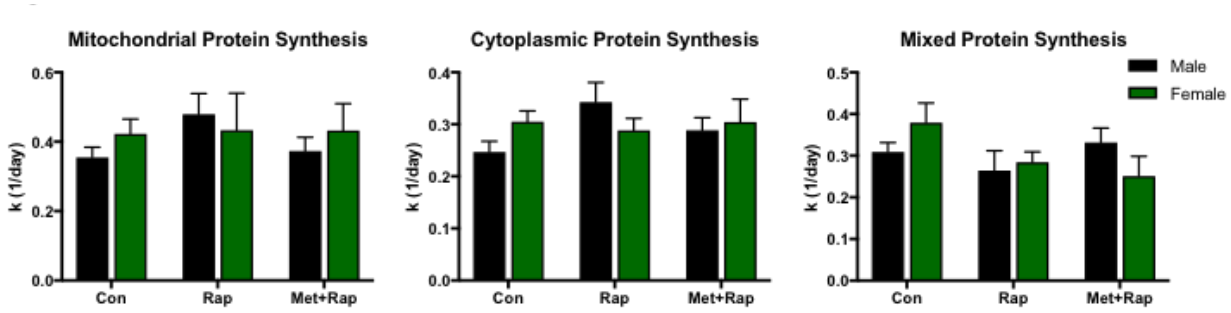

Figure 2.4. Liver Protein Synthesis in MITO, CYTO, and MIX Fractions. Protein synthesis rate constant values are presented from each protein fraction in the liver tissue. There were no significant differences in protein synthesis between treatments or sexes in any of the three protein fractions.

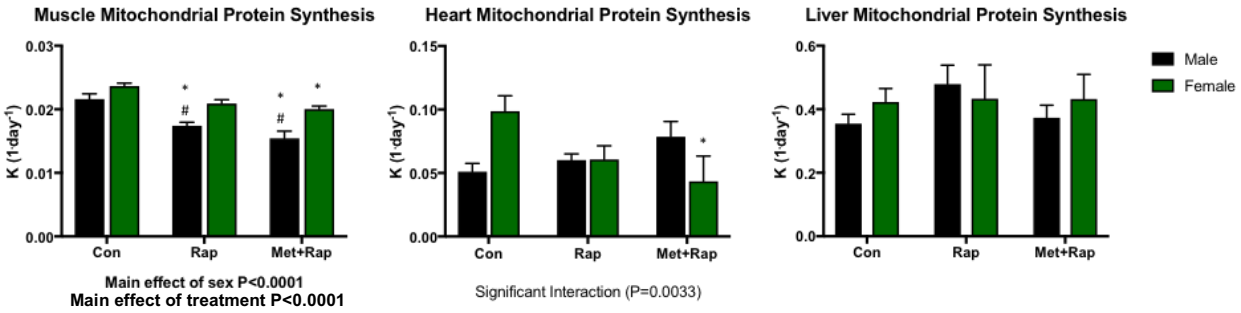

Figure 2.5 Tissue Specific Effects of Rap and Met+Rap Treatment on MITO Protein Synthesis. MITO protein synthesis is presented from the skeletal muscle, heart, and liver. There was a main effect of treatment and main effect of sex in the skeletal muscle, but not in heart or liver. There was a significant interaction of treatment and sex in the heart tissue, and Met+Rap significantly lowered MITO protein synthesis compared to Con in female mouse heart. There was no effect of treatment or sex on MITO protein synthesis in the liver. * denotes $P<0.05$ compared to corresponding control within sex. # denotes $P<0.05$ compared to corresponding value from the other sex within a treatment.

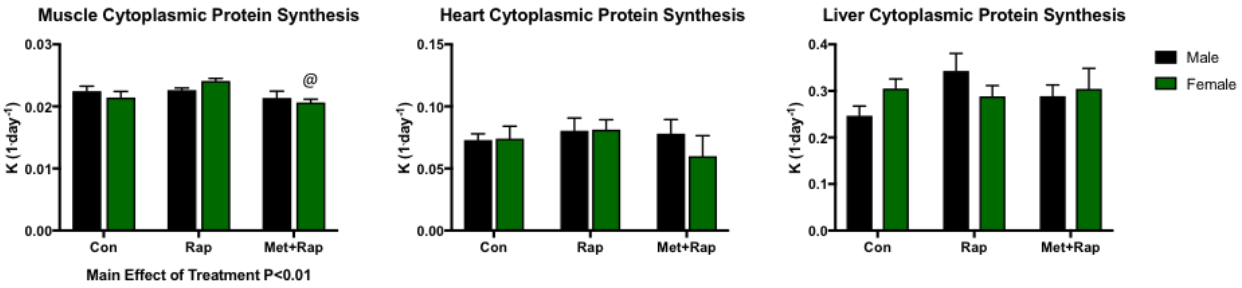

Figure 2.6 Tissue Specific Effects of Rap and Met+Rap Treatment on CYTO Protein Synthesis. CYTO protein synthesis is presented from the skeletal muscle, heart, and liver. There was a main effect of treatment in the skeletal muscle, but not in heart or liver. @ denotes $P<0.05$ compared to corresponding Rap-treated animals within sex.

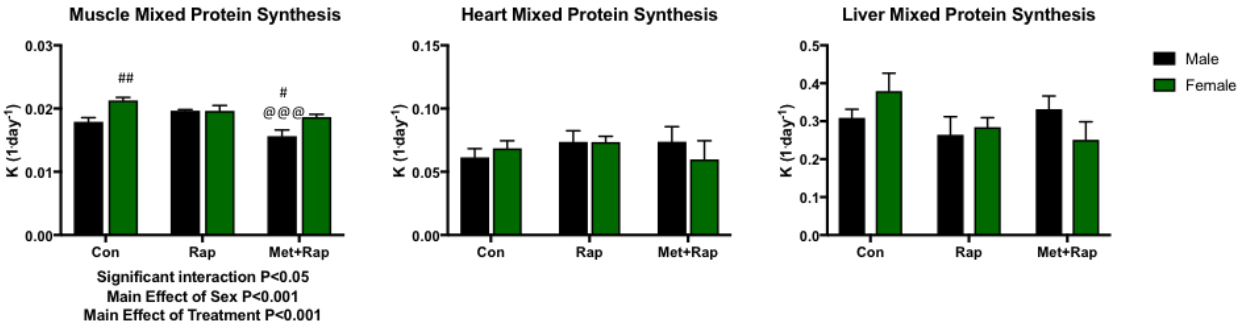

Figure 2.7 Tissue Specific Effects of Rap and Met+Rap Treatment on MIX Protein Synthesis. MIX protein synthesis is presented from the skeletal muscle, heart, and liver. There was a main effect of treatment, a main effect of sex, and an interaction of treatment and sex in the skeletal muscle, but no significant differences in the heart or liver. There was no effect of treatment or sex on MIX protein synthesis in the liver. # denotes P<0.05 compared to corresponding value from the other sex within a treatment. ## denotes P<0.01 compared to corresponding value from the other sex within a treatment. @@@ denotes P<0.001 compared to corresponding Rap-treated animals within sex.

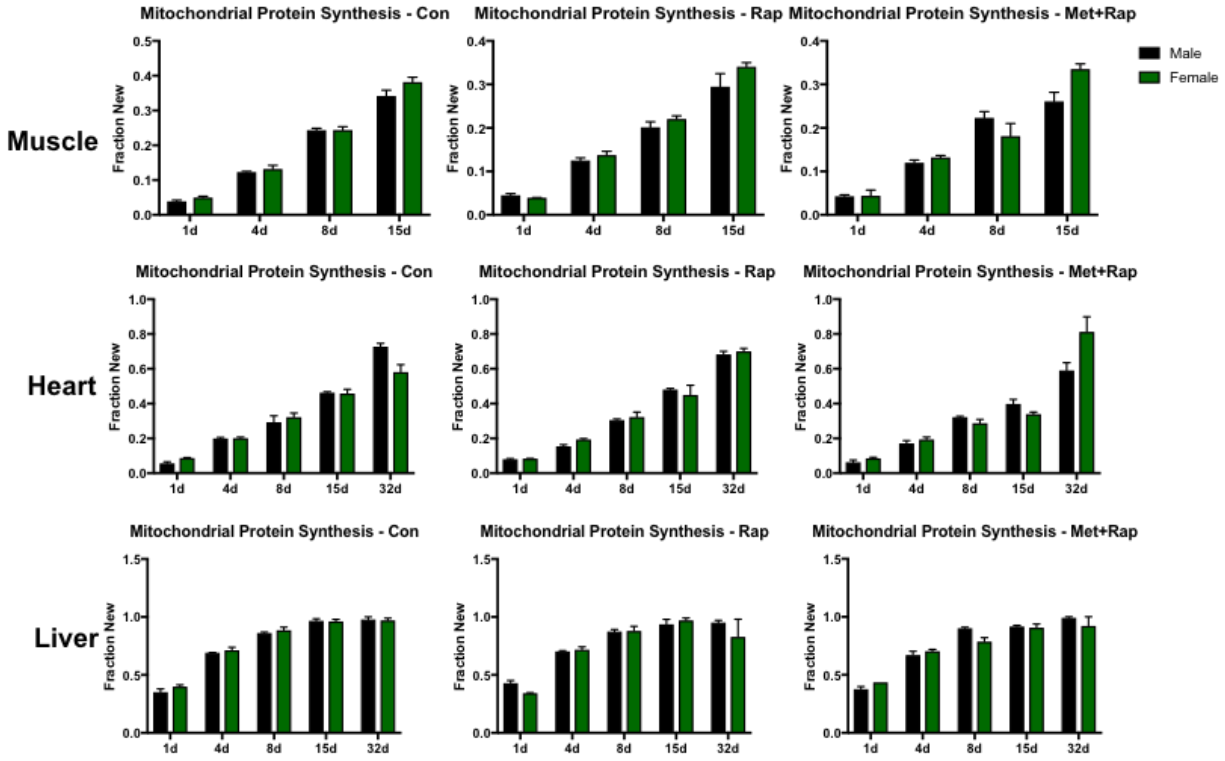

Figure 2.8 Tissue and Sex Specific Effects of Rap and Met+Rap Treatment on MITO Protein Synthesis. MITO protein synthesis values are presented from each treatment in each tissue over time. These data were not statistically analyzed. These data were used to look for different responses between treatments and sexes in each of the three tissues. Met+Rap treatment elicited sex-specific differential responses in the muscle, heart, and liver of 15d animals.

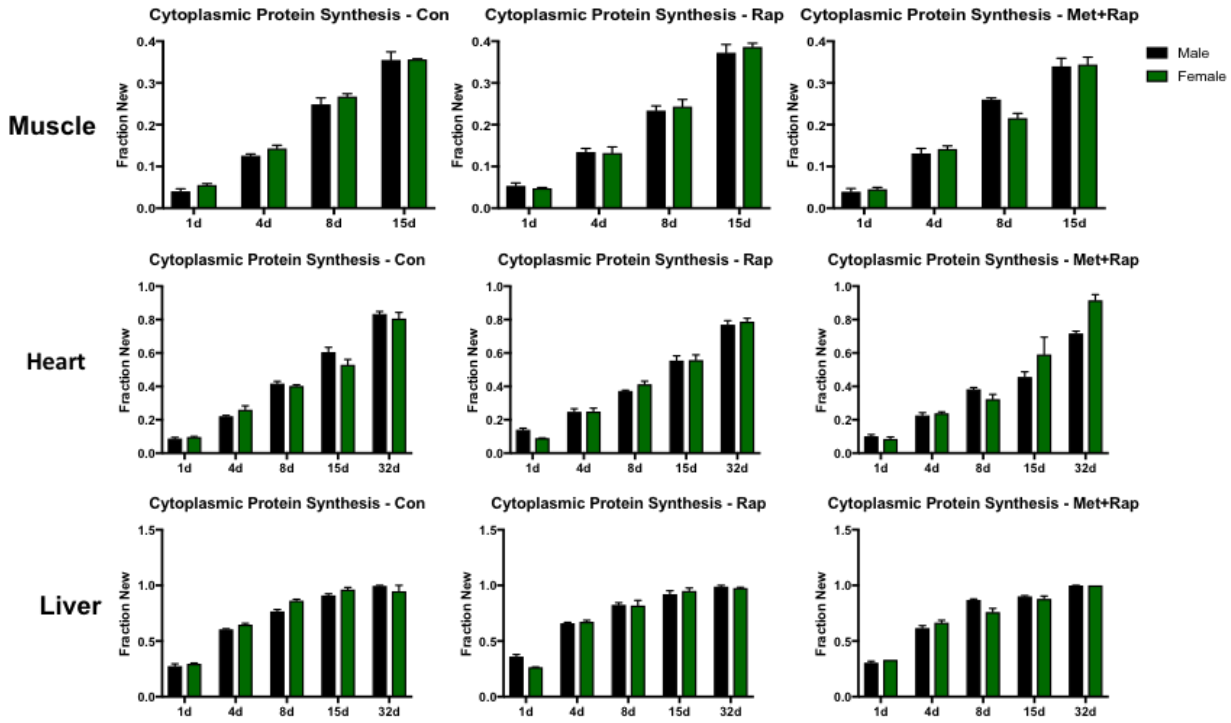

Figure 2.9 Tissue and Sex Specific Effects of Rap and Met+Rap Treatment on CYTO Protein Synthesis. CYTO protein synthesis values are presented from each treatment in each tissue over time. These data were not statistically analyzed. These data were used to look for different responses between treatments and sexes in each of the three tissues.

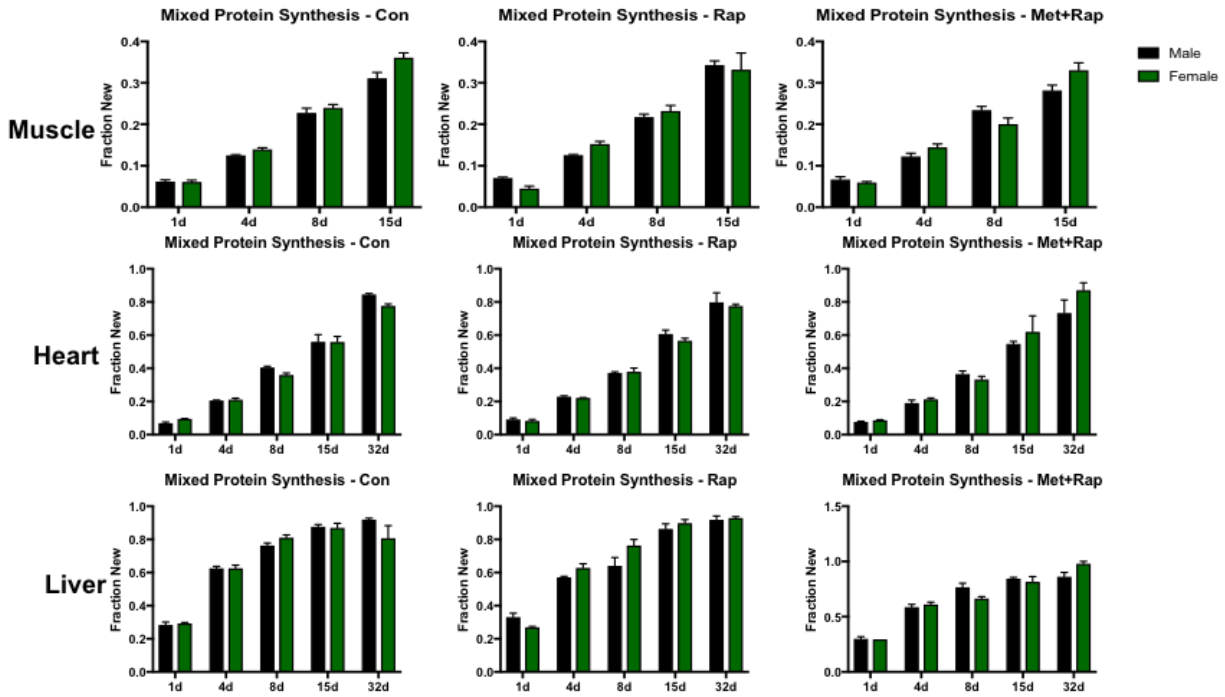

Figure 2.10 Tissue and Sex Specific Effects of Rap and Met+Rap Treatment on MIX Protein Synthesis. MIX protein synthesis values are presented from each treatment in each tissue over time. These data were not statistically analyzed. These data were used to look for different responses between treatments and sexes in each of the three tissues.

CHAPTER 3 – TISSUE-SPECIFIC CHANGES IN PROTEIN SYNTHESIS FOLLOWING METFORMIN+RAPAMYCIN TREATMENT IN OLDER MICE

Loss of protein homeostasis (proteostasis) is a hallmark of the aging process (96). Proteostasis refers to protein localization, concentration, folding, and turnover. Despite the extensive cellular proteostasis network, aging leads to an accumulation of misfolded, oxidized, and aggregated proteins (13, 165), contributing to a decline in cellular function and viability (165). Because enzymatic capacity for protein repair is limited (158), protein synthesis and degradation (i.e., turnover) are essential for proteome maintenance. However, both protein synthesis (133), and breakdown (126) are energetically costly processes. As such, protein turnover requires efficient energy production to maintain cellular function (116). Thus, interventions that can maintain cellular proteostasis during aging may have a positive influence on cell viability, and potentially organismal function. Data from our laboratory have demonstrated that in some long-lived murine models, mitochondrial protein synthesis is increased compared to control animals (36-38, 51, 106, 108).

Chronic rapamycin (Rap) treatment extends median and maximal lifespan (45, 55, 112). Specifically, when administered daily starting at nine months of age, Rap treatment increases median lifespan of male mice by 10% and female mice by 18% (112). Short term treatment with Rap inhibits mTOR complex 1 (mTORC1) thereby reducing cap-dependent protein translation as well as cell cycle progression (30, 169). Despite inhibition of cap-dependent translation, some transcripts have been shown to be selectively translated, including nuclear-encoded mitochondrial genes (152, 201). Additionally, long-term treatment with Rap has an inhibitory effect of mTORC2 and this

inhibition has a negative consequence on glucose handling (90). Because Rap treatment has a negative impact on glucose metabolism and does not extend median lifespan to the same extent in male and female animals, it may not be the best option for translation to humans.

Multiple models of slowed aging exhibit a sexual dimorphism in lifespan, with females living longer than males in most cases (119). No precise mechanisms have been described to explain the sexual dimorphism in slowed aging models. One investigation revealed that chronic daily Rap treatment has sexually dimorphic effects on gene expression patterns (191). Specifically, Rap treatment induced a calorie restriction (CR)-like pattern of gene expression in the liver of female, but not male, mice (191). One purported mechanism for the difference in male and female lifespan is mTORC2, as the rapamycin-insensitive companion of mTOR (Rictor) knockout model significantly reduced male lifespan but did not influence female lifespan (89).

The ITP program has also shown that the anti-diabetes drug metformin in combination with rapamycin (Met+Rap) increases male and female lifespan compared to control animals (161). The mechanism through which Met+Rap contributes to increased longevity has not been investigated. However, the intracellular signaling associated with Met+Rap treatment is thought to be similar to that of CR, as both activate the 5' AMP-activated protein kinase (AMPK) while inhibiting the mechanistic target of rapamycin complex 1 (mTORC1) (17, 137, 151). Lifelong and short-term CR are known to have positive effects on glucose metabolism, as CR decreases insulin resistance (181, 187). Although chronic Rap and CR have similar intracellular signaling patterns, long term Rap treatment has negative effects on glucose handling (90), making co-treatment with the

anti-hyperglycemia drug metformin potentially beneficial. Accordingly, Met+Rap appears to extend median lifespan to a greater extent than Rap alone (55, 161). Furthermore, Met+Rap equally extends the lifespan of male and female mice, compared to Rap treatment increasing female lifespan to a greater extent than male lifespan (55, 112, 113, 161).

There is evidence that long-lived species have better maintenance of proteostasis in response to stress compared to control (128). Interventions that help maintain cellular proteostasis may therefore have a positive impact on lifespan. We have assessed proteostatic mechanisms by measuring cellular protein synthesis rates and cellular proliferation to account for protein synthetic contributions to cellular growth or remodeling (106). Previous work from our laboratory demonstrates that life-long CR or chronic Rap treatments, both of which extend lifespan in murine models, are characterized by maintained proteostatic mechanisms compared to controls (106). Additionally, reduced rates of protein synthesis improve the fidelity of translation, while also preventing the accumulation of unfolded proteins. Thus, maintenance of proteostasis may be a shared characteristic of long-lived species (51).

To our knowledge, however, no investigation has assessed how the lifespan-extending Met+Rap treatment influences protein synthesis in older animals. Thus, the purpose of the current investigation was to determine how 8 weeks of Met+Rap treatment in older (17 mo) mice influences protein synthesis in the skeletal muscle, heart, and liver compared to control. We hypothesized that Met+Rap would decrease protein synthesis compared to control treated animals. We further hypothesized that Met+Rap would decrease protein synthesis to the same extent in both male and female mice.

METHODS

Animals & Experimental Design

All procedures and conditions for the housing and care of the animals was approved by the Animal Care and Use Committee at the University of Michigan. Thirty-six (18 male, 18 female) genetically heterogeneous UM-HET3 mice for this investigation. The UM-HET3 model are genetically heterogeneous mice that were crossbred from two different F1 strains: (BALB/cByJ x C57BL/6J) F1 females (JAX stock 10009) and (C3H/HeJ x DBA/2J) F1 males (JAX stock 10004), as previously described (35). Seventeen-month old mice were housed 3-per cage on a 12h light-dark cycle. Eighteen mice (9M/9F) were randomized into control diet group (Purina 5LG6, CON) or Metformin (Met)+Rap encapsulated into control diet group (1000 ppm Met and 14 ppm Rap), as in the Interventions Testing Program (161). Control and Met+Rap diets were administered for 8 weeks (56 days) in total (Figure 1). Mice received stable isotopic labeling with deuterium oxide (D_2O) for one day (1d), four days (4d), or eight days (8d) (Figure 1). Specifically, after 48 days of treatment, a subset of mice received a 99% D_2O injection with subsequent access to drinking water enriched with 8% D_2O, and those mice were sacrificed after 8 days of labeling (8d). At 52 days of treatment, a second subset of mice received a 99% D_2O injection with subsequent access to drinking water enriched with 8% D_2O, and those mice were sacrificed after 4 days of labeling (4d). At 55 days of treatment, a final subset of mice received a 99% D_2O injection with subsequent access to drinking water enriched with 8% D_2O, and those mice were sacrificed after one day of labeling (1d). All mice received 8 full weeks of treatment. We aimed to have all animals reach a total body water enrichment of ~5%, as previously described (38, 127). Animals were euthanized using carbon dioxide following a 18h fast. At each time point, 6 mice (3 male

and 3 female) were sacrificed for each treatment (CON and Met+Rap) group. We collected the liver, heart, and gastrocnemius from each animal to assess isotope incorporation into proteins and DNA, and plasma and bone marrow to assess precursor enrichment (described in detail below).

Subcellular Fractionation of Tissues

Excised skeletal muscle, heart, and liver samples were snap-frozen in liquid nitrogen and subsequently powdered in liquid nitrogen. Powdered samples were further homogenized using zirconium oxide beads in a bullet blender with mitochondrial isolation buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH=7.5) as previously described (36, 38, 109). Homogenized samples were spun at 800 x *g* for 10 minutes at 4°C. The pellet from this spin was washed and resuspended as the mixed-protein fraction (MIX). The supernatant was transferred to a new tube and spun at 10,000 x *g* for 30 minutes at 4°C. The pellet from the 10,000 x *g* spin was washed and resuspended as the mitochondrial-enriched protein fraction (MITO). The supernatant from the 10,000 x *g* spin was split into two volumes with one volume being precipitated in 4% sulfosalicylic acid for one hour on ice and one volume being frozen for use in western blotting applications. Following the precipitation, the solution was centrifuged at 16,000 x *g* for 10 minutes at 4°C. The resultant pellet was washed and resuspended as the cytoplasmic-enriched fraction (CYTO). All pellets were solubilized in 1M NaOH at 50°C, and hydrolyzed in 6M HCl for 24h at 120°C. DNA from tissue was extracted using a commercially available kit (QIamp DNA Mini, Qiagen, California). DNA was extracted from bone marrow by separating the isolated bone marrow in half and adding 500µl of ATL buffer and incubating overnight at 56°C. Bone marrow and DNA were then hydrolyzed

overnight at 37°C with nuclease S1 and potato acid phosphatase. Hydrolyzed DNA was analyzed using GC-MS after derivation procedures described below.

Preparation of Analytes for GC-MS Analysis

After each protein fraction was hydrolyzed, cation exchange chromatography was used to isolate positively charged amino acids. Amino acids were eluted from the chromatography resin using 4M NH_4OH. Purified amino acids were the. dried on a vacuum centrifuge (SpeedVac Savant 110, Thermo, Rockford, IL). Dried amino acid pellets were resuspended in 1mL of molecular biology grade water, and 500µl of the resuspended amino acids were subjected to our standard derivation procedure. Specifically, acetonitrile, 1M potassium phosphate, and pentafluorobenzyl bromide were added to the amino acid solution and incubated at 100°C for one hour. Ethyl acetate was used to extract the pentafluorobenzyl derivatives which were dried under N_2. Dried derivatives were resuspended in 700µl of ethyl acetate and analyzed using a 7890A gas chromatograph coupled to 5975C mass spectrometer with a DB-5MS GC column (30 m x 0.25 mm x 0.25 µm; all from Agilent), as previously described in detail by our laboratory (38, 109, 111).

To prepare the DNA samples for GC-MS analysis, DNA hydrolysates were reacted with pentafluorobenzyl hydroxylamine hydrochloride and glacial acetic acid. Following incubation at 100°C for thirty minutes, samples were acetylated by the addition of acetic anhydride and 1-methylimidazole. Dichloromethane was used to extract the derivatives, which were dried under vacuum, resuspended in ethyl acetate, and subsequently analyzed on a 7890A gas chromatograph coupled to 5975C mass spectrometer using a DB-17 Column (Agilent). Deuterium incorporation into deoxyribose was calculated as

previously described (21, 38, 109). Briefly, the fractional molar isotope abundances at m/z 435 (M0) and 436 (M1) of pentafluorobenzyl triacetyl derivatives of deoxyribose were quantified using Agilent Mass Hunter software. Enrichment was calculated as the ratio of M1 abundance to M0+M1 abundance minus background enrichment.

Deuterium enrichment of the total body water pool was used to estimate the alanine precursor enrichment based upon mass isotopomer distribution analysis (MIDA (38, 109, 111). Body water enrichment was measured in 125µl of evaporated plasma samples from each animal as previously described (38). Briefly, plasma samples were placed onto inverted plastic microcentrifuge caps with a rubber o-ring. Tubes were then sealed and placed onto a heat block at 80°C overnight. Evaporated water from the plasma was captured in the top of the tubes. The evaporated water then underwent proton exchange with 10M NaOH and acetone overnight. Finally, proton exchanged samples were extracted in hexanes into anhydrous sodium sulfate before being transferred into chromatography vials for analysis on a 7890A gas chromatograph coupled to 5975C mass spectrometer using a DB-17 Column (Agilent).

Protein Synthesis Calculations

Protein synthesis was calculated based on the precursor-product relationship. Briefly, protein synthesis was calculated from the precursor enrichment that was determined from the deuterium enrichment in the body water pool and then adjusted using Mass Isotopomer Distribution Analysis (56, 125). The fraction of new proteins was calculated for each time point (1, 4, or 8 days) and each fraction (MITO, CYTO, and MIX).

Statistics

Graphpad Prism 7 was used to complete statistical analyses for this investigation. Two-way analysis of variance (ANOVA) tests were used to compare differences in protein synthesis (fraction new) between treatments and sexes. Where appropriate, multiple comparison adjustments were made using a Tukey adjustment. Significance was accepted as $P<0.05$ for these experiments.

RESULTS

Skeletal Muscle Protein Synthesis

On day 1, there was a main effect of treatment ($P<0.01$) on MITO protein synthesis (Figure 3.2). CYTO protein synthesis was not different between any groups on day 1 (Figure 3.2). There was a tendency for a main effect of treatment on protein synthesis in the MIX fraction on day 1 ($P=0.06$; Figure 3.2). On day 4, MITO protein synthesis was not different between sexes or between Met+Rap and control (Figure 3.3). There was a main effect of treatment ($P<0.05$) on CYTO protein synthesis at day 4 (Figure 3.3). MIX protein synthesis was not different between groups on day 4 (Figure 3.3). On day 8, MITO protein synthesis was not different between groups. There was a main effect of treatment for both CYTO ($P<0.001$) and MIX ($P<0.05$) protein synthesis on day 8. Met+Rap treatment significantly ($P<0.01$) reduced MIX protein synthesis compared to control in the female mice only on day 8 (Figure 3.3).

Heart Protein Synthesis

On day 1, there were no significant differences between treatment or sex on protein synthesis in any of the three protein fractions (Figure 3.4). On day 4, there were no significant differences between treatment or sex on protein synthesis in the MITO or

CYTO protein fractions. However, on day 4 there was a main effect of treatment ($P<0.05$) on protein synthesis in the MIX fraction (Figure 3.5). There was a main effect of sex ($P<0.05$) for MITO protein synthesis on day 8. However, there were no significant differences between treatment or sex on protein synthesis in the CYTO or MIX protein fractions on day 8 (Figure 3.6).

Liver Protein Synthesis

On day 1, there were no significant differences between treatment or sex on protein synthesis in the MITO or MIX protein fractions (Figure 3.7). However, there was a main effect of treatment ($P<0.05$) on CYTO protein synthesis on day 1. On day 4, there were no significant differences between treatment or sex on protein synthesis in any of the three protein fractions (Figure 3.8). Additionally, on day 8, there were no significant differences between treatment or sex on protein synthesis in any of the three protein fractions (Figure 3.9).

DISCUSSION

The purpose of this investigation was to determine how the known lifespan-extending treatment Met+Rap influences protein synthesis in a tissue- and sex-specific manner in older mice. We found that Met+Rap treatment did not change mitochondrial protein synthesis in the skeletal muscle, heart, and liver tissue of older mice compared to control. We also report that Met+Rap treatment significantly decreased cytoplasmic or mixed fraction protein synthesis in the skeletal muscle, heart, and liver. These data are the first to demonstrate that Met+Rap treatment does not alter mitochondrial protein synthesis in older animals. Further, these data suggest that Met+Rap treatment may

extend lifespan by contributing to proteostasis in multiple tissues, similar to other lifespan-extending treatments (106).

Potential Mechanisms for Lifespan-Extension by Met+Rap Treatment

Treatments that prevent the age-related decline in proteostasis prolong lifespan in lower organisms (78). Additionally, rodents with increased maintenance of proteostasis have increased lifespan compared to rodents with limited resources allocated to maintaining proteostasis (128). Although limited data are available regarding the effect of Met+Rap treatment on proteostasis and cellular function during the aging process, the effects of the individual components (Rap and Met) have been better characterized (34, 38, 55, 102, 122, 195). Table 3.1 contains a brief summary of how Met, Rap, or Met+Rap treatments influence the so-called seven pillars of aging research (77).

No studies have investigated the intracellular signaling associated with Met+Rap treatment directly, but both Met alone and Rap alone inhibit mTORC1 signaling and activity (121), while Met alone activates AMPK activity (200). mTORC1 specifically is known to regulate cap-dependent protein translation (192), cell growth and proliferation (140), cell cycle progression (44), autophagy (105), senescence (105), nutrient sensing (184), and metabolism (140). AMPK also has diverse regulation including: cellular respiration (65), autophagy (174), cellular proliferation (174), cell polarity (53), and AMPK inhibits mTORC1 (61). Loss of function in each of these listed pathways contributes to an aged phenotype (77, 96). Thus, Met+Rap treatment influences aging-related processes, which contributes to the positive effects of Met+Rap on lifespan

Maintenance of proteostasis in aging tissues prevents damage accumulation, thereby preserving cellular function (81). As stated above, we show that Met+Rap did not

alter mitochondrial protein synthesis in liver, heart, or skeletal muscle of older mice. Met+Rap treatment should reduce protein synthesis and cellular proliferation by inhibiting the mTORC1 pathway and activating the AMPK pathway (121, 151). Thus, the lack of an effect of Met+Rap treatment on protein synthesis rates suggest newly synthesized proteins are more likely allocated to replacing existing structures rather than cell growth (47, 51).

Reduced rates of protein synthesis can also contribute to the integrity of the proteome. Specifically, aging is associated with a decrease in protein chaperones, such as heat shock factors and the nascent peptide-associated complex, which help to facilitate proper folding of nascent polypeptides (80, 115). Thus, reduced rates of cap-dependent translation can facilitate proper protein folding, by preventing overload of the nascent peptide-associated complex, potentially reducing protein aggregation (147). However, in each of the three tissues, Met+Rap treatment did not decrease mitochondrial protein synthesis compared to control. These data are in line with a previous investigation that reported mitochondrial proteins are selectively translated despite inhibited mTOR signaling (201). The selective translation of nuclear-encoded mitochondrial genes also increased mitochondrial function and prolonged lifespan (201). Turnover of mitochondrial proteins during the aging process can prevent age-related decline in mitochondrial function, as oxidative damage to mitochondrial proteins can prevent their degradation (20). Thus, our data that Met+Rap treatment in older mice does not decrease mitochondrial protein synthesis compared to control suggest that mitochondrial proteins continue to be degraded and resynthesized.

Tissue Specific Effects of Met+Rap Treatment

It is important to note that both the heart and skeletal muscle are primarily post-mitotic tissues, while the liver continues to turnover (85). Furthermore, no investigations have examined tissue specific effects of Met+Rap treatment. However, experiments using Rap alone or Met alone potentially provide insight into how Met+Rap treatment effects different tissues. Long-term Met treatment increased lifespan and induced similar changes in gene expression in both the skeletal muscle and liver (102). A more recent investigation reported that Met treatment elicits tissue-specific in gene expression that improve cellular function with age (86). Additionally, Met treatment increased gene expression of cytoprotective processes, such as mitochondrial genes and DNA damage repair genes (86). Overall, it appears that Met treatment has inconsistent effects in multiple tissues, regardless of proliferative capacity.

Tissue specific assessments of Rap treatment are also limited, especially with respect to proteostasis. One investigation found that Rap treatment increases mitochondrial regulators of proteostasis in muscle but not liver of marmosets (94). Six months of Rap treatment induces tissue-specific effects on proteasome function as well, increasing proteasome activity in the brain but decreasing proteasome activity in adipose tissue (132). Data from our laboratory suggest that following 12 weeks of Rap treatment, both skeletal muscle and heart tissue have no change or a slight reduction in protein synthesis compared to control animals (38). The data from the current investigation reveal that Met+Rap treatment elicits tissue-specific differences in protein synthesis. Although there are no clear patterns of tissue-specific changes with Met+Rap, Met, or Rap treatments, the tissue-specific differences in protein synthesis responses from each tissue may be due to the proliferative capacity of the three cell types studied (143). More

specifically, liver tissue is proliferative, and we observed an effect of Met+Rap on protein synthesis after a single day of D_2O labeling, and we also show that the protein pool was fully turned over by 8d. We observed that Met+Rap treatment decreased cytoplasmic and mixed protein synthesis in the muscle and heart on 4d or 8d of labeling, and these tissues turnover more slowly. Thus, Met+Rap treatment may increase the contribution of protein synthesis to maintaining the proteome in a tissue-specific manner, potentially revealing mechanisms for lifespan-extending interventions.

Perspectives and Conclusions

We demonstrate for the first time in older mice (17mo) that 8 weeks of Met+Rap treatment did not change mitochondrial protein synthesis in skeletal muscle, heart, or liver. However, Met+Rap treatment decreased cytoplasmic and mixed protein synthesis compared to control. Additionally, we did not observe a sexually dimorphic effect of Met+Rap treatment on protein synthesis in 17 month old, genetically heterogeneous mice. Furthermore, these data suggest a potential relationship between the protein synthetic contribution to maintaining the proteome and the positive effect of Met+Rap treatment on lifespan-extension.

TABLES AND FIGURES

Table 3.1. Brief Overview of the Effects of Metformin, Rapamycin, or Metformin+Rapamycin Treatments on the Seven Pillars of Aging.

Pillar of Aging	Metformin Treatment	Rapamycin Treatment	Met+Rap Treatment
Macromolecular Damage	↓DNA Damage (118) ↓Protein Damage (129) ↓Lipid Damage (8) ↓Global Oxidative Damage (41)	↓DNA Damage(138)	↓Oxidative Stress (150)
Epigenetics	↑ Methylation Potential (31, 199)	↓ "Aging Signature" (177)	Unknown
Inflammation	↓ Inflammasome Assembly (93)	↓ Inflammasome Assembly (82)	Unknown
Adaptation to Stress	↑ Integrated Stress Response (62)	↑ Integrated Stress Response (88)	Unknown
Proteostasis	↑ (175)	↑ (22)	Unknown
Stem Cells & Regeneration	↑ Neural Stem Cell Regeneration (42)	↑ Stem Cell Maintenance (49)	Unknown
Metabolism	↓ Mitochondrial Respiration & ↑ Glucose Metabolism (4)	↓ Glucose Utilization & ↑ Fatty Acid Oxidation (153)	↓ Cancer Cell Viability (117, 193)
Lifespan	↑ Median 5.8% ↑ Maximal 7% (102)	↑ Median 10-18% ↑ Maximal 9-14% (55, 112)	↑ Median 23% ↑ Maximal 10-17% (161)

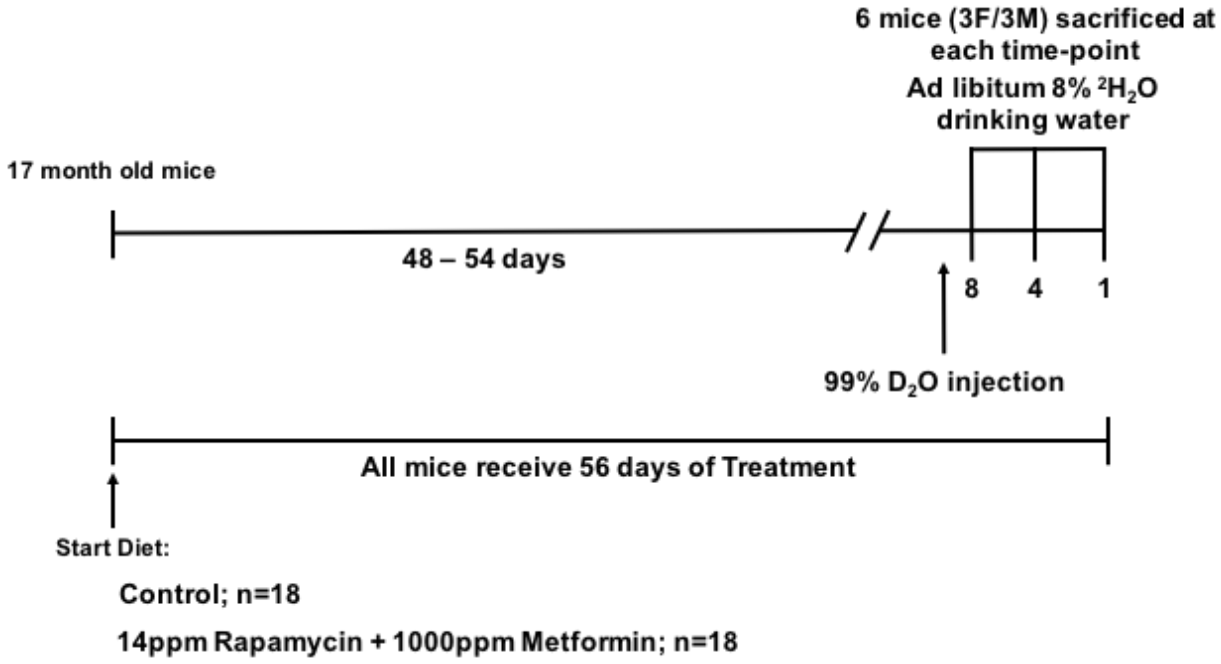

Figure 3.1. Study Timeline and Experimental Conditions. Thirty-six UM-HET3 mice were randomized into two groups. One group received Met+Rap encapsulated into the diet, the other consumed Con (5LG6) Chow. Animals began treatment at ~17 mo of age, and received 8 full weeks of treatment. All animals received an intraperitoneal injection with 99% D2O the day before receiving ad libitum access to drinking water enriched to 8% with D2O. Animals were then sacrificed one day later (1d), four days later (4d), or eight days later (8d).

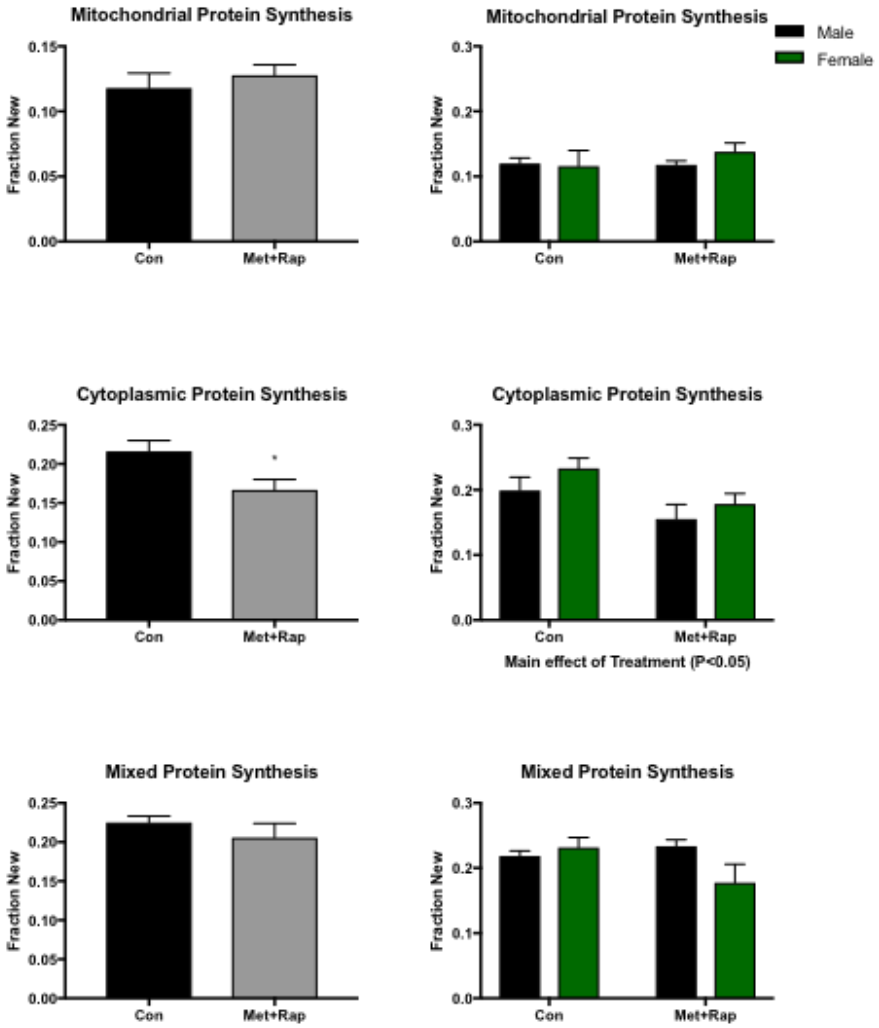

Figure 3.2. Skeletal Muscle Protein Synthesis in MITO, CYTO, and MIX Fractions at 4d of D_2O Labeling. There was no effect of Met+Rap treatment on MITO or MIX protein synthesis when both sexes were combined, while Met+Rap lowered CYTO protein synthesis compared to Con. There was also a main effect of treatment in the CYTO fraction when the data were separated by treatment and by sex. * denotes $P<0.05$ compared to corresponding Con value.

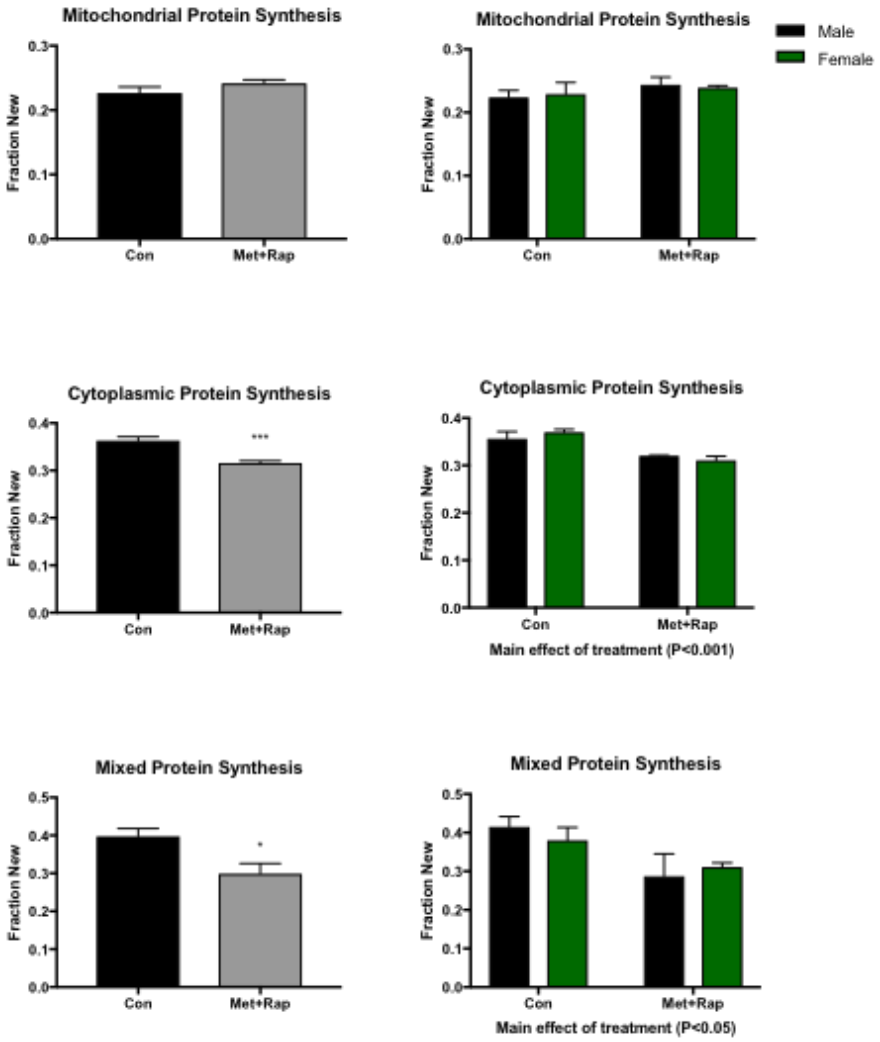

Figure 3.3. Skeletal Muscle Protein Synthesis in MITO, CYTO, and MIX Fractions at 8d of D_2O Labeling. There was no effect of Met+Rap treatment on MITO protein synthesis when both sexes were combined, while Met+Rap lowered CYTO and MIX protein synthesis compared to Con. There was also a main effect of treatment in the CYTO and MIX fractions when the data were separated by treatment and by sex. * denotes P<0.05 compared to corresponding Con value. *** denotes P<0.001 compared to corresponding Con value.

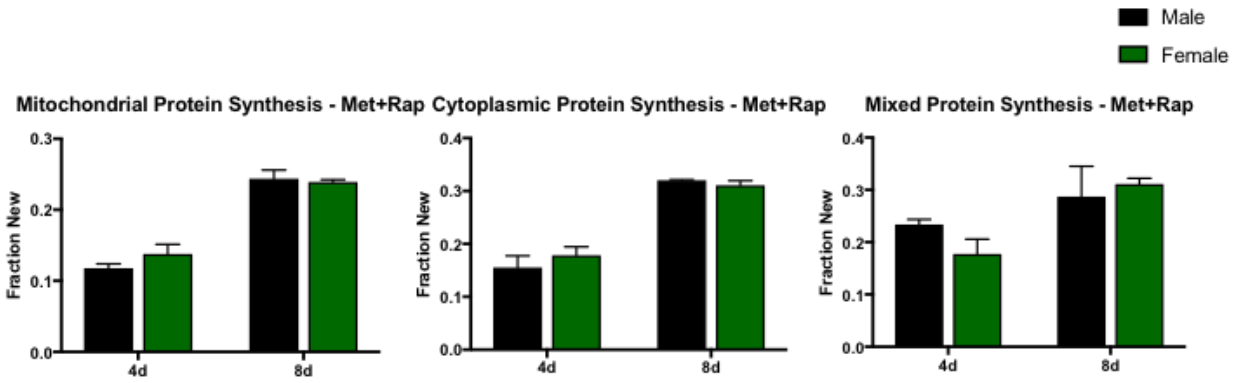

Figure 3.4. Skeletal Muscle Protein Synthesis following Met+Rap Treatment in MITO, CYTO, and MIX Fractions at 4d and 8d of D_2O Labeling. There were no differences in how Met+Rap treatment changed protein synthesis in either sex or any fraction over time.

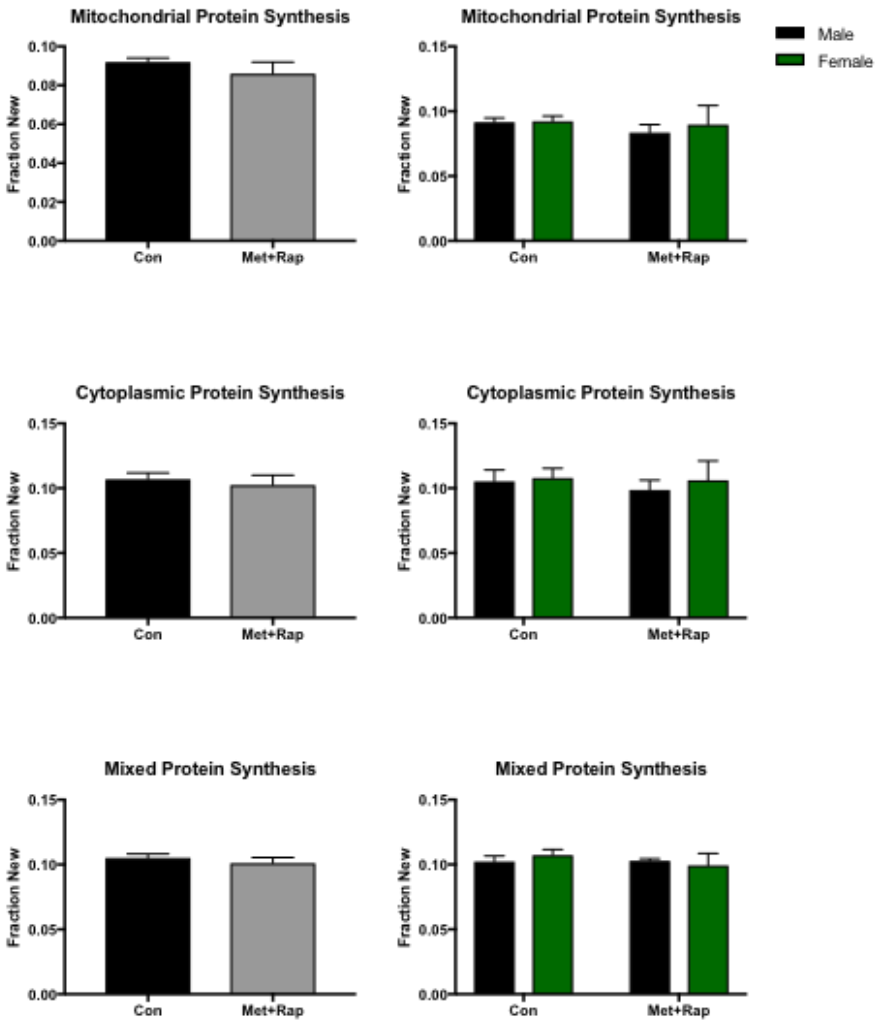

Figure 3.5. Heart Protein Synthesis in MITO, CYTO, and MIX Fractions at 1d of D_2O Labeling. There was no effect of Met+Rap treatment on MITO, CYTO, or MIX protein synthesis when both sexes were combined. There were no significant main effects when the data were separated by treatment and by sex.

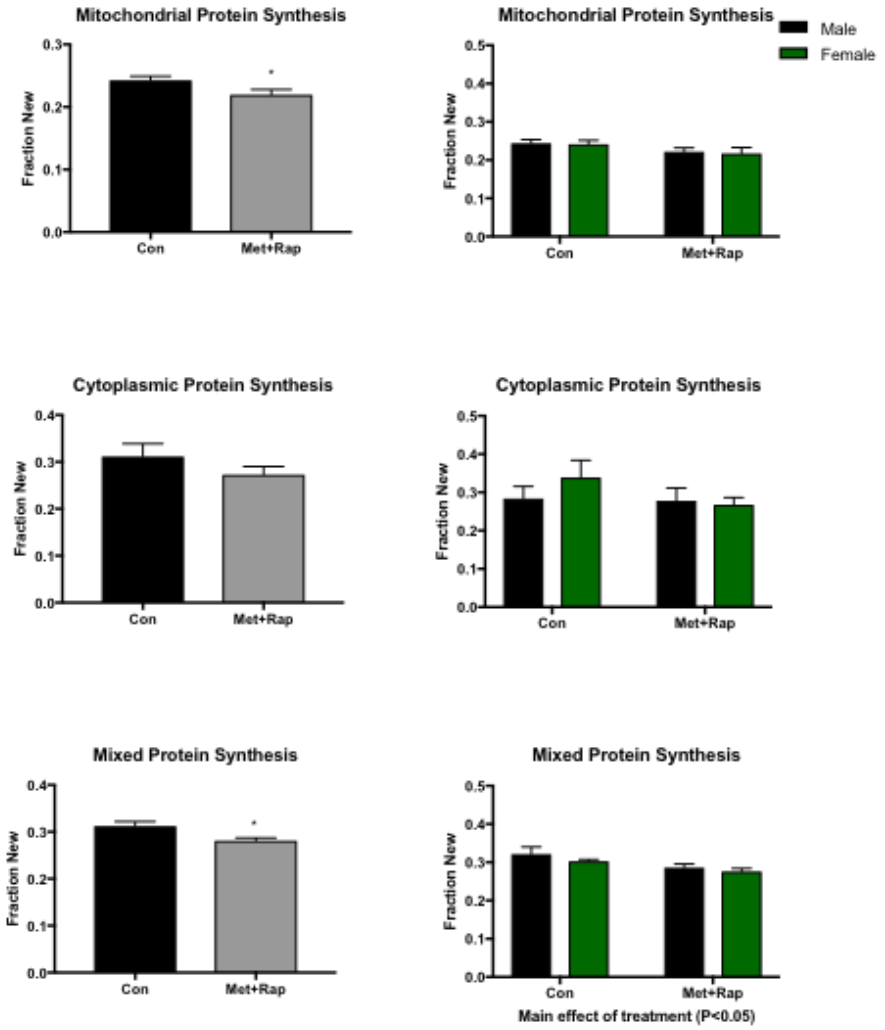

Figure 3.6. Heart Protein Synthesis in MITO, CYTO, and MIX Fractions at 4d of D_2O Labeling. There was no effect of Met+Rap treatment on CYTO protein synthesis when both sexes were combined, while Met+Rap lowered MITO and MIX protein synthesis compared to Con. There was also a main effect of treatment in the MIX fraction when the data were separated by treatment and by sex. * denotes P<0.05 compared to corresponding Con value.

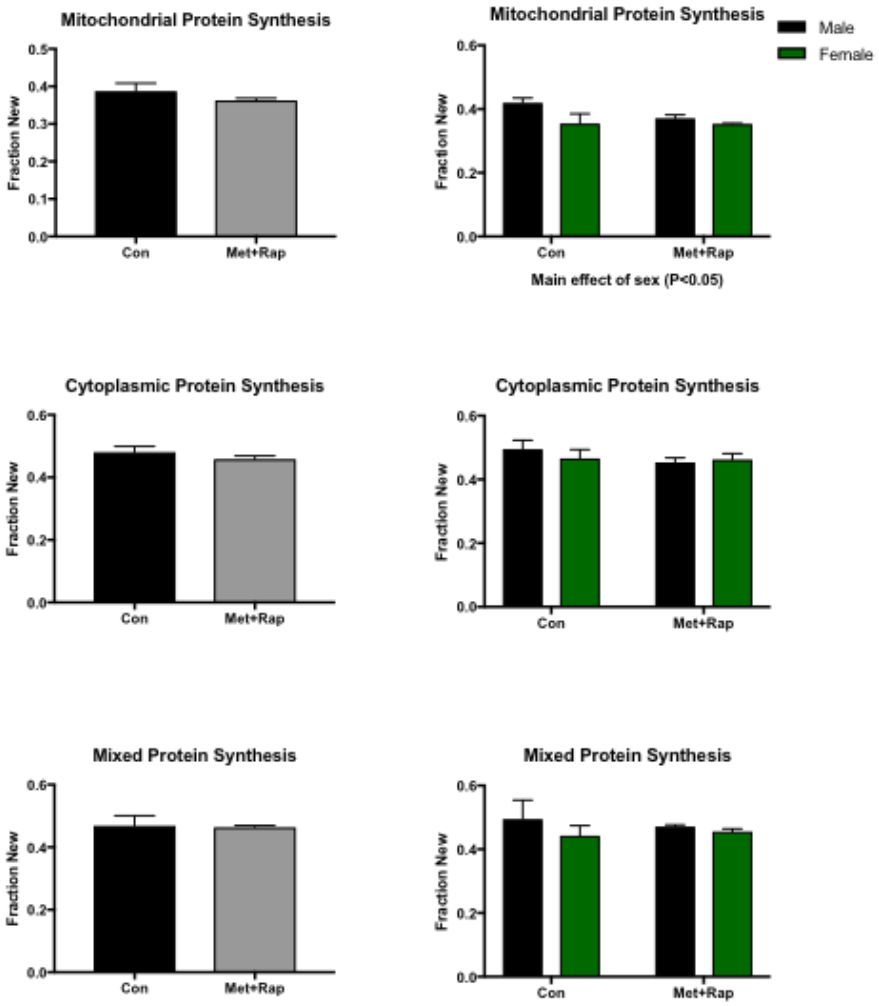

Figure 3.7. Heart Protein Synthesis in MITO, CYTO, and MIX Fractions at 8d of D_2O Labeling. There was no effect of Met+Rap treatment on MITO, CYTO, or MIX protein synthesis when both sexes were combined. There was a main effect of sex in the MITO fraction when the data were separated by treatment and by sex.

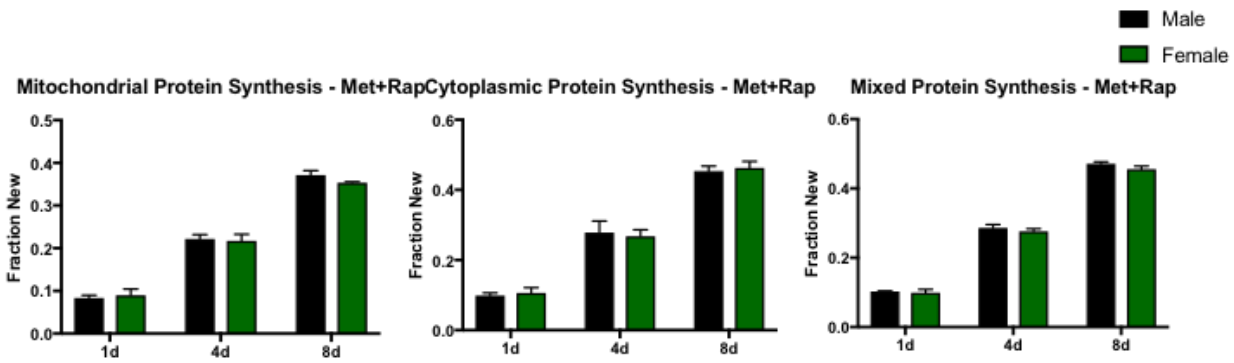

Figure 3.8. Heart Protein Synthesis following Met+Rap Treatment in MITO, CYTO, and MIX Fractions at 1d, 4d, and 8d of D_2O Labeling. There were no differences in how Met+Rap treatment changed protein synthesis in either sex or any fraction over time.

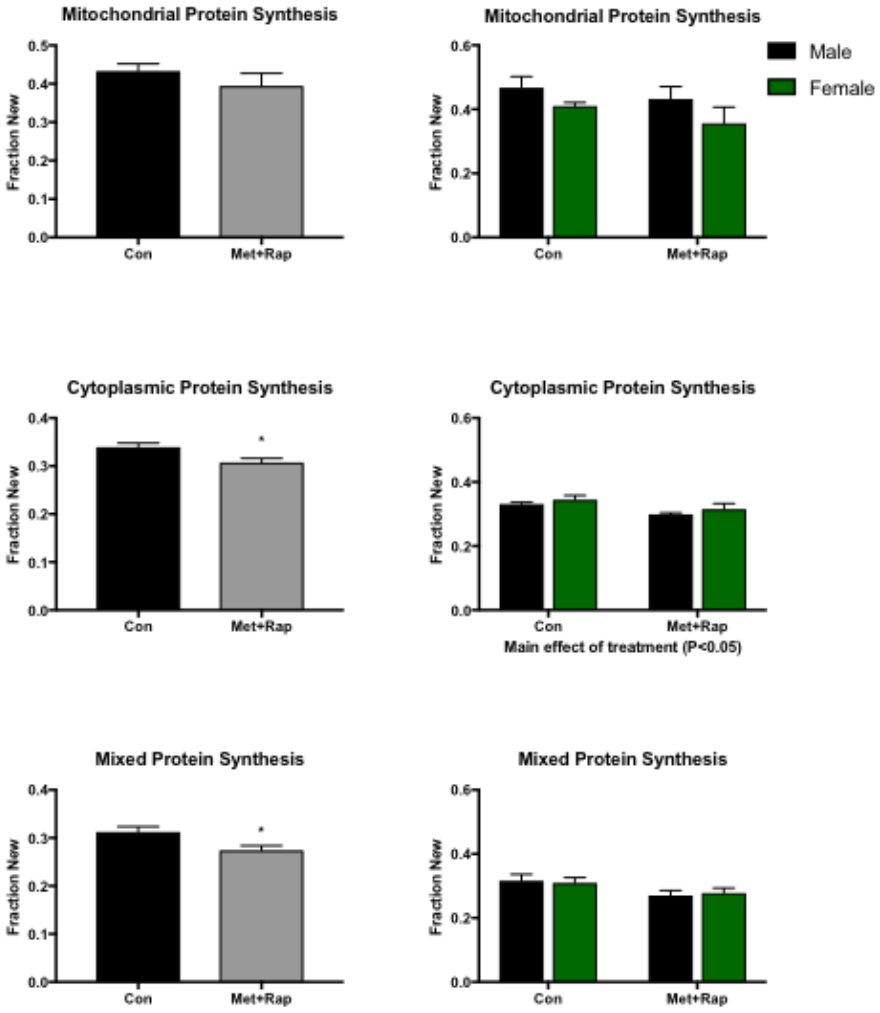

Figure 3.9. Liver Protein Synthesis in MITO, CYTO, and MIX Fractions at 1d of D_2O Labeling. There was no effect of Met+Rap treatment on MITO protein synthesis when both sexes were combined, while Met+Rap treatment lowered CYTO and MIX protein synthesis compared to Con. There was a main effect of treatment in the CYTO fraction when the data were separated by treatment and by sex. * denotes $P<0.05$ compared to corresponding Con value.

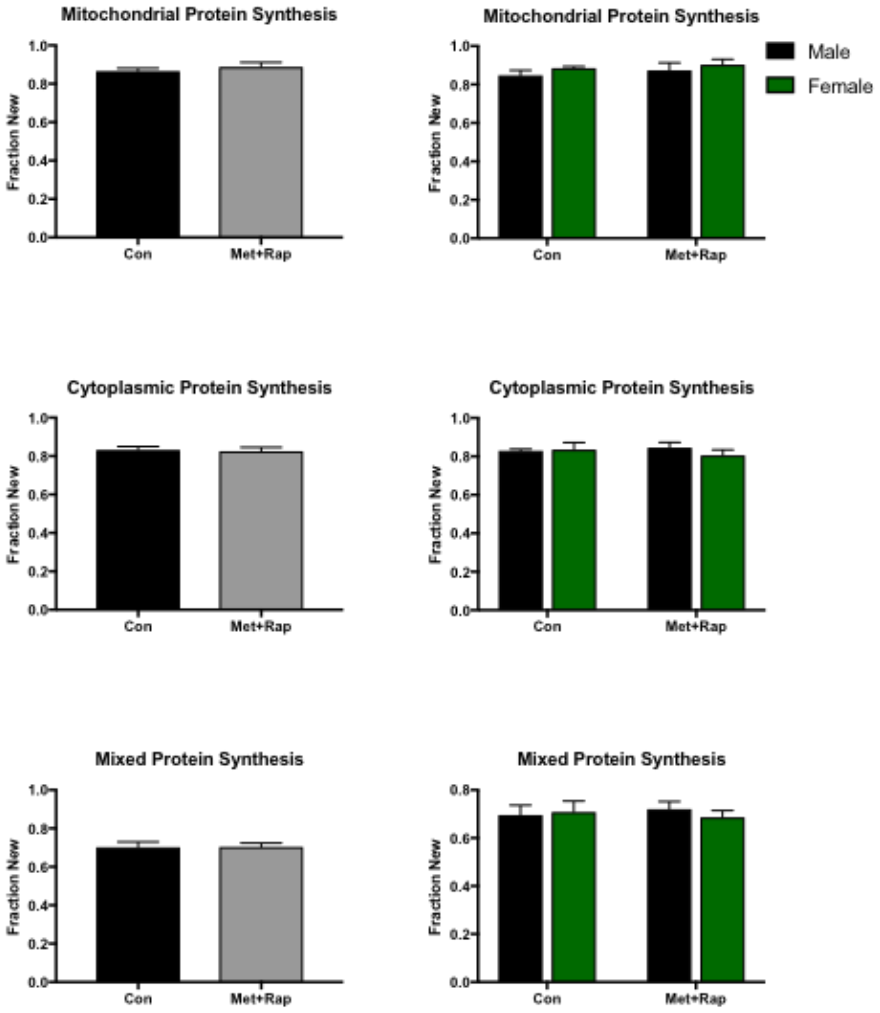

Figure 3.10. Liver Protein Synthesis in MITO, CYTO, and MIX Fractions at 4d of D_2O Labeling. There was no effect of Met+Rap treatment in any fraction when the data were combined. There were no main effects of sex or treatment when the data were separated by sex.

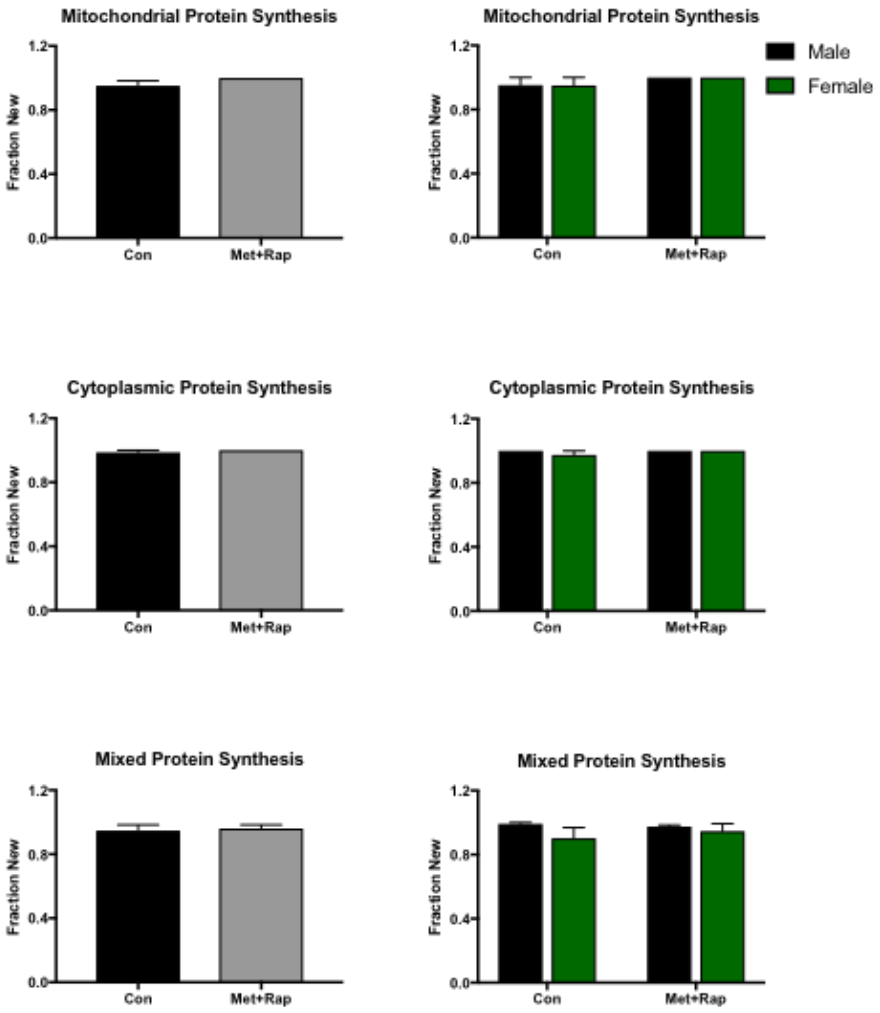

Figure 3.11. Liver Protein Synthesis in MITO, CYTO, and MIX Fractions at 4d of D_2O Labeling. There was no effect of Met+Rap treatment in any fraction when the data were combined. There were no main effects of sex or treatment when the data were separated by sex.

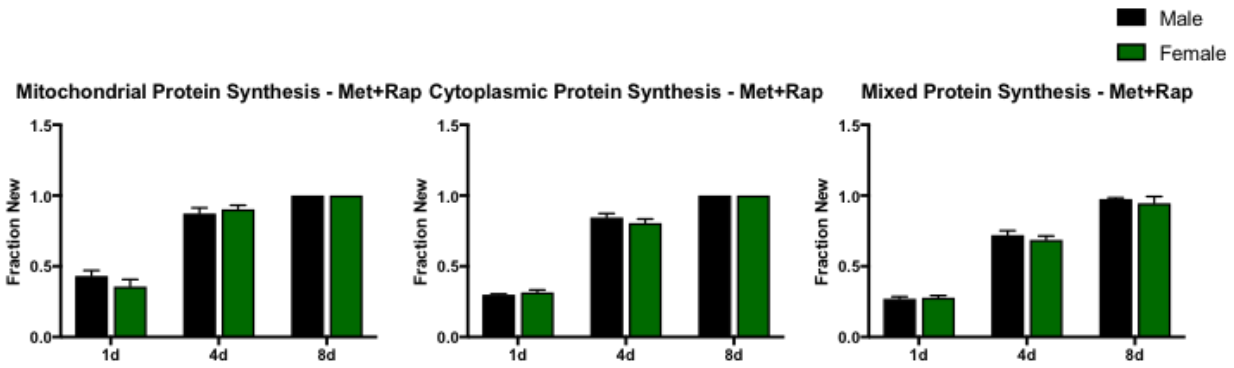

Figure 3.12. Liver Protein Synthesis following Met+Rap Treatment in MITO, CYTO, and MIX Fractions at 1d, 4d, and 8d of D_2O Labeling. There were no differences in how Met+Rap treatment changed protein synthesis in any fraction over time.

CHAPTER 4 – RAPAMYCIN AND METFORMIN IN COMBINATION WITH RAPAMYCIN TREATMENTS ALTER PROTEIN TURNOVER AND CELLULAR PROLIFERATION IN CULTURED SKELETAL MYOTUBES

Aging is a primary non-modifiable risk factor for most chronic diseases (142). Given that by 2050 the population of individuals over the age of 65 will account for 20-40% of the global population (54), identifying interventions that slow the aging process, or slow the age-related decline in cellular function, is an important area of study. Aging is characterized by an accumulation of damaged cellular constituents, including essential protein structures and enzymes. Accumulation of damaged proteins leads to reduced cellular function (154), and enzymatic protein repair mechanisms are limited (158). However, multiple long-lived species have increased mechanisms to maintain protein homeostasis (proteostasis) compared to controls (128).

Our laboratory demonstrated that increasing protein synthesis as an energetic tradeoff for cellular proliferation is a proteostatic mechanism shared by several long-lived murine models (38, 106). Interventions that restrict growth and increase protein turnover may help slow the aging process. Inhibition of the mechanistic target of rapamycin complex 1 (mTORC1) pathway reduces cellular growth by blocking cell cycle progression (30, 44). mTORC1 inhibition also blunts cellular protein translation (30, 44). Multiple investigations have demonstrated that some mRNA are selectively translated despite the effect of mTORC1 inhibition on cap-dependent translation, and these translation products improve cellular stress responses and longevity (48, 144, 152, 201). Thus, interventions that blunt mTORC1 activity may slow the aging process.

The macrolide rapamycin (Rap) is known to inhibit the kinase function of mTORC1 (14, 99, 140). The National Institute on Aging's Interventions Testing Program (NIA-ITP) demonstrated that Rap alone and in combination with the anti-diabetes medication metformin (Met+Rap), increases median lifespan (55, 113, 161). Metformin is known to activate the 5' AMP-activated protein kinase (AMPK), potentially through moderate inhibition of the mitochondrial complex 1 (97), however the precise mechanism has not been confirmed. In the NIA-ITP, Rap alone increased median lifespan by 10-13% in males and 18-21% in females (112). However, Met+Rap increased both male and female median lifespan by ~23% (161).

Rapamycin and metformin have each been shown to activate autophagic flux (155, 166, 176). Autophagy is a catabolic process that recycles intracellular components through lysosomal acidification (28, 183). Autophagic flux declines during the normal aging process (28). Autophagy is required for maintaining intracellular amino acid levels during the lifespan of yeast (3, 123). Autophagy is required for the lifespan-extending effects of lifelong calorie restriction (CR) in *C. elegans* (67). Reduced autophagy may blunt protein synthesis rates through decreased amino acid concentrations. However, limited data have directly assessed the contributions of autophagic flux to protein synthesis rates (75). Autophagy also contributes to degradation of dysfunctional organelles (e.g., mitochondria) to maintain cellular homeostasis (196). Mitochondrial specific autophagy (mitophagy) may directly contribute to mitochondrial proteostasis by isolating damaged portions of the organelle through mitochondrial fission for degradation and recycling (1, 100, 149). To date, however, it is unclear if the beneficial effects of Rap

or Met+Rap treatment on median lifespan are dependent upon autophagic or mitophagic flux.

It is currently unknown how Rap or Met+Rap treatments alter mechanisms of proteostasis, or if autophagic and mitophagic flux are dispensable for altering mechanisms of proteostasis in response to these treatments. Therefore, the purpose of this investigation was to: 1) assess protein turnover and cellular proliferation during treatment with Rap or Met+Rap treatments, and 2) determine how autophagy and mitophagy contribute to protein synthesis, protein breakdown, and cellular proliferation during treatment with Rap or Met+Rap *in vitro*. We hypothesized that Rap or Met+Rap treatments would decrease protein synthesis and protein breakdown while also reducing cellular proliferation. We further hypothesized that Rap and Met+Rap treatments would increase protein:DNA (pro:DNA) ratio, which is a representation of proteostatic mechanisms, compared to control. We also hypothesized that autophagy and mitophagy would be essential for the proteostatic effects of Rap or Met+Rap treatments.

METHODS

Experimental Design and Reagents

C2C12 myotubes were used for each of the following experiments. The C2C12 cell line has been used extensively in our laboratory, and is widely utilized as a model system for skeletal myotubes. Furthermore, we have previously published stable isotope data using the C2C12 cell line (110). For each experiment described below, C2C12 myoblasts were obtained from American Type Culture Collection (ATCC; Manassas, VA) and grown in growth medium (Dulbecco's modified eagle medium (DMEM) + 10% fetal bovine serum + 1% penicillin/streptomycin) in a humidified 37°C, 5% CO_2 incubator. For

both synthesis and breakdown experiments, myoblasts were plated on to 100mm culture plates, grown to approximately 90% confluence, and then differentiated into myotubes using differentiation medium (DM; DMEM containing 2% horse serum and 1% penicillin/streptomycin) for 5 days. During differentiation, medium was replaced every 48h. All treatments were diluted in growth medium prior to adding to plates. Rapamycin was dissolved in DMSO to create a 500μM stock solution, and treatments were applied to the cells at a concentration of 5nM. Metformin was dissolved in DMEM to create a 20mM stock solution, and was sterile filtered prior to being mixed with growth medium to prepare a 2mM solution. Bafilomycin-A1 (Baf) was dissolved in DMSO and used at a oncentration of 100nM. Mdivi-1, a small molecular inhibitor of mitochondrial division (23), was also dissolved in DMSO to a concentration of 10μM. Each of the concentrations selected for use in the following experiments was determined by completing concentration-response experiments to identify the lowest effective dose. Specifically, 5nM Rap was the lowest dose to inhibit RPS6, 2mM Met was the lowest dose to activate AMPK, 100nM Baf was the lowest dose to inhibit autophagic flux, and 10μM Mdivi-1 was the lowest dose to inhibit mitochondrial fission.

Following differentiation, growth medium was added to the control cells (Con), growth medium containing 0.01% DMSO was applied to the vehicle-treated cells (DMSO), and Rap, Met+Rap were applied to treated cells. Rap and Met+Rap without inhibitors were administered to myotubes in one set of experiments, and were also administered in combination with either Baf (e.g., B-Met+Rap) or mdivi-1 (e.g., M-Rap) in subsequent experiments, as described in detail below. All treated cells were harvested using mechanical lysis, and lysates from each plate were divided for the assessment of protein

synthesis or breakdown as well as DNA synthesis or breakdown. An overall experimental timeline is presented in Figure 1.

Protein and DNA Synthesis Experiments

To measure protein and DNA synthesis in C2C12 myotubes, treatments were prepared in growth medium as listed above and supplemented with 10% sterile deuterium oxide (D_2O). Cells were treated in triplicate for each experimental condition at all time points. To assess protein and DNA synthesis in cultured myotubes, C2C12 myotubes were treated for 24 hours with Rap (5nM), or Met+Rap (2mM+5nM) as well as Con and DMSO, and cells were harvested at 4h and 24h in triplicate. Each treatment was applied in growth medium enriched with 10% deuterium oxide to measure the incorporation of deuterium into alanine or deoxyribose as described in detail below.

To assess the contribution of autophagy to protein synthesis rates during Rap and Met+Rap treatments, Baf was used to pre- and co-treat C2C12 myotubes. Four hours prior to the start of the stable isotope labeling, plates that were to be treated with Rap, Met+Rap, or Baf were pre-treated with 100nM Baf. At the end of the pre-treatment, cells were rinsed twice with sterile PBS, and treatments (B-Rap, B-Met+Rap, Baf) were applied with Baf co-treatment in growth medium enriched with 10% deuterium oxide. Control and DMSO cells did not receive Baf at any point during the experiment. The experiment to assess the contributions of mitophagy to protein synthesis was completed as the autophagy experiment, replacing Bafilomycin pre- and co-treatment with Mdivi-1 pre- and co-treatment.

Protein and DNA Breakdown Experiments

To measure protein breakdown in C2C12 myotubes, C2C12 myoblasts were plated onto 100mm plates. Differentiation media for the breakdown experiments consisted of DMEM, 2% horse serum, antibiotics, and the media was enriched to 15% with sterile D_2O. DM was replaced every 48 hours during differentiation over the course of 5 days. Following differentiation, cells were rinsed twice with sterile PBS, and then treated for 24 hours with Rap or Met+Rap as well as Con medium and DMSO and cells were harvested at 0h, 4h, and 24h in triplicate. Each treatment was applied in unenriched growth medium. Treatment concentrations for Rap, Met+Rap, DMSO, Con, Baf, and Mdivi-1 were the same as in the synthesis experiments. For the autophagy and mitophagy inhibition experiments, cells were pre- and co-treated with Baf, or Mdivi-1 as listed in the synthesis experiment above. A triplicate set of cells were harvested at the time of switching medium from 15% enriched to unenriched to determine the starting protein and DNA enrichments for the experiments (i.e., 0h time point). To prevent recycling of tracer from rapidly degraded proteins, media was removed after one hour of treatment, cells were rinsed with sterile PBS, and fresh, D_2O-free media plus treatments was added to rinsed cells.

Preparation of Protein Analytes for Mass Spectrometric Analyses

For all isotope experiments, following 4 and 24h of treatment, cells were harvested in 1.2mL of mitochondrial isolation buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH=7.5), with 200µL of the lysate saved to extract cellular DNA and 1.0mL of the lysate used to isolate mitochondrial and cytosolic enriched protein fractions using differential centrifugation techniques as previously described (111). Specifically, following a slow speed spin (800*g*), the mitochondrial-enriched fraction was

spun at 10,000*g* for 30 minutes, and the supernatant from the mitochondrial spin was split in half. 400 µl of the supernatant from the mitochondrial spin was precipitated using a one-hour incubation with 14% sulfosalicylic acid and a 16,000g spin for 10 minutes, and the other ~400µl frozen at -80°C for future use. The mitochondrial (MITO) and cytoplasmic (CYTO) protein pellets were solubilized in 1M NaOH at 50°C for 15 minutes, and hydrolyzed in 1.5mL 6M HCl for 24 hours at 120°C. Samples then underwent cation exchange chromatography using Dowex resin (AW50, BioRad, Hercules, CA, USA). Amino acids were eluted from the ion exchange column with 4M NH_4OH and dried under vacuum. Amino acid hydrolysates were resuspended in molecular biology grade water and 500 µl of these resuspensions were derivatized in 500 µl acetonitrile, 50 µl 1 M KH_2PO_4 (pH = 11), and 20 µl of pentafluorobenzyl bromide. Samples were then incubated at 100°C for 1 hour and then extracted using ethyl acetate. The organic layer was removed and dried by N_2 and samples were reconstituted in 400 µl of ethyl acetate for analysis. The pentafluorobenzyl-N,N-di(pentafluorobenzyl) derivative of alanine was analyzed on an Agilent 7890A GC coupled to an Agilent 5975C MS as previously described (110).

Preparation of Nucleic Acid Analytes for Mass Spectrometric Analyses

For the assessment of cellular DNA synthesis, we extracted total cellular DNA using a commercially available kit (Qiagen DNA Mini, Qiagen, Valencia, CA, USA) (111). DNA was eluted from the columns contained within the kit using molecular biology grade water warmed to 56°C. Isolated DNA was hydrolyzed overnight (>12h) with nuclease S1 and acid phosphatase from potato, as previously described (38). For derivatization, the pentafluorobenzyl-hydroxylamine hydrochloride derivative was resuspended in 70 µl of

ethyl acetate, placed into tapered glass inserts, and analyzed on an Agilent 7890A GC coupled to an Agilent 5975C MS as previously described (37, 38).

Calculations

The media enrichment for each plate was used to calculate the precursor enrichment for protein and DNA synthesis by using the appropriate mass isotopomer distribution analysis (MIDA) adjustment. Protein and DNA fractional synthesis rates (FSR, %/hr) were calculated by dividing the fraction of newly synthesized proteins or DNA at the time of cell harvest by the duration, in hours, of the treatment period. Mitochondrial and cytoplasmic pro:DNA ratios were calculated by dividing the respective protein fractional synthesis rates by the corresponding DNA fractional synthesis rate from the same plate. Fractional breakdown rates (FBR) for both protein fractions and DNA were calculated based on the fractional change from the starting enrichment (0h) for each treatment over time. The pro:DNA breakdown ratio was calculated by dividing fractional breakdown rates for protein and DNA.

Statistics

Statistical analyses were performed using Prism v7 (GraphPad Software, Inc. La Jolla, CA, USA). One-way analysis of variance (ANOVA) tests with Tukey's multiple comparison adjustments were used to determine the effect of treatment on protein synthesis, DNA synthesis, pro:DNA, protein breakdown rates, or DNA breakdown rates for the synthesis and breakdown experiments individually. One-way ANOVAs were also used to test for differences between Rap, B-Rap, and M-Rap or Met+Rap, B-Met+Rap, and M-Met+Rap to determine if inhibiting autophagy or mitochondrial fission alter proteostatic mechanisms during Rap or Met+Rap treatments. All treatments for these

experiments were diluted in DMSO. Thus, we performed non-parametric t-tests for all non-vehicle control (Con) samples compared to DMSO samples. There were no significant differences between Con and DMSO for protein synthesis, DNA synthesis, pro:DNA, protein breakdown, or DNA breakdown values. Significance was accepted as $P<0.05$ with additional notations for $P<0.01$, $P<0.001$, and $P<0.0001$ as needed. All data are presented as Mean±SEM.

RESULTS

Protein Synthesis, DNA Synthesis, and pro:DNA

Rapamycin and Met+Rap treatments did not change MITO protein synthesis compared to DMSO after 4h (Figure 4.2). However, Rap and Met+Rap treatments significantly lowered ($P<0.0001$) MITO protein synthesis compared to DMSO after 24h (Figure 4.3). Additionally, Met+Rap treatment significantly lowered MITO protein synthesis ($P<0.0001$) compared to Rap at 24h (Figure 4.3). Met+Rap lowered CYTO protein synthesis after 4h of treatment compared to DMSO ($P<0.01$) and Rap ($P<0.01$; Figure 4.2). After 24h, Met+Rap lowered CYTO protein synthesis compared to DMSO ($P<0.001$) and Rap ($P<0.01$), and Rap was not different from DMSO ($P=0.084$; Figure 4.3). Rapamycin and Met+Rap did not alter DNA synthesis after 4h of treatment compared to DMSO (Figure 4.2). Rap ($P<0.001$) and Met+Rap ($P<0.001$) significantly lowered DNA synthesis compared to DMSO after 24h (Figure 4.3).

We utilize the ratio of protein synthesis to DNA synthesis (pro:DNA) to examine whether changes in the former are linked to the existing proteome or to cell proliferation. A change in protein synthesis linked to the proteome is interpreated as an adaptive mechanism for proteostasis. Rap and Met+Rap did not significantly alter MITO pro:DNA

after 4h (Figure 4.2). However, after 24h, Rap significantly increased MITO pro:DNA compared to DMSO ($P<0.01$) and Met+Rap ($P<0.05$; Figure 4.3). Similarly, Rap and Met+Rap did not significantly alter CYTO pro:DNA at 4h compared to DMSO (Figure 4.2). After 24h, Rap significantly increased CYTO pro:DNA compared to DMSO ($P<0.01$) and Met+Rap ($P<0.01$; Figure 4.3).

Protein Breakdown, DNA Breakdown, and pro:DNA

Rap and Met+Rap did not significantly alter MITO protein FBR after 4h of treatment (Figure 4.4). However, after 24h, Rap significantly lowered MITO protein FBR compared to DMSO ($P<0.001$; Figure 4.5). Met+Rap lowered MITO protein FBR compared to DMSO ($P<0.0001$) and was also significantly lowered compared to Rap ($P<0.01$) at 24h (Figure 4.5). Rapamycin and Met+Rap significantly lowered CYTO protein FBR compared to DMSO ($P<0.001$, and $P<0.01$, respectively) after 4h of treatment (Figure 4.4). After 24h of treatment, Rap lowered CYTO protein FBR compared to DMSO ($P<0.001$), while Met+Rap lowered CYTO protein FBR compared to DMSO ($P<0.0001$) and Rap ($P<0.01$; Figure 4.5). Neither Rap, nor Met+Rap treatment altered DNA FBR compared to DMSO at either timepoint (Figures 4.4 and 4.5). There were no significant changes in MITO or CYTO pro:DNA measurements during the breakdown experiments (Figures 4.4 and 4.5).

Contributions of Autophagy and Mitophagy to Protein Breakdown

Bafilomycin-A1 did not alter MITO protein FBR compared to DMSO after 4h (Figure 4.6). However, Baf significantly lowered ($P<0.001$) MITO protein FBR compared to DMSO after 24h (~29%; Figure 4.6). In the CYTO fraction, Baf increased CYTO protein FBR compared to DMSO after 4h ($P<0.01$; Figure 4.6). However, after 24h, Baf lowered CYTO protein FBR compared to DMSO ($P<0.05$; Figure 4.6).

MITO protein FBR was not altered by treatment with Mdivi-1 after 4h or 24h compared to DMSO (Figure 4.7). Similarly, treatment with Mdivi-1 did not alter CYTO protein FBR compared to DMSO at 4h or 24h (Figure 4.7).

Inhibition of Autophagosome Degradation and pro:DNA

Bafilomycin and Rap co-treatment did not change MITO pro:DNA compared to rapamycin alone or DMSO after 24h (Figure 4.9). However, B-Rap significantly increased CYTO pro:DNA compared to Rap ($P<0.001$) and DMSO ($P<0.0001$) after 24h. MITO and CYTO pro:DNA ratios calculated from breakdown experiments were not different between any treatment at 24h (Figure 4.11). Baf treatment did not alter MITO or CYTO pro:DNA at 4h (data not shown).

Inhibition of Mitochondrial Fission and pro:DNA

M-Met+Rap CYTO pro:DNA was significantly lower than Rap alone ($P=0.0427$), as well as M-Rap ($P=0.0234$; Figure 4.10). M-Rap treatment significantly lowered ($P=0.0242$) MITO pro:DNA compared to Rap alone (Figure 4.10). M-Met+Rap treatment significantly lowered MITO pro:DNA compared to Rap alone and Met+Rap alone ($P=0.0006$, and 0.0253, respectively). However, when pro:DNA ratios were calculated from the fractional breakdown rates, there were no significant differences between any treatments and DMSO (Figure 4.11).

DISCUSSION

These are the first data to demonstrate that the lifespan-extending treatments Rap and Met+Rap alter mitochondrial and cytoplasmic protein turnover (synthesis and breakdown), and that inhibition of autophagy or mitochondrial fission alter the Rap-induced decrease in protein turnover. These are the first data to demonstrate that the

lifespan-extending treatment Met+Rap does not have an influence on the pro:DNA during assessments of protein synthesis or breakdown. In accordance with our hypothesis, Rap and Met+Rap treatments decreased protein synthesis and protein breakdown, and concomitantly decreased cellular proliferation. We show, for the first time, that inhibition of autophagic flux decreased mitochondrial protein breakdown rates (29%), with less of an effect on cytoplasmic protein breakdown. These data demonstrate that autophagy is responsible for ~20-30% of mitochondrial protein degradation in cultured skeletal myotubes. Mitochondrial fission contributes to mitochondrial protein degradation when autophagy is activated by Met+Rap, but not Rap treatment. Together, these data suggest that Rap treatment increase one aspect of proteostatic mechanisms, and that while autophagy and mitochondrial fission contribute to cellular proteostasis, they are not essential processes for the beneficial effects of Rap treatment on the protein turnover component of proteostasis in cultured skeletal myotubes.

Rap and Met+Rap Treatment Alter Protein Turnover

Proteostasis is a coordination of processes designed to maintain a functional proteome. Protein turnover is the balance between protein synthesis and protein degradation, and is a key mechanism of proteostasis, as enzymatic protein repair systems are limited (13, 106). We utilized D_2O to isotopically label proteins and DNA in cultured skeletal myotubes and assess protein and DNA synthesis and breakdown. In the current investigation, Rap, but not Met+Rap treatment, increased mitochondrial and cytoplasmic pro:DNA, an indication of how protein turnover contributes to proteostasis. The lack of an effect of Met+Rap on pro:DNA was surprising, as we hypothesized that Met+Rap would also increase pro:DNA ratio. We arrived at this hypothesis because

Met+Rap treatment increases median lifespan to a greater extent than Rap alone (161), and increased protein synthesis as a mechanism of proteostatic maintenance is a shared characteristic of long-lived models (51). Although we did not detect increased pro:DNA following Met+Rap treatment in the current investigation, loss of proteostasis is only one of seven pillars of aging (96). Thus, while Met+Rap did not increase pro:DNA in the current investigation, Met+Rap treatment may reduce the accumulation of intracellular damage, reduce inflammation, contribute to stem cell regeneration, or improve one of the other pillars of aging to increase lifespan. Furthermore, investigations using Met+Rap treatment are limited. Investigating how Rap alone and Met alone influence proteostatic processes in other tissues or cells will provide insight into the potential mechanisms of Met+Rap on proteostasis.

Individually, both Rap treatment and Met treatment increase mechanisms of proteostasis, and have been demonstrated to reduce age-related decline in physiological function (94, 172). One previous investigation in mouse skeletal muscle and in cultured cells concluded that Rap treatment significantly reduces mitochondrial biogenesis, as measured by transcription of mitochondrial genes (29). However, data from the current investigation, refute that Rap has a negative impact on mitochondrial biogenesis. Specifically, in the current study we found that Rap treatment significantly increased the mitochondrial pro:DNA compared to control (Figure 4.3). Additionally, previous data from our laboratory revealed that 12 weeks of Rap treatment increased mitochondrial biogenesis and pro:DNA in young mice (38). We have previously reported that mitochondrial protein synthesis is a more appropriate measure of mitochondrial biogenesis than gene transcription and protein content measures (107).

Together, our data demonstrate that Rap and Met+Rap treatments lowered protein synthesis rates and protein breakdown rates compared to control. We also found that Rap and Met+Rap treatments reduced DNA synthesis and DNA breakdown compared to control. These data suggest that Rap and Met+Rap treatments influence cellular proteostasis through a reduction in protein turnover as well as cellular proliferation, allowing the cells to allocate energetic resources to maintaining existing protein structures. Specifically, the reduced DNA synthesis and breakdown measured following Rap and Met+Rap treatments indicate less change in total cell content, and thus the total protein pool that must undergo turnover is reduced. Therefore, Rap and Met+Rap increase the protein turnover component of proteostasis by reducing cellular proliferation. Interestingly, previous data suggest that rapamycin treatment accelerates protein breakdown (197); however, rapamycin is an allosteric inhibitor of the proteasome (124). Although rapamycin treatment stimulates autophagy, its inhibition of the proteasome system may slow protein breakdown as we show in the current study. Thus, interventions that reduce cellular proliferation without reducing protein synthesis are ideal candidates for improving cellular maintenance of proteostasis, and therefore potentially lifespan.

Contributions of Autophagy to Protein Turnover

Autophagic flux is a complex process, that is responsible for bulk degradation of cellular components (130). Autophagy was initially described as a cellular response to starvation (123). Specifically, autophagic flux increases during periods of low energy availability to recycle cellular components into energy sources (114). For skeletal muscle, autophagic flux is essential for maintaining both glucose and amino acid concentrations, as well as mitochondrial function and overall muscle integrity during the aging process

(19). In the current experiment, we found that inhibiting autophagic flux consistently reduced mitochondrial protein breakdown rates, but transiently increased cytoplasmic protein breakdown rates (Figure 4.6). These data are surprising, as cytoplasmic proteins are primarily degraded by the ubiquitin proteasome system (33, 92), while mitochondria are degraded primarily through organelle-specific autophagy (188). However, acute autophagic inhibition stimulates the ubiquitin-proteasome system (UPS (95).

The effect of Baf treatment on pro:DNA demonstrate that autophagic flux does not contribute to the increased protein turnover component of proteostasis associated with Rap or Met+Rap treatments (Figures 4.10 and 4.111). However, Baf treatment has a profound effect on cytoplasmic pro:DNA during co-treatments with Rap and Met+Rap (B-Rap and B-Met+Rap; Figure 4.10). There were limited effects of B-Rap and B-Met+Rap on cytoplasmic protein synthesis rates, while B-Rap and B-Met+Rap robustly lowered DNA synthesis rates (Figure 4.8). The significant decrease in DNA synthesis following Baf, B-Rap, and B-Met+Rap treatments is the result of Baf, Rap, and Met all inhibiting cell cycle progression (30, 185, 198). Together, the significant increases in cytoplasmic pro:DNA synthesis during B-Rap and B-Met+Rap treatment in combination with no change of the cytoplasmic pro:DNA breakdown suggest that cytoplasmic proteins are degraded and resynthesized despite autophagic inhibition. These data suggest that additional mechanisms contribute to cytoplasmic protein turnover, demonstrating the complexity of the proteostasis network.

One potential explanation for the robust decrease in DNA synthesis measured during treatment with Baf is an increase in apoptosis. Previous data demonstrate that application of Baf to mesangial cells caused mitochondrial depolarization and apoptosis

(186). The loss of mitochondrial membrane potential could be due to the potassium ionophore-like effect of Baf seen at high doses (167). In addition to mitochondrial depolarization, previous studies report an intricate relationship between autophagic and apoptotic signaling (40). Further investigation is needed to verify the mechanism through which autophagic inhibition alters cellular proliferation and proteostasis.

Contributions of Mitochondrial Fission to Protein Turnover

Mitochondrial fission is a multi-step process that is required to regulate mitochondrial morphology as well as maintain a functional mitochondrial reticulum (190). The outer mitochondrial membrane is constricted by dynamin-related protein 1 (Drp-1) a GTPase, which is recruited to the outer mitochondrial membrane by the fission-1 (Fis1) protein or mitochondrial fission factor (Mff) (173). Mdivi-1 is a small molecule inhibitor of Drp-1 (194). Mdivi-1 treatment has been shown to have a protective role against excessive mitochondrial fission in neurons (135). Mdivi-1 is a reversible inhibitor of mitochondrial complex 1, which increases oxidant production in the cell. Thus, the increased mitochondrial protein degradation rate we observed during Mdivi-1 treatment alone may be due to increased oxidation of proteins. Specifically, oxidative modifications to proteins increase the degradation of these proteins through the 20S proteasome system (2). During periods of moderate oxidative stress, autophagic flux is also activated (43). Thus, Mdivi-1 treatment may have increased proteasomal degradation of proteins as well as potentially increasing autophagic flux, both contributing to the increased protein turnover found in the current study.

However, Drp-1 knockout is embryonically lethal, as Drp-1 is required for synapse formation during development (64). Genetic defects in the function of Drp-1 have been

demonstrated to increase resistance to apoptosis as well as autophagic flux (168). Parkin is an E3 ubiquitin ligase that is recruited to the outer mitochondrial membrane (OMM) to initiate the mitophagy process by ubiquitinating OMM proteins, leading to the recruitment of p62 and LC-3 (163). This clustering of p62 and LC-3 at ubiquitin-tagged proteins on the OMM an essential first step of mitophagy (148). Drp-1 is required for mitophagy in a Parkin-independent manner (70). Regardless of how mitophagy is initiated, it contributes to turnover of mitochondrial proteins (39, 171). To our knowledge, no investigations have reported the specific effect of Mdivi-1 treatment on protein synthesis nor breakdown rates.

The findings from the current study demonstrate that Mdivi-1 treatment in cultured skeletal myotubes does not alter the rate of mitochondrial or cytoplasmic protein degradation (Figure 4.7). When myotubes received M-Rap or M-Met+Rap treatments, which activate autophagy but inhibit mitochondrial fission, mitochondrial protein breakdown rates were increased compared to Rap and Met+Rap treatments alone (Figure 4.9). These data suggest that mitochondrial fission is not a limiting step for mitochondrial protein degradation during Rap and Met+Rap treatments. Specifically, if mitochondrial fission were a primary process preceding mitochondrial protein degradation, Mdivi-1 treatment would prevent or extremely reduce mitochondrial protein degradation. Additionally, recent data indicate that mitochondrial proteins are not exclusively degraded through autophagic flux, which helps explain the increased protein breakdown rate despite inhibition of mitochondrial fission in the current investigation (57, 188). We also found that for pro:DNA measured by synthesis and by breakdown, Mdivi-1 co-treatment did not increase pro:DNA compared to control, nor did it lower the pro:DNA compared to control or Rap or Met+Rap treatments alone. These findings suggest that Rap and

Met+Rap treatments influence components of proteostasis by diverse mechanisms, as mitochondrial fission does not limit mitochondrial protein turnover in cultured skeletal myotubes.

Perspectives and Conclusions

Overall, our data suggest that treatments that reduce cellular proliferation and protein synthesis rates increase pro:DNA, indicating increased allocation of protein synthesis towards maintaining cellular structures. We show for the first time, in vitro, that Met+Rap, a lifespan-extending treatment, does not increase pro:DNA in cultured skeletal myotubes despite robustly lowering protein synthesis and cellular proliferation. We also demonstrate that autophagy is not the only process responsible for the positive effects of Rap treatment on mitochondrial or cytoplasmic protein turnover. Additionally, we demonstrate that mitochondrial fission is not a limiting process for mitochondrial protein degradation. The data from this investigation provide important mechanistic insights into how Rap and Met+Rap treatments influence protein turnover as well as the pro:DNA ratio.

FIGURES

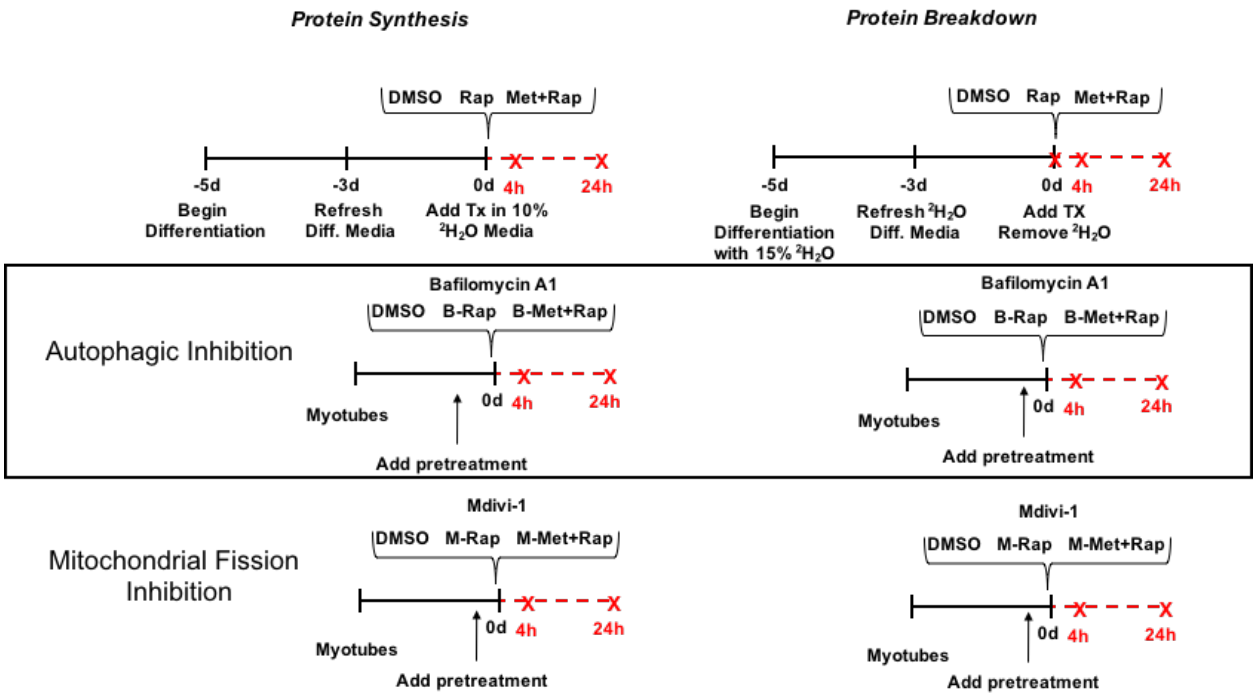

Figure 4.1. Study Timeline and Experimental Layout. Protein synthesis experimental layouts are presented on the left side of the figure. C2C12 myotubes were treated with DMSO, Rap, or Met+Rap for 24h in growth medium containing 10% D2O. Cells were harvested at 4h and 24h in triplicate. For the autophagy inhibition experiment, myotubes were pre-treated with Baf for four hours (except DMSO), and then received co-treatment with Baf for 24h. Cells were harvested at 4h and 24h. The same experimental design was used for the mitochondrial fission inhibition experiment, replacing Baf with Mdivi-1. For protein breakdown experiments (right panels), myoblasts were differentiated in media containing 15% D2O. Treatments were applied in media containing no D2O for 24h, with cells being harvested at 4h and 24h. For the autophagic inhibition and mitochondrial fission inhibition experiments, Baf and Mdivi-1 pre-treatments contained 15% D2O. At the start of treatments, all D2O was removed and treatments were applied in media free from added D2O. Cells were treated for 24h and harvested at 0h, 4h, and 24h.

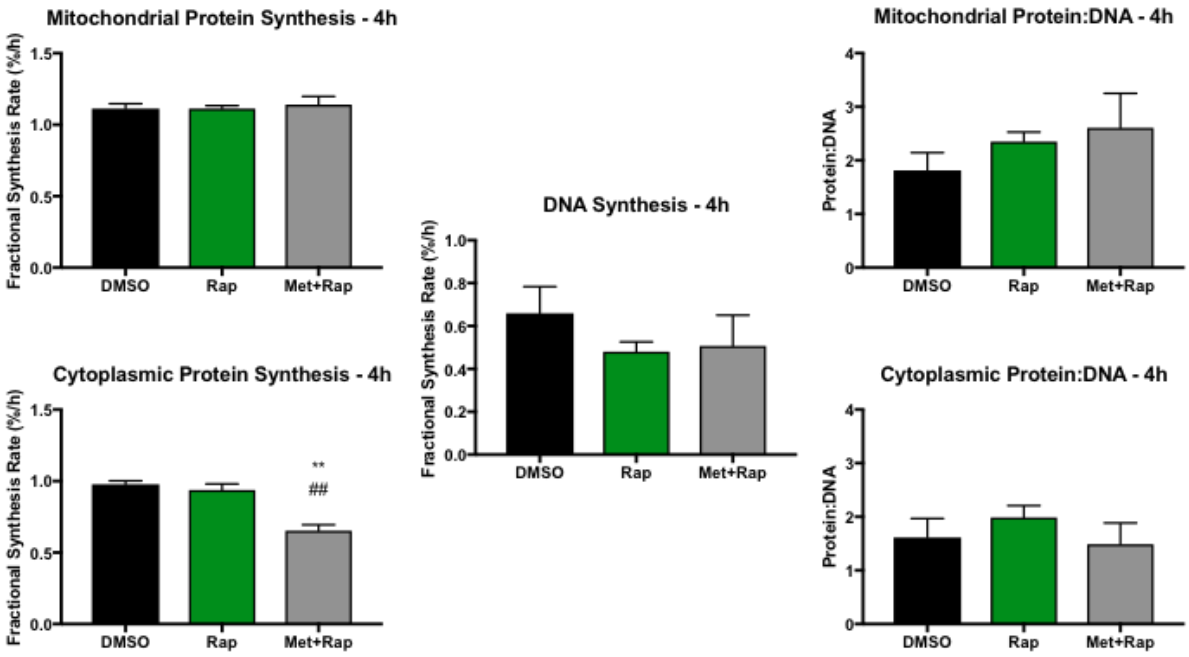

Figure 4.2. Protein Synthesis, DNA Synthesis, and Protein:DNA for MITO and CYTO Fractions after 4h of Rap or Met+Rap Treatments. In the CYTO fraction, Met+Rap lowered protein synthesis rate compared to Rap and DMSO treated cells. Cellular DNA synthesis rates were not different between treatments. There were no treatment differences in Protein:DNA for either fraction. ** denotes P<0.01 compared to corresponding DMSO value. ## denotes P<0.01 compared to corresponding Rap-treated value.

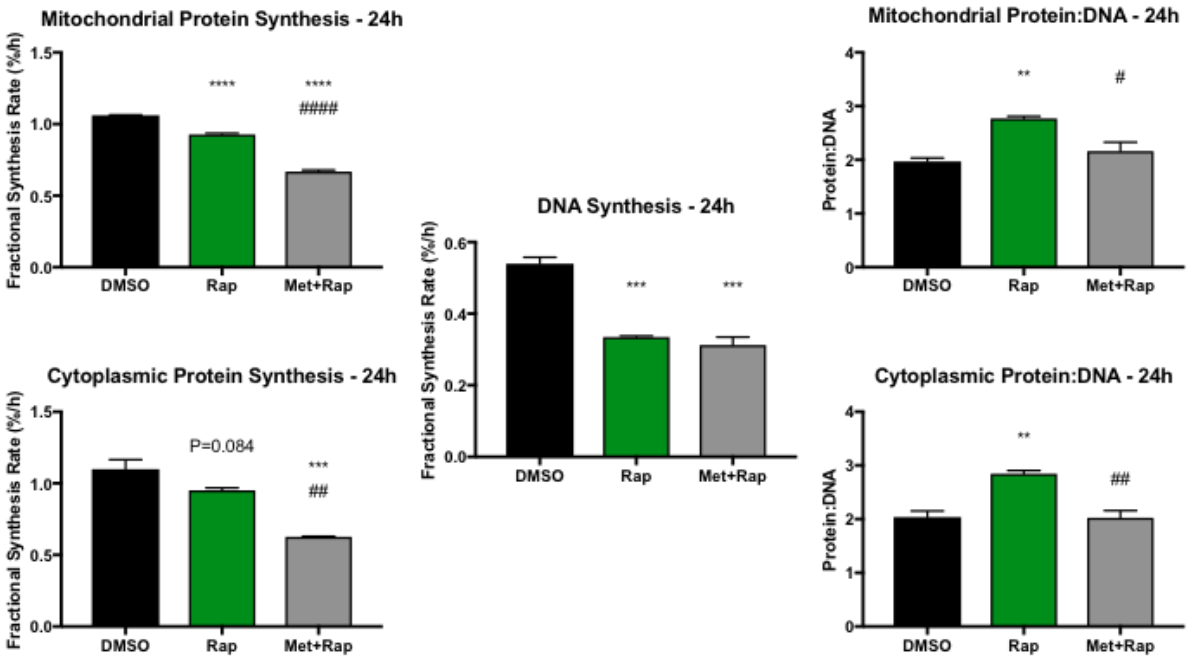

Figure 4.3. Protein Synthesis, DNA Synthesis, and Protein:DNA for MITO and CYTO Fractions after 24h of Rap or Met+Rap Treatments. Both Rap and Met+Rap lowered MITO protein synthesis rate compared to DMSO treated cells, and Met+Rap lowered MITO protein synthesis rate compared to Rap treated cells. Met+Rap lowered CYTO protein synthesis rate compared to both Rap and DMSO treated cells. Both Rap and Met+Rap treatments lowered cellular DNA synthesis rates compared to DMSO. In both MITO and CYTO fractions Rap treatment increased protein:DNA compared to DMSO and compared to Met+Rap treated cells. ** denotes $P<0.01$ compared to corresponding DMSO value. *** denotes $P<0.001$ compared to corresponding DMSO value. # denotes $P<0.05$ compared to corresponding Rap-treated value. ## denotes $P<0.01$ compared to corresponding Rap-treated value. #### denotes $P<0.0001$ compared to corresponding Rap-treated value.

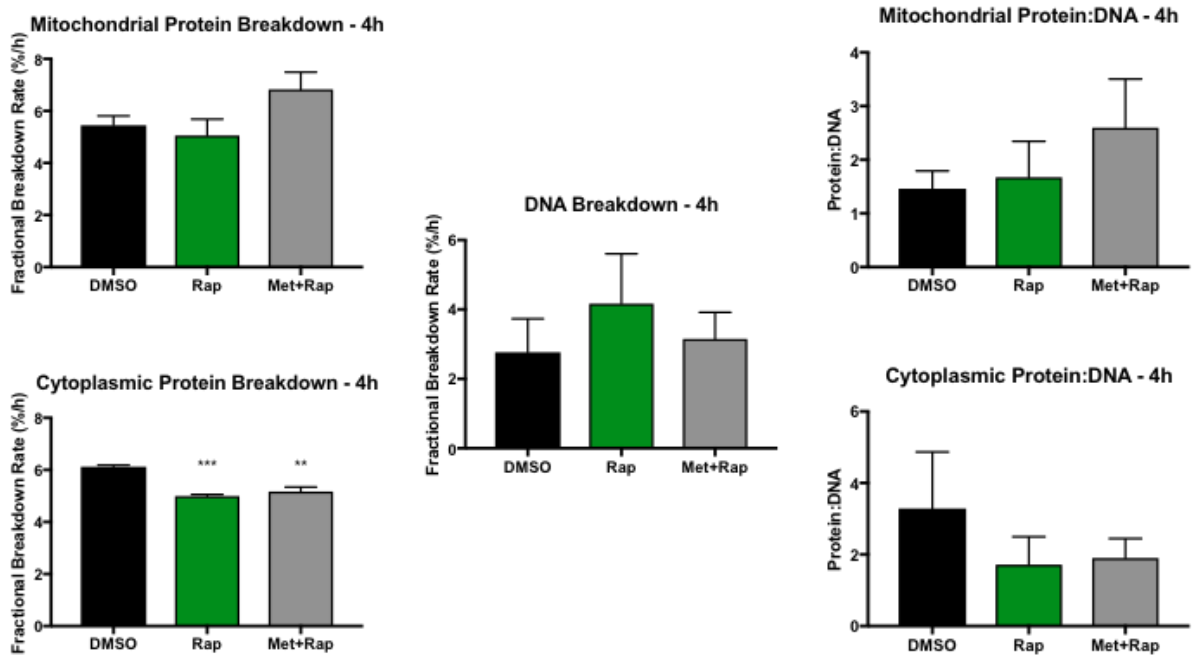

Figure 4.4. Protein Breakdown, DNA Breakdown, and Protein:DNA for MITO and CYTO Fractions after 4h of Rap or Met+Rap Treatments. Both Rap and Met+Rap lowered CYTO protein breakdown rate compared to DMSO treated cells, but did not change MITO protein breakdown. There were no significant differences in DNA breakdown or protein:DNA with any treatment. ** denotes P<0.01 compared to corresponding DMSO value. *** denotes P<0.001 compared to corresponding DMSO value.

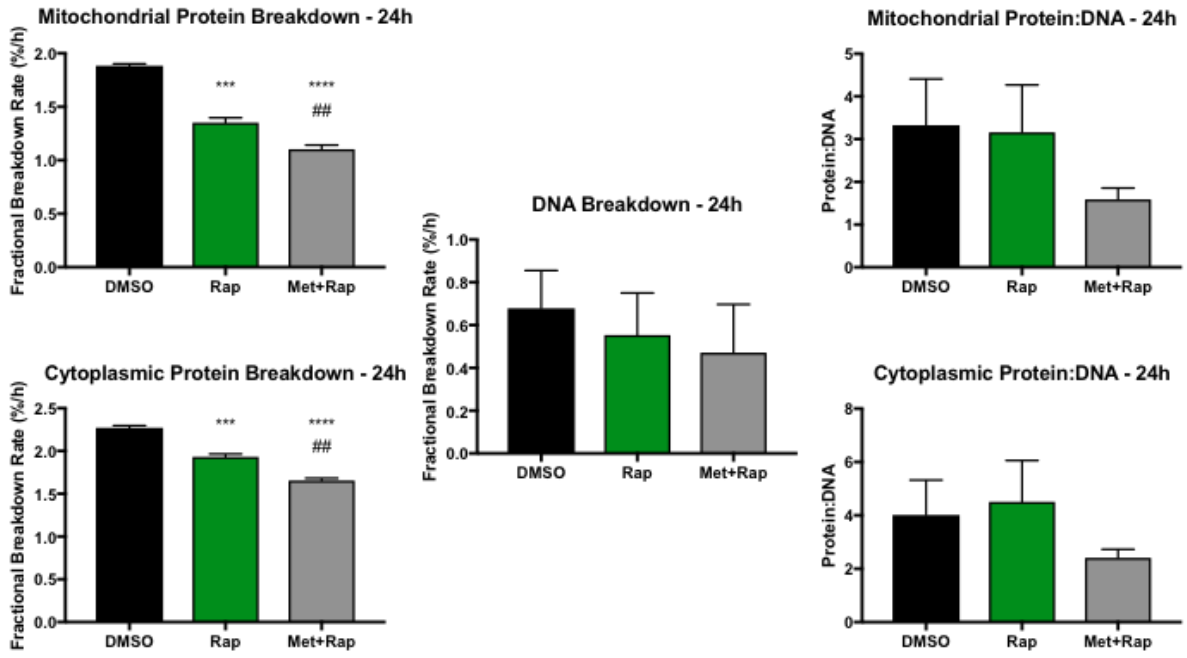

Figure 4.5. Protein Breakdown, DNA Breakdown, and Protein:DNA for MITO and CYTO Fractions after 24h of Rap or Met+Rap Treatments. Both Rap and Met+Rap lowered MITO and CYTO protein breakdown rates compared to DMSO treated cells, and Met+Rap lowered both MITO and CYTO protein breakdown rates compared to Rap treated cells. There were no significant differences in DNA breakdown or protein:DNA with any treatment. *** denotes $P<0.001$ compared to corresponding DMSO value. **** denotes $P<0.0001$ compared to corresponding DMSO value. ## denotes $P<0.01$ compared to corresponding Rap treated cells.

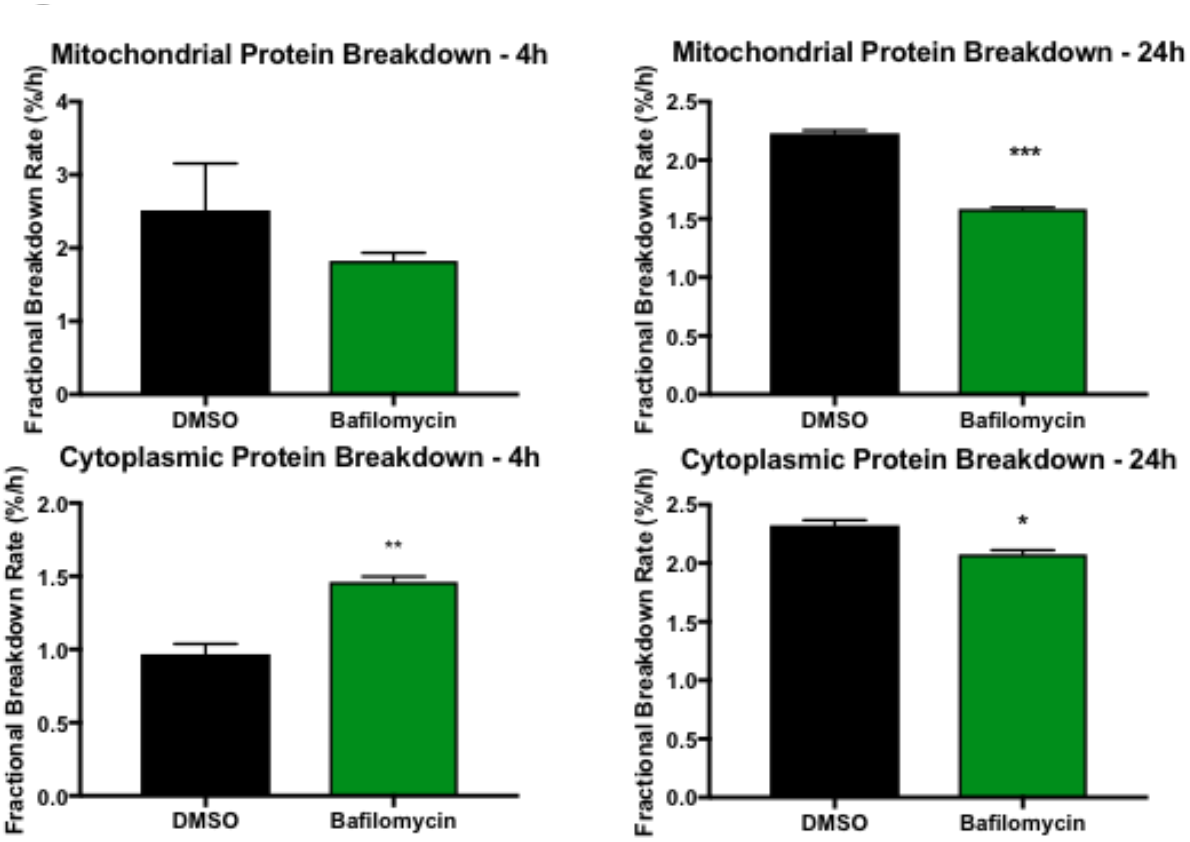

Figure 4.6. MITO and CYTO Protein Breakdown during Autophagic Inhibition. There was no effect of Baf treatment on MITO protein breakdown at 4h, while Baf treatment increased CYTO protein breakdown rate at 4h compared to DMSO. However, Baf treatment lowered MITO and CYTO protein breakdown rates at 24h. * denotes $P<0.05$ compared to corresponding DMSO value. ** denotes $P<0.01$ compared to corresponding DMSO value. *** denotes $P<0.001$ compared to corresponding DMSO value.

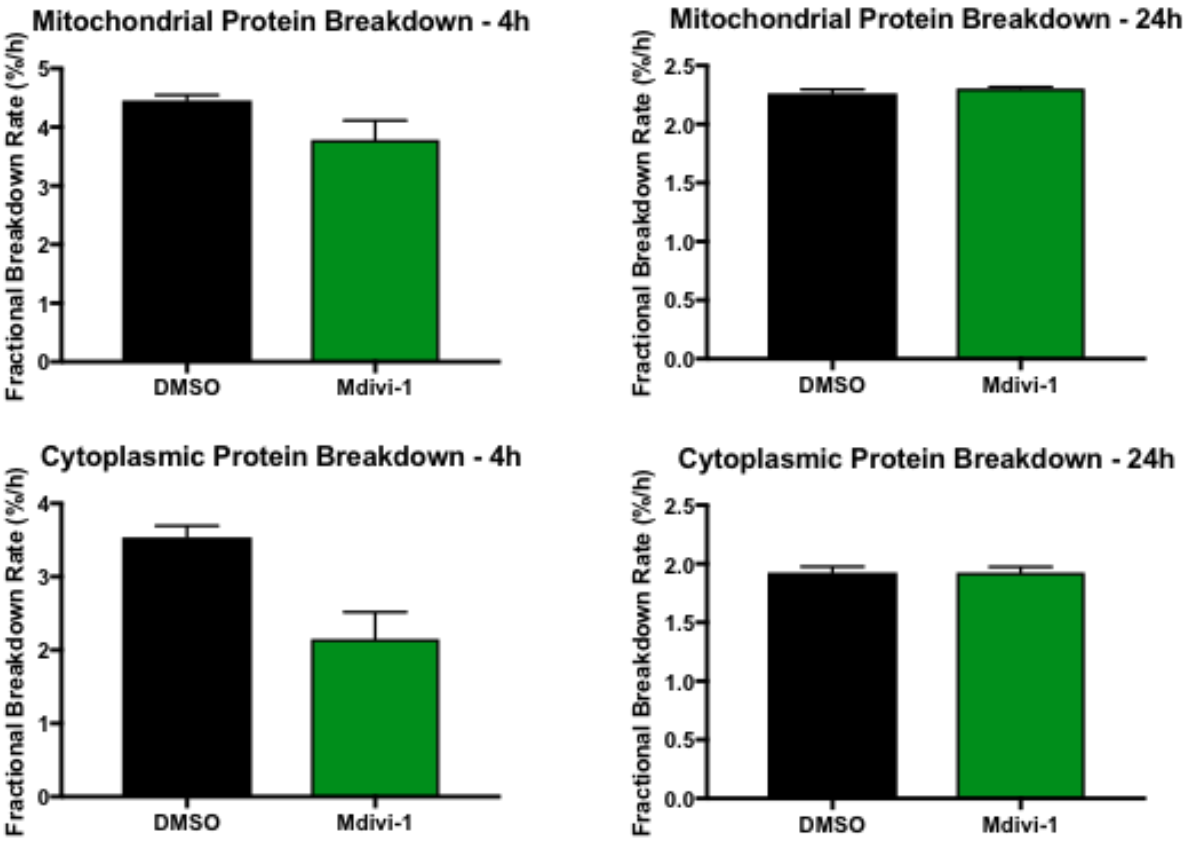

Figure 4.7. MITO and CYTO Protein Breakdown during Inhibition of Mitochondrial Fission. There was no effect of Mdivi-1 treatment on MITO or CYTO protein breakdown rates compared to DMSO at 4h or 24h.

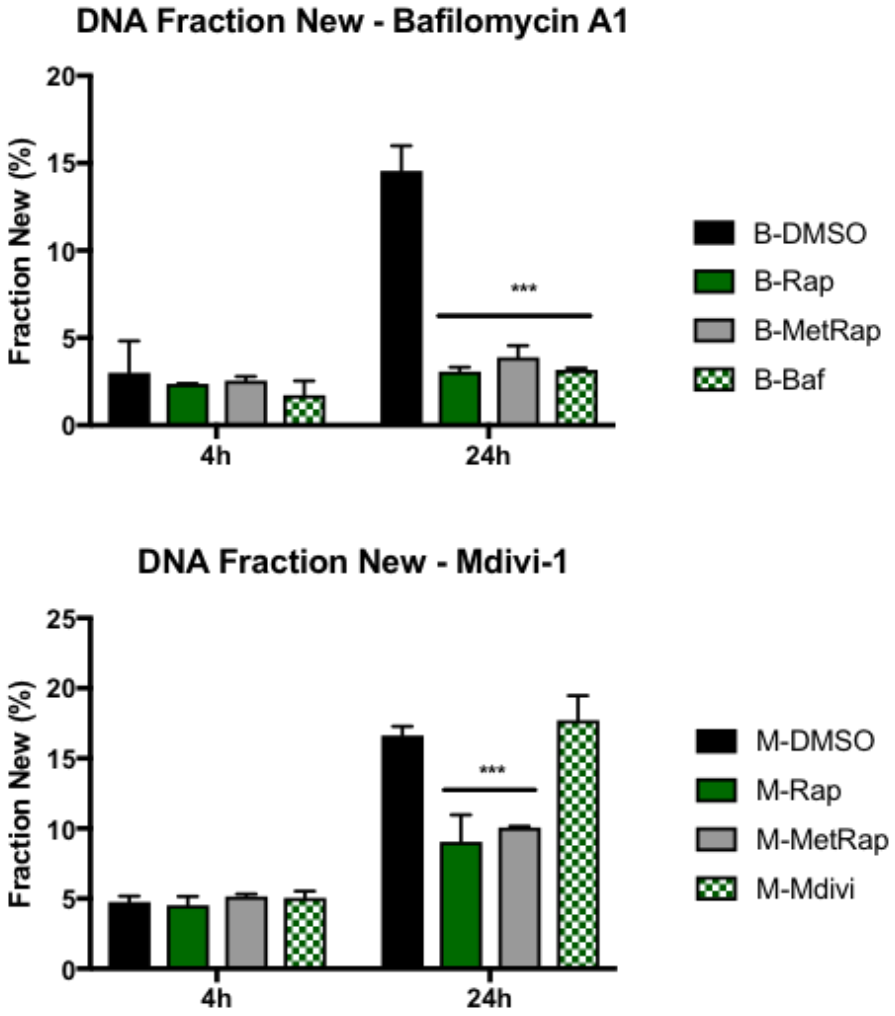

Figure 4.8. Effect of Baf and Mdivi-1 co-treatment with Rap and Met+Rap on DNA synthesis. There were no significant differences in DNA synthesis with any treatment at 4h. After 24h, B-Rap, B-Met+Rap, and Baf treatments lowered DNA synthesis compared to DMSO. While M-Rap and M-Met+Rap lowered DNA synthesis compared to DMSO and to Mdivi-1 alone. *** denotes $P<0.001$ compared to corresponding DMSO treated cells.

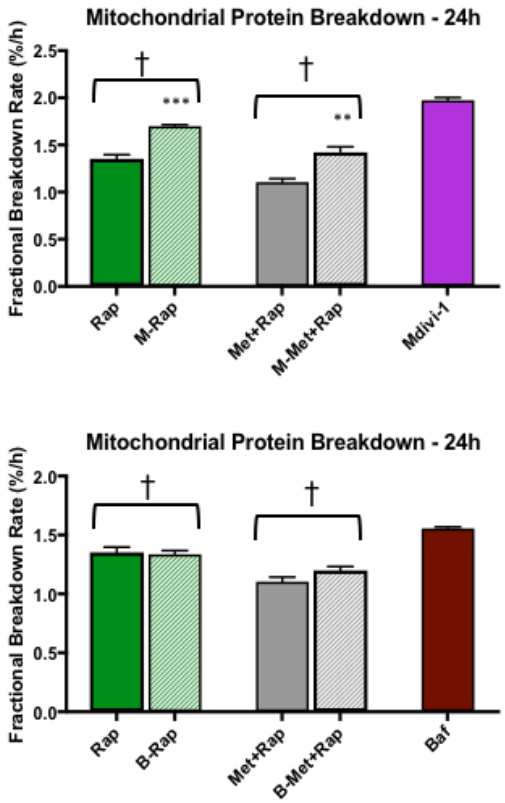

Figure 4.9. Effect of Baf and Mdivi-1 co-treatment with Rap and Met+Rap on MITO protein breakdown rates after 24h. Co-treatment of cells with Mdivi-1 and Rap (M-Rap) increased MITO protein breakdown rate compared to Rap alone. Mdivi-1 and Met+Rap co-treatment (M-Met+Rap) also increased MITO protein breakdown rate compared to Met+Rap alone. Mdivi-1 treatment alone had the highest MITO protein breakdown rate of the 5 groups. There were no effects of Baf co-treatment on MITO protein breakdown rates. Baf treatment alone had the highest MITO protein breakdown rate of the 5 groups. ** denotes $P<0.01$ compared to corresponding Rap or Met+Rap treated cells. *** denotes $P<0.001$ compared to corresponding Rap or Met+Rap treated cells. † denotes $P<0.05$ compared to Mdivi-1 or Baf treated cells.

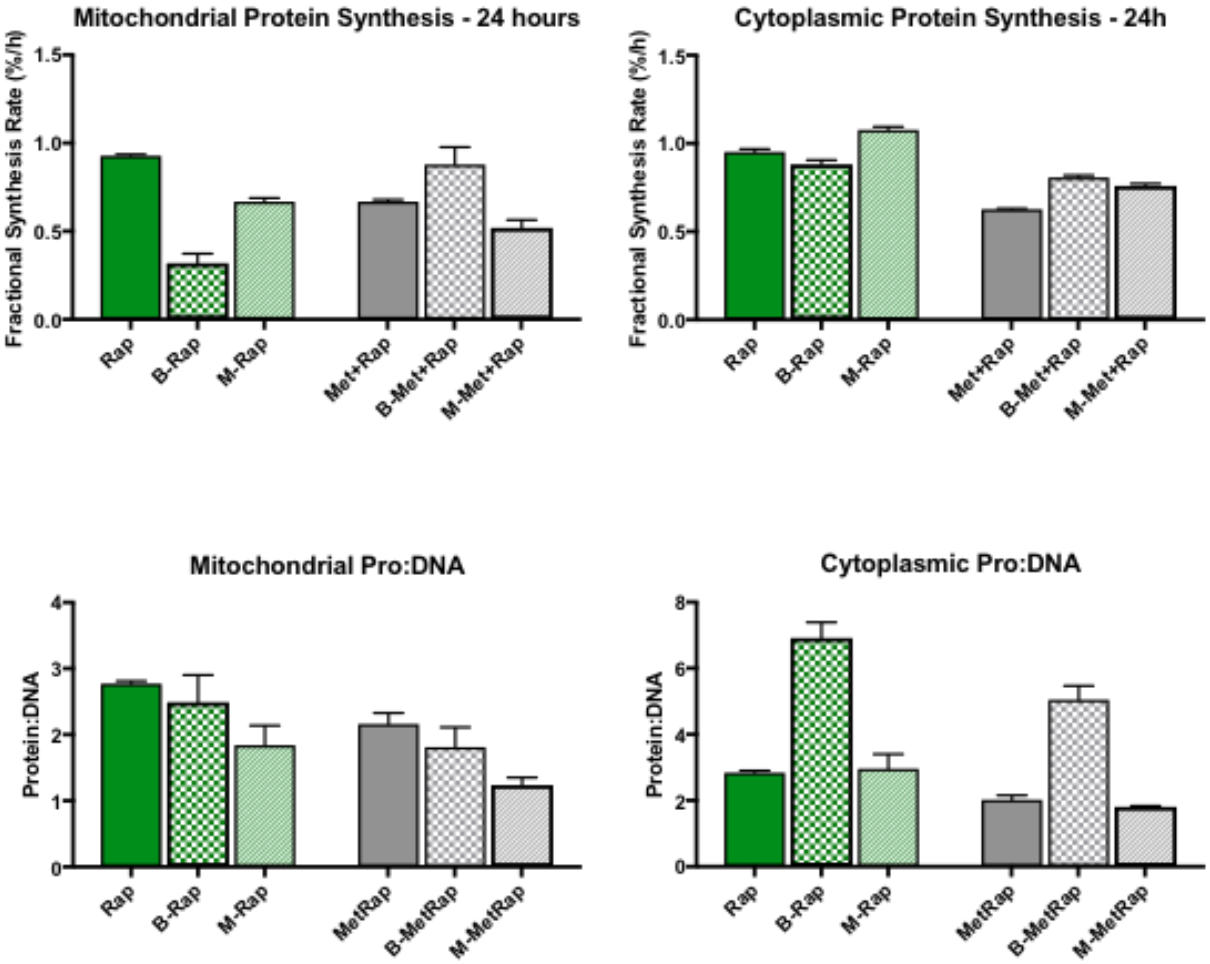

Figure 4.10. Effect of Baf and Mdivi-1 co-treatment with Rap and Met+Rap on MITO and CYTO protein:DNA after 24h. M-Rap treated cells lowered MITO protein:DNA compared to Rap treated cells. M-Met+Rap treated cells lowered MITO protein:DNA compared to Met+Rap treated cells. * denotes $P<0.05$ compared to corresponding Rap or Met+Rap treated cells.

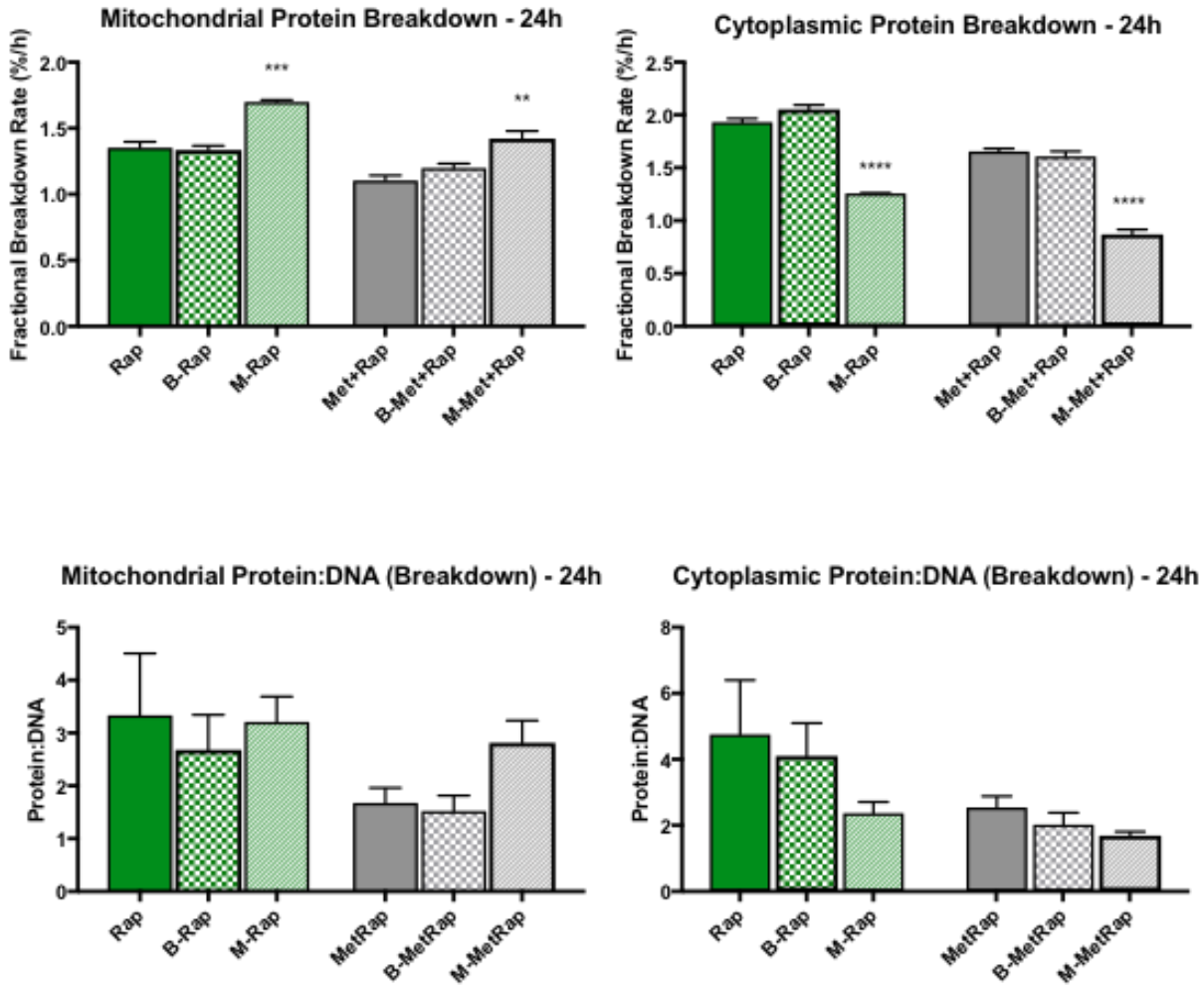

Figure 4.11. Effect of Baf and Mdivi-1 co-treatment with Rap and Met+Rap on MITO and CYTO protein:DNA after 24h. M-Rap treated cells lowered MITO protein:DNA compared to Rap treated cells. M-Met+Rap treated cells lowered MITO protein:DNA compared to Met+Rap treated cells. * denotes $P<0.05$ compared to corresponding Rap or Met+Rap treated cells.

CHAPTER 5 – OVERALL CONCLUSIONS

In the current collection of experiments, we measured the effects of rapamycin (Rap) and metformin (Met) in combination with Rap (Met+Rap) on protein synthesis in three tissues from young animals, the effect of Met+Rap treatment on protein synthesis in three tissues from old animals, and finally the effect of Rap and Met+Rap on protein synthesis and breakdown in cultured skeletal myotubes. Collectively, these experiments demonstrate that the lifespan-extending treatments Rap and Met+Rap have an age- and tissue-specific effect on mitochondrial protein synthesis. We also demonstrate that inhibition of autophagy or mitochondrial fission do not prevent the positive effects of Rap and Met+Rap on protein turnover, in vitro. The purpose of this chapter is to discuss the age-related effects of Met+Rap treatment, and subsequently discuss insights gained from cultured skeletal myotubes treated with Rap and Met+Rap.

Both Rap and Met+Rap treatments are known to inhibit the mTORC1 signaling pathway while activating AMPK signaling (38, 121). In isolation, such a signaling response would be expected to significantly decrease protein synthesis. Specifically, mTORC1 regulates cap-dependent translation through its phosphorylation of 4E-BP1 (169). Additionally, by phosphorylating ribosomal protein S6 kinase, mTORC1 regulates ribosome biogenesis, another contributing process to translational regulation (63). Despite the inhibition of cap-dependent protein translation by 4EBP1, increased 4EBP activity in D. melanogaster allows for translation of certain mRNA transcripts (201), in particular mitochondrial related transcripts.

Age-Related Differences in Met+Rap Treatment

To our knowledge, there are few published investigations that have examined Met+Rap treatment (26, 117, 145, 161). Three investigations examined the influence of Met+Rap treatment on cancer cell proliferation, while another demonstrated that Met+Rap treatment increases the median and maximal lifespan of male and female mice (161). Thus, the two in vivo experiments from this dissertation provide the first evidence that Met+Rap treatment causes age-related differences in protein synthesis in genetically heterogeneous mice.

We demonstrate that protein synthesis is higher in older animals in all three fractions of the heart (Figures 5.1 and 5.2) and liver (Figures 5.3 and 5.4), and in the cytoplasmic and mixed fraction of the skeletal muscle (Figures 5.5 and 5.6) compared to young animals. It is important to note, however, that these data are from two separate experiments, although conditions were controlled to be similar in both experiment. Within each experiment, however, Met+Rap treatment did not alter mitochondrial protein synthesis in old mice compared to control. However, Met+Rap treatment significantly reduced mitochondrial protein synthesis in the skeletal muscle of young mice. One potential explanation for higher protein synthesis rates in old mice compared to young mice is that cytochrome p450 detoxification enzyme function declines with age as the result of post-translational modification (180). Thus, it is possible that the Met+Rap treatment was not fully processed in older mice.

Previous investigations suggest that liver protein turnover declines with age (178). However, we show that protein synthesis rates are higher in the liver of older mice compared to young mice. In support of increased liver protein turnover during aging, we revealed that mitochondrial proteins in the old animal liver were fully turned over by day

8, compared to day 15 in the young animals. Our data suggest that protein turnover increases with age, a potential mechanism through which liver cells prevent the age-related accumulation of protein damage (11, 139). Specifically, cellular protein damage is removed by protein turnover because enzymatic protein repair is limited (158). Furthermore, we demonstrate that Met+Rap treatment in the old animals did not reduce protein synthesis in any fraction of the liver, while Met+Rap lowers protein synthesis in the young animal liver tissue. Considering that there is an age-related decline in proteostasis (16, 165), our data support the potential use of Met+Rap treatment to slow or reverse this age-related decline in proteostasis.

Tissues within an organism have divergent functions as well as different rates of protein turnover (52). Specifically, liver proteins turn over more rapidly than heart and skeletal muscle proteins (52). Aging is associated with an increased accumulation of damaged proteins in the liver (24), heart (103), and skeletal muscle (9). We found that Rap and Met+Rap treatments lowered protein synthesis in all three fractions of the skeletal muscle of young mice, but Met+Rap treatment only lowered cytoplasmic and mixed protein fractions in old mice. Decreased protein synthesis rates may increase the fidelity of translation. More specifically, slowed translation rates allow for more extensive co-translational chaperone binding to the nascent polypeptide chain, improving the folding of proteins and therefore reducing the protein load on the cell (147). Furthermore, slower rates of translation accompanied by improved protein folding reduce the potential for oxidative modifications to proteins (91). Thus, the lower protein synthesis rates following Met+Rap treatment in both age mice may improve protein folding to prevent oxidative damage to proteins.

Mitochondria are the primary source of reactive oxygen species (ROS) in the cell. Rapamycin and Metformin have differential effects on ROS production. Specifically, Rap treatment decreases mitochondrial ROS production in culture (50), while Met treatment increases ROS production in healthy cells (18). Although ROS carries a negative connotation, low levels of ROS production can contribute to increased lifespan through a mitochondrial hormetic response (131). Additionally, low levels of ROS that may be produced during Rap or Met+Rap treatment would increase the translation of stress response proteins. Such low-level translation would not be detectable with our bulk measurements. We have previously reported that the assessment of protein synthesis in subcellular compartments can be biased by highly abundant, rapidly synthesized proteins (110).

ROS-induced protein unfolding may activate the unfolded protein response, which can inhibit protein translation through eif2a (146). Additionally, H_2O_2 treatment in vitro decreases overall protein translation but a subset of stress response proteins exhibit increased translation (146). Accordingly, our findings of no change or slight decreases in mitochondrial protein synthesis in multiple tissues following Met+Rap treatment in vivo suggest a potential preferential translation of certain proteins. However, to better understand the specific translational landscape following Rap and Met+Rap treatments, we are currently assessing the translatome in vitro using the ribosome profiling approach (Wolff et al, in preparation). With the data collected from the ribosome profiling experiments, we will be able to identify the transcriptional changes in response to Rap and Met+Rap treatment as well as the translational changes. These data will provide insight into cellular responses to Rap and Met+Rap treatments.

Approximately 99% of mitochondrial proteins are encoded in the nuclear genome, requiring coordination between nuclear DNA transcription and mitochondrial DNA transcription to reach the proper stoichiometry of nuclear:mitochondrial genes (60). Rapamycin treatment induces mitonuclear imbalance, activating the mitochondrial unfolded protein response (60). Although no data are available regarding how Met treatment or Met+Rap treatment would influence mitonuclear balance, Met+Rap extends lifespan, and may therefore elicit mitonuclear imbalance to contribute to increased lifespan (72). The activation of the mitochondrial unfolded protein response that results from mitonuclear imbalance has been reported to be a distinct mechanism by which lifespan is extended (72). Further investigation of how Met+Rap treatment may influence mitonuclear balance to contribute to cellular proteostasis is needed, but represents a potential mechanism through which Met+Rap treatment extends lifespan.

In Vitro Insights into Mechanisms of Protein Turnover following Rap or Met+Rap

Our in vitro experiments were designed to 1) examine how Rap and Met+Rap treatments influence protein turnover in cultured skeletal myotubes as a potential representation for skeletal muscle, and 2) to determine if autophagic flux or mitochondrial fission were essential for the effects of Rap or Met+Rap on protein turnover. We found that both Rap and Met+Rap treatment lower mitochondrial and cytoplasmic protein synthesis, as well as lowering cellular proliferation. Reductions in cellular proliferation allow for the allocation of protein synthetic processes towards maintaining cellular structures, as opposed to cell proliferation. We quantify this contribution of protein synthesis to maintaining cellular structures by relating the amount of protein synthesis to the amount of DNA synthesis (pro:DNA). We demonstrated that Rap, but not Met+Rap

treatment significantly increased the pro:DNA ratio for mitochondrial and cytoplasmic proteins. Further, we demonstrated that inhibition of autophagy or mitochondrial fission did not change how Rap or Met+Rap influence mitochondrial pro:DNA synthesis nor breakdown, while inhibiting autophagy robustly increased cytoplasmic pro:DNA synthesis, but not cytoplasmic pro:DNA breakdown. Thus, our in vitro findings suggest that while mitochondrial fission may not be essential for the effect of Rap and Met+Rap on protein turnover, the role of autophagy is not completely clear and must be further investigated.

As stated above, Met+Rap treatment is novel, and not well studied to date. The data from these dissertation experiments reveal that Met+Rap treatment robustly lowers mitochondrial protein synthesis of cultured skeletal myotubes after 24 hours, but that Met+Rap treatment maintained mitochondrial protein synthesis at the same level as control animals in the skeletal muscle from both young and old mice. We also report that Met+Rap treatment lowered DNA synthesis in the cultured myotubes, although the concomitant reductions in protein synthesis and DNA synthesis with Met+Rap treatment did not increase the pro:DNA ratio in vitro. Thus, although speculative, we hypothesize that if Met+Rap treatment slightly reduced the measured DNA synthesis in our young or old mice, we would detect an increase in the mitochondrial pro:DNA in vivo. Increased mitochondrial pro:DNA following the lifespan-extending Met+Rap treatment would also be consistent with previous findings from our laboratory, as we have reported that increased mitochondrial pro:DNA is a shared characteristic of long-lived murine models (106).

Final Summary

To summarize, our overall objective for these experiments was to 1) determine if there are differences in how Rap and Met+Rap treatment influence protein synthesis in young mice, 2) examine how Met+Rap treatment influences protein synthesis in older mice, and 3) determine the mechanisms through which Rap and Met+Rap treatment influence protein turnover. Collectively, the data presented here reveal that lifespan-extending treatments exert age- and tissue-specific effects on protein synthesis in genetically heterogeneous mice. Further, we demonstrate, in vitro, that Rap and Met+Rap treatments reduce protein synthesis and protein breakdown, while also reducing cellular proliferation. Thus, both Rap and Met+Rap treatments appear to contribute to cellular proteostasis in vivo and in vitro.

FIGURES

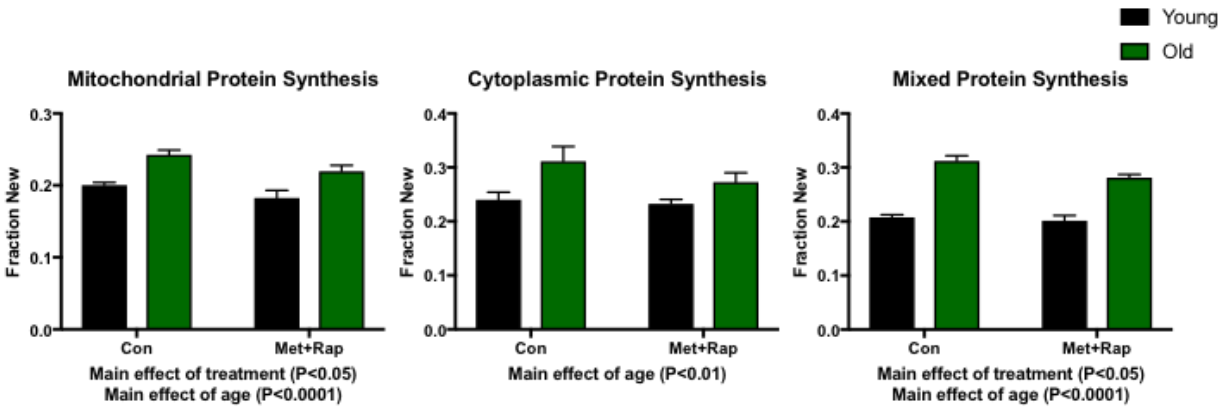

Figure 5.1. The effect of 8 weeks of Met+Rap treatment on mitochondrial, cytoplasmic, and mixed protein synthesis in the heart of young and old mice following 4d of D_2O labeling.

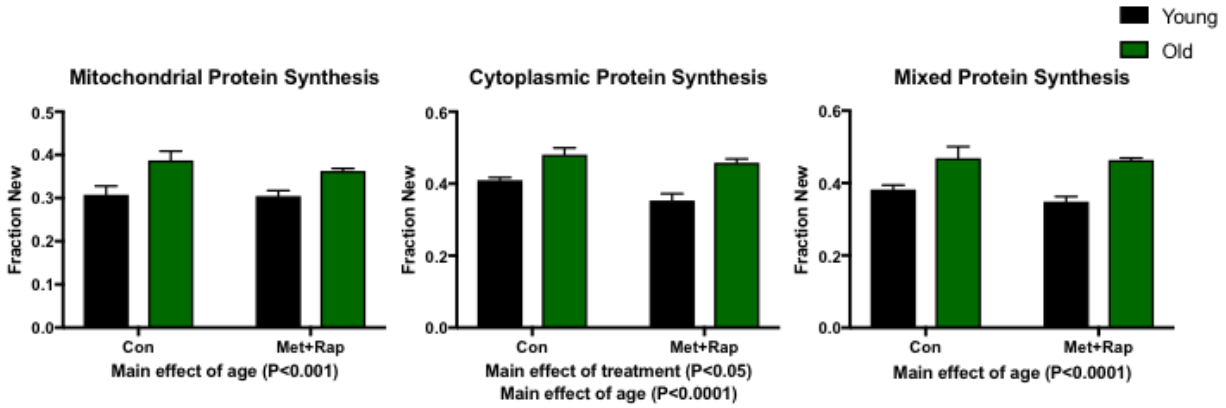

Figure 5.2 The effect of 8 weeks of Met+Rap treatment on mitochondrial, cytoplasmic, and mixed protein synthesis in the heart of young and old mice following 8d of D_2O labeling.

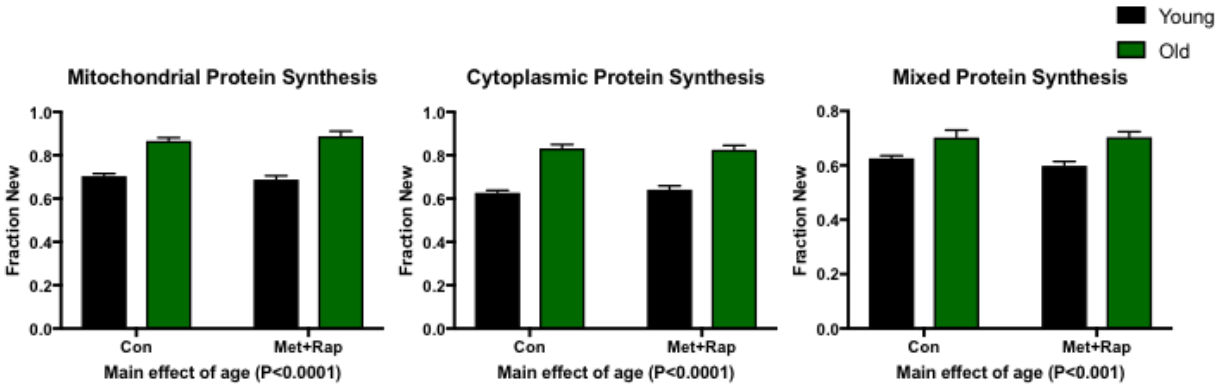

Figure 5.3 The effect of 8 weeks of Met+Rap treatment on mitochondrial, cytoplasmic, and mixed protein synthesis in the liver of young and old mice following 4d of D_2O labeling.

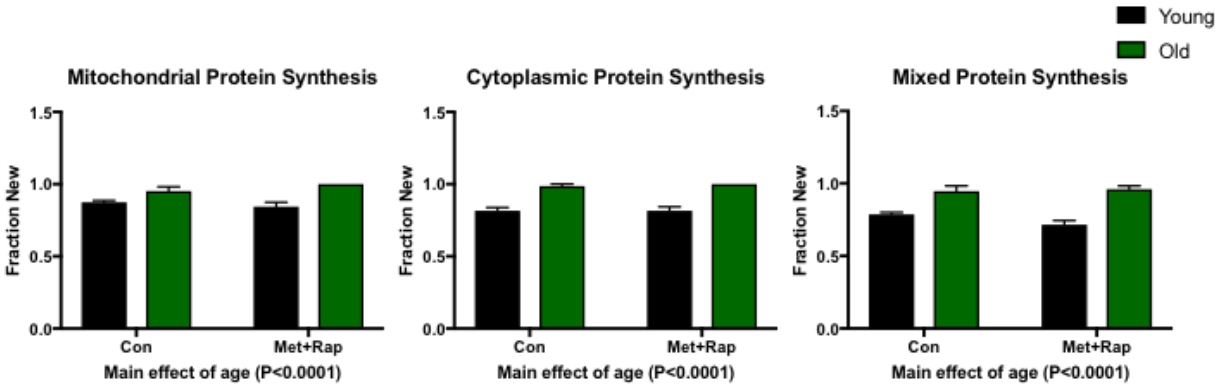

Figure 5.4 The effect of 8 weeks of Met+Rap treatment on mitochondrial, cytoplasmic, and mixed protein synthesis in the liver of young and old mice following 8d of D_2O labeling.

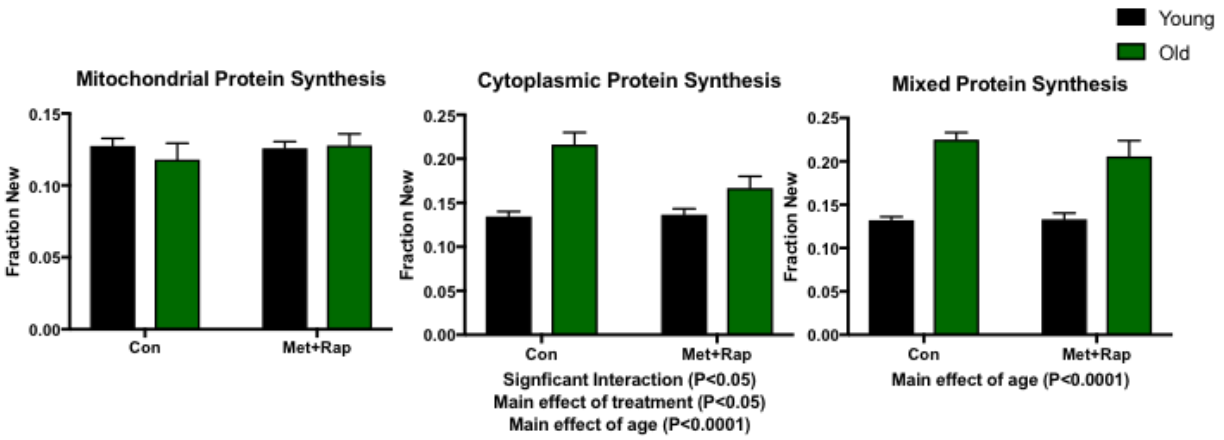

Figure 5.5 The effect of 8 weeks of Met+Rap treatment on mitochondrial, cytoplasmic, and mixed protein synthesis in the skeletal muscle of young and old mice following 4d of D_2O labeling.

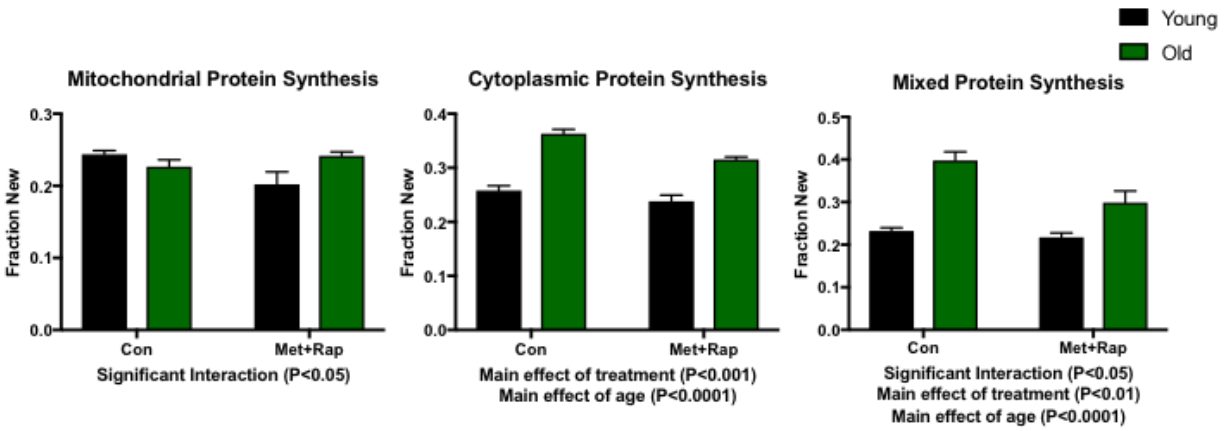

Figure 5.6 The effect of 8 weeks of Met+Rap treatment on mitochondrial, cytoplasmic, and mixed protein synthesis in the skeletal muscle of young and old mice following 8d of D_2O labeling.

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