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A novel D_2O tracer method to quantify RNA turnover as a biomarker of *de novo* ribosomal biogenesis, in vitro, in animal models, and in human skeletal muscle

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Abstract

Current methods to quantify in vivo RNA dynamics are limited. Here, we developed a novel stable isotope (D_2O)-methodology to quantify RNA synthesis (i.e. ribosomal biogenesis) in cells, animal models, and humans. Firstly, proliferating C2C12 cells were incubated in D_2O-enriched media and myotubes (±) 50ng.ml^{-1} IGF-1. Secondly, rat quadriceps [untrained n=9; 7-wks interval-"like" training n=13] were collected after ~3-wks D_2O (70-Atom%) administration, with body-water enrichment (BWE) monitored via blood sampling. Finally, 10 (23±1y) men consumed 150ml D_2O followed by 50ml/wk and undertook 6-wks resistance-exercise (RE; 6×8 repetitions, 75%-1RM 3/wk) with BWE monitored by saliva sampling and muscle biopsies (for determination of RNA synthesis) 0-3-6-wks. Ribose mole percent excess (r-MPE) from purine nucleotides was analyzed via GC-MS/MS. Proliferating C2C12 cells r-MPE exhibited a rise-to-plateau while IGF-1 increased myotube RNA from 76±3ng/ul to 123±3ng/ul and r-MPE by 0.39±0.1% (both $P<0.01$). After 3-wks, rat quadriceps r-MPE had increased to 0.25±0.01% ($P<0.01$) and was greater with running-exercise (0.36±0.02%; $P<0.01$)). Human muscle r-MPE increased to 0.06±0.01% and 0.13±0.02% at 3/6-wks respectively equating to synthesis rates of ~0.8%/d, increasing with RE to 1.7±0.3%/d ($P<0.01$) and 1.2±0.1%/d ($P<0.05$) at 3/6-wks, respectively. Therefore, we have developed and physiologically validated a novel technique to explore ribosomal biogenesis in a multi-modal fashion.

Introduction

Cellular protein content is under constant renewal to maintain cellular homeostasis. Typically, the balance between protein synthesis and breakdown remains relatively stable, yet under conditions of growth, atrophy or cellular proliferation, rapid and significant changes in protein content and cell size occur (5). With protein synthesis rates determined by the number (translational capacity) and activity (translational efficiency) of ribosomes (27, 39), the ribosome provides a primary point of control in cellular homeostasis; yet our understanding of dynamic ribosome metabolism is poorly understood. Ribosomal biogenesis is the product of the coordinated synthesis of multiple ribosomal RNA's (rRNA) and proteins. In being an energy consuming process, ribosomal biogenesis is tightly regulated by multiple signaling pathways responsive to nutrition, hormones and mechanical activity (22). However, basal rates of ribosomal biogenesis and ribosome half-life across tissues are for the most part largely undescribed. With the demands to modulate and maintain protein content varying considerably across cell types, such as rapidly dividing single cells, to the coordinated maintenance repair of multicellular organs, regulation of ribosome pools is likely to be tightly linked with protein metabolism (10). Furthermore, when the coordinated control of ribosome biogenesis becomes unregulated it can be the source of many conditions such as cancer (30).

Skeletal muscle is one of the body's most plastic tissues, undergoing substantial and rapid hypertrophy or atrophy under conditions of functional overload, disuse or malnutrition (2, 9, 17). Understanding these processes is of great importance as preservation of muscle mass and function throughout life is crucial in preventing

disability and maintaining quality of life- particularly in advanced ageing (16, 31). In being a post-mitotic tissue, muscle mass is controlled by the balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). Many acute changes in MPS (< 5h) are accompanied by the activation or suppression of proteins in the mTORc1 pathway (1), modulating translational efficiency rather than translational capacity (6). However, prolonged exposure to muscle loading modifies RNA content – increasing with hypertrophy (3, 12, 34) and decreasing with atrophy (15). As such, ribosomal biogenesis is thought to be central to muscle mass regulation.

With rRNA comprising 80% of total RNA, changes in RNA concentration are thought to be indicative of changes in the balance of ribosome synthesis and breakdown. However in addition to relying on long-term interventions, efficient extraction and normalization to muscle weight introducing variability, measures of concentration do not inform on dynamic RNA metabolism, plus changes in RNA synthesis naturally precede those of content. Past measures of RNA synthesis have typically relied on the incorporation of modified nucleotides such as [3H]-uridine or 5-bromouridine. However the use of these techniques is limited and generally cannot be used in whole animals due to their mutagenic nature. Alternatively, stable isotope tracers offer a safe method for use in humans and measures of RNA synthesis using these have been made (8, 14). Yet their applicability in human research has been limited due to numerous caveats, including variable and complex salvage pathways, infusions and time limits (<24h) resulting in a lack of methods to determine RNA synthesis rates, particularly in tissues with slow renewal rates (like skeletal muscle). Heavy water (D_2O) provides alternate routes in the measurement of substrate metabolism and can overcome some limitations

associated with other stable isotope methods. In being easily administered and with the precursor pool being maintained over weeks to months, we and others have made long-term cumulative measures of muscle protein synthesis (32, 40), with many other substrates measured in a range of different tissues including DNA (23, 29). Deuterium is incorporated via nucleotide *de novo* synthesis, overcoming previous limitations of nucleotide analogues and thus similarly creates a viable route in the measurement of RNA synthesis. Here, we developed sensitive GC-MS/MS universally applicable methods for the measurement of RNA synthesis including in slower turning over tissues requiring only minimal D_2O consumption; we validate these methods in cell cultures, pre-clinical models and humans and in a cell type of contemporary interest i.e. skeletal muscle.

Materials and Methods

Cell culture

Murine C2C12 myoblasts passage nos. 5-7; ECACC, Salisbury, UK were seeded and maintained in Dulbecco's modified Eagle's medium as previously described (7) containing 10% fetal bovine serum, amphoteracin B (1%), penstreptomycin (1%) and 4mM L-glutamine (Sigma-Aldrich, UK). Sterile 70% deuterium and U-^{13}C-Glucose were added to DMEM at required enrichments and distributed amongst wells for labeling consistency. In proliferating cells, media was changed every 48h and cells scraped at required time points. At 90% confluency, cells were differentiated by reducing serum concentrations to 2% with RNA synthesis stimulated 6 days after differentiation with IGF1 50 ng.ml^{-1}.

Animals

Mixed population of females and males n=22 of high responder to training (HRT) rats for aerobic training were used for the study. Rats originated from the generations 17 and 18 of selection for their training response, and were 9.2 ± 3.0 months old at the start of the experiment (21). All experimental procedures described in this study protocol were approved by the Animal Care and Use Committee of the Southern Finland, license number ESAVI-2010-07989/Ym-23, STH 534A (21.9.2010) and complements ESAVI/1968/04.10.03/2011, PH308A (30.3.2011) & ESAVI/722/04.10.07/2013, PH275A (1.3.2013). All experiments were conducted in accordance with the Guidelines of the European Community Council directive 86/609/EEC. Rats were kept in air-conditioned rooms single-housed, at an ambient temperature of 21 ±2 °C and the relative humidity at 50 ± 10 %. Artificial lighting provided light cycles of 12 h light/12 h total darkness. Commercially available pelleted rodent diet (R36, Labfor/Lantmännen, Malmö, Sweden) and the tap water (from the municipal water system of Jyväskylä) was available *ad libitum* for rats throughout the study. The energy content of the feed was 1260 kJ/100 g (300.93 kcal/100g). The feed contained raw protein 18.5%, raw fat 4.0%, NFE (nitrogen free extracts) 55.7%, fiber 3.5%, ash 6.3%, and water <12%. Rats received a gavage of 7.2 ml/kg of 70% D_2O for the remaining 3 wks of the 7 wk training period, with drinking water enriched to 2% to maintain body water enrichment. Body water enrichment was determined from blood samples collected at necropsy and used to represent the average enrichment throughout; although variability may occur over time, enriched drinking water minimizes these effects. Interval Training consisted of Warm-up for 5 min, at 50-60% of maximum speed (individually speed for each rat) +

149 running for 15 min: 3 min at 85 - 90%, 2 min pauses at 50%, repeated for 3 times;
150 inclination 15^{o} uphill. Training was done 3 times per week, with one-day rest between
151 (if possible). 48 hrs after the last training bout animals were anaesthetized with carbon
152 dioxide and killed by cardiac puncture and thereafter immediately necropsied. Left
153 quadriceps were rapidly exposed, removed and immediately frozen by complete
154 immersion in liquid nitrogen.
155
156 **Subject characteristics and ethics**
157 Ten healthy younger (23±1y, BMI: 24±1) men were recruited as previously described
158 (3). All subjects provided their written, informed consent to participate after all
159 procedures and risks (in relation to muscle biopsies, blood sampling etc.) were
160 explained. Following inclusion to the study, subjects were studied over a 6-week period.
161 After baseline bilateral biopsies, subjects provided a saliva and blood sample then
162 consumed 150 ml D_2O (70 atom%; Sigma-Aldrich, Poole, UK) to label the body water
163 pool to ~0.2% APE which was maintained with weekly top-up boluses (50 $ml.wk^{-1}$).
164 Thereafter subjects performed progressive unilateral RET 3/wk at 75% 1RM, with
165 additional bilateral biopsies taken at 3 and 6 wks to monitor RNA incorporation. Blood
166 was collected at 0, 3 and 6 weeks to follow deuterium incorporation into peripheral
167 blood mononuclear cells, isolated using Histopaque (Sigma). For the temporal
168 monitoring of body water enrichment, each participant provided a saliva sample on RET
169 visits >30 min after their last meal or drink, with extra samples taken ~3 h after weekly
170 50 ml boluses to ensure that body water enrichment was accurately represented.
171 Samples were collected in sterile plastic tubes and immediately cold centrifuged at
172 16,000g to remove any debris that might be present; they were then aliquoted into 2ml

glass vials and frozen at -20°C until analysis. This study was approved by The University of Nottingham Ethics Committee and complied with studies conducted in accordance with the declaration of Helsinki and registered as clinical trials (clinicaltrials.gov registration no. NCT02152839).

Media and body water enrichment

The deuterium enrichment was measured in media collected from cell culture plates and plasma from rats by incubating 100µl of each sample with 2µl of 10 M NaOH and 1µl of acetone for 24 h at room temperature. Following incubation the acetone was extracted into 200µl of n-heptane, and 0.5µl of the heptane phase was injected into the GC-MS/MS for analysis. A standard curve of known D_2O enrichment was run along side the samples for calculation of enrichment. Human body water enrichment was extracted by heating 100 µl saliva in an inverted 2 ml autosampler vial for 4 h at 100°C. Vials were then placed upright on ice to condense extracted body water and transferred to a clean autosampler vial ready for injection. A total of 0.1µl body water was injected into a high-temperature conversion elemental analyzer (Thermo Finnigan; Thermo Scientific, Hemel Hemp- stead, United Kingdom) connected to an isotope ratio mass spectrometer (Delta V Advantage; Thermo Scientific)

Protein-bound alanine muscle fraction enrichment and calculation of FSR

Myofibrillar protein was isolated from human *VL* muscle biopsies and rat quadriceps by homogenizing 30–50 mg muscle in ice-cold homogenization buffer, rotated for 10min, and the supernatant collected after centrifugation at 13,000 g for 5 min at 4°C. The myofibrillar pellet was solubilized in 0.3 M NaOH and separated from the insoluble

197 collagen by centrifugation, and the myofibrillar protein was precipitated using 1 M
198 perchloric acid (PCA). Protein-bound amino acids were released using acid hydrolysis
199 by incubating in 0.1 M HCl in Dowex H+ resin slurry overnight before being eluted
200 from the resin with 2 M NH4OH and evaporated to dryness; amino acids were then
201 derivatised as their N-methoxycarbonyl methyl esters. Dried samples were suspended in
202 60 µl distilled water and 32 µl methanol, and following vortex, 10 µl pyridine and 8 µl
203 methylchloroformate were added. Samples were vortexed for 30 s and left to react at
204 room temperature for 5 min. The newly formed N- methoxycarbonylmethyl ester amino
205 acids were then extracted into 100 µl chloroform. A molecular sieve was added to each
206 sample for ~20 s before being transferred to a clean glass gas chromatography insert,
207 removing any remaining water by size exclusion absorption. Human protein-bound
208 alanine enrichment was determined by gas chromatography: pyrolysis:isotope ratio
209 mass spectrometry (Delta V Advantage Thermo Finnigan, Thermo Scientific, Hemel
210 Hempstead, UK), with rat protein-bound alanine enrichment determined by gas
211 chromatography tandem mass spectrometry (TSQ 8000 Thermo Finnigan, Thermo
212 Scientific, Hemel Hempstead, UK) alongside a standard curve of known DL-Alanine-
213 2,3,3,3-d4 enrichment to validate measurement accuracy of the machine. Myofibrillar
214 MPS was calculated from the incorporation of deuterium-labeled alanine into protein,
215 using the enrichment of body water [corrected for the mean number of deuterium
216 moieties incorporated per alanine (3.7) and the dilution from the total number of
217 hydrogens in the derivative (i.e., 11)] as the surrogate precursor labeling between
218 subsequent biopsies. The equation used was

$$FSR = -Ln\left(\frac{1 - \left[\frac{(APEala)}{(APEp)}\right]}{t}\right)$$

where APEala equals deuterium enrichment of protein-bound alanine, APEp indicates mean precursor enrichment over the time period, and t is the time between biopsies.

RNA extraction, digestion and derivatisation

To extract RNA, ~20-30mg of muscle was homogenized in extraction buffer (5µl/mg) containing 0.1 M Tris-HCL pH 8, 0.01 M EDTA pH 8 and 1M NaCL. Proteinase K was added to a final concentration of 50 µg/µl and placed at 55^{o}C for ~2 hrs with occasional mixing until complete digestion had occurred. For cell culture, each well was scraped in 200 µl of extraction buffer and PBMC's homogenized in 200 µl of extraction buffer. To the extractions an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, inverted several times to mix and the upper aqueous layer removed to a clean eppendorf after centrifugation at 13000 rpm for 10 min. To remove additional protein an equal volume of chloroform:isoamyl alcohol (24:1) was added to the aqueous layer and repeated as above. To precipitate RNA, an equal volume of isopropanol was added to the aqueous layer, inverted several times and centrifuged at 13000 rpm for 20 min. The pellet was washed 3 times in 70% ethanol; air-dried, re suspended in 22 µl of molecular biology and digested with 5 µl of 375 mM sodium acetate (pH 4.8), 750 µM $ZnSO_4$ containing 0.5 units of nuclease S1 and 0.25 units potato acid phosphatase and placed at 37^{o}C overnight. Hydrolysates were then reacted with 10 µl of O-benyzylhyrdoxylamine (2% w/v) and 7.5 µl of acetic acid at 100^{o}C for 30 min. Samples were allowed to cool at room temperature before the addition of 10µl of 1-methyimidazole and 100 µl of acetic anhydride. The reactions are transferred to a boiling tubed and quenched by the addition of 2 ml ddH2O. The newly formed derivatives were extracted by the addition of 750 µl dichloromethane (DCM) vortex

mixed and phases allowed to separate. By pre wetting the tip with DCM, the lower layer is removed to a clean boiling tube and the procedure repeated. DCM extracts were then dried and re suspended in 40 µl of ethyl acetate for GC-MS/MS analysis.

GC-MS/MS instrument conditions and fractional synthesis rate calculation

To measure RNA enrichment 2 µl of sample was injected into a trace 1310 gas chromatograph connected to TSQ 8000 triple quadrupole GC-MS/MS (Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK). Samples were injected on splitless mode with inlet temperature at 280^{o}C. GC ramp conditions were 120^{o}C for 1 min, ramp to 280 ^{o}C at 10 ^{o}C /min and hold for 3 min. Selected reaction monitoring (SRM) was performed for the mass to charge ratios m/z of 273.1-111.1 and 274.1-112.1 representing the M and M^{+1} ions with a collision induced dissociation energy of 6. Enrichment was calculated as (M^{+1}/ (M + M^{+1}) with the mole percent excess (MPE) expressed as difference from unlabeled D_2O free samples. Fractional synthesis rates were calculated as FSR(%.d^{-1}) = (r-MPE)/[(p-MPE) x t] x 100 where r-MPE is the excess enrichment of bound ribose, p-MPE is the mean precursor enrichment over the time period and t is the time between samples. In cell culture and rat studies, p-MPE was calculated as the water enrichment multiplied by the amplification factor of 2.098 determined in cells. In human studies, p-MPE was taken as the ribose PBMC enrichment measured over the labeling period. Samples were run in triplicate alongside standard curves of known ribose standards, with the average of both peaks were used in the results. Additionally, unlabeled samples were injected in different quantities to determine abundance effects.

Statistical Analysis

Descriptive statistics were produced for all data sets to check for normal distribution (accepted if $P>0.05$) using a Kolmogorov-Smirnov test. All data are presented as means ± SEM. Differences between the effects of interval training and control on RNA synthesis in rates were analyzed by t-test. All other data sets were analyzed by repeated measures one-way or two-way ANOVA with a Bonferroni correction using GraphPad Prism (La Jolla, CA) Software Version 5. Correlations were assessed using Pearsons product moment correlation coefficient. The α level of significance was set at $P<0.05$.

Results

GC-MS/MS chromatography and SRM transitions

Addition of O-benyzylhyrdoxylamine and 4 acetyl groups to ribose produces a derivative with a molecular weight of 423.1 (Fig 1A). Upon gas chromatography the derivative produces two peaks representing the cis-trans isomers formed due to the anomeric carbon of ribose (Fig 1B). Full scan MS analysis of the derivative produces a most abundant fragment with best chromatography of 273.1, with second fragmentation producing a most abundant fragment of 111.1. Analysis of this transition is highly selective and produces GC-MS/MS spectra with very low background, detecting standard enrichments as little as 0.02 APE (Fig 1C). Further this SRM encompasses all backbone carbons of ribose confirmed by +5 enrichment from U-^{13}C-Glucose incorporation (Fig 1D).

Deuterium incorporation into RNA bound ribose

The MPE of ribose (Fig 2A) extracted from purine nucleotides of RNA from C2C12 cells increased linearly with increasing concentrations of media D_2O enrichment being 0%, 1.6±0.08%, 4.1±0.1%, 9.5±0.15%, 18.8±0.18% at media concentrations of 0% 0.67%, 1.9%, 4.6% and 8.9% respectively. Linear regression revealed that on average 2.1 ^{2}H are incorporated into purine ribose during synthesis of new RNA. PBMC'S from human subjects showed an increase in MPE to 0.37±0.04% and 0.42±0.04% (both $P<0.01$), revealing the average accessible hydrogen's to be 2.6±0.2 (Fig 2B).

Validation of deuterium incorporation in RNA *in vitro*

C2C12 cell number increased from 0.083±0.001 million per well to 1.2 ±0.03 million after 117 h of proliferation (Fig 3A), whilst RNA MPE followed a rise to plateau relationship from 0.28±0.03% after 15 h and progressed to 0.52±0.02% by 117 h (Fig 3B). In response to IGF1 treatment, RNA content increased by 27.5 h from 76.1±3 $ng.ul^{-1}$ to 123.4±3 $ng.ul^{-1}$ (Fig 3C). Similarly, RNA MPE significantly increased in control to 0.15 ±0.01% at 27.5 h, with the increase in IGF1 treatment being significantly greater (Fig 3D).

RNA Synthesis in rat muscle *in vivo*

After 3 weeks of continuous D_2O administration, RNA MPE significantly increased to 0.25±0.01% in control and was significantly greater with interval training to 0.36±0.01% P<0.001 (Fig 4A). The calculated RNA FSR was 0.97±0.05 $\%.d^{-1}$ with a significant increase in response to interval training to 1.3±0.05 $\%.d^{-1}$ P<0.001 (Fig 4B). Rat quadriceps MPS showed a correlation with quadriceps RNA synthesis % of r^2=0.17 and P=0.05

RNA synthesis in human muscle

The MPE from RNA bound ribose increased in rest legs to 0.064 ±0.01% and 0.137 ±0.02% at 3 and 6 weeks respectively (P<0.001). In RET legs, the MPE increased to 0.125 ±0.02% and 0.211 ±0.01% at 3 and 6 week respectively (P<0.001), being greater than rest at both time points (P<0.005). Corrected for PBMC RNA enrichment an FSR of 0.86 ±0.1%.d^{-1} and 0.78 ±0.1%.d^{-1} was determined in rest legs at 3 and 6 weeks respectively. In RET legs the RNA FSR was significantly greater than rest being 1.69

323 ±0.2 %.d^{-1} ($P<0.01$) and 1.24 ±0.1%.d^{-1} ($P<0.05$) at 3 and 6 weeks respectively. Human

324 VL MPS was highly correlated with VL RNA synthesis $P=0.009$ and an $r^2=0.32$.

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Discussion

We have developed and validated D_2O-based methods for the measurement of *in vitro* and *in vivo* RNA synthesis that can be used safely and effectively in humans and with the potential for application to any tissue- this is a step forward from current practices in providing methods for long term measures of RNA synthesis in humans, particularly those of slow turnover pools such as skeletal muscle. RNA content is closely linked to cellular metabolism, with ribosomal biogenesis being required for cellular proliferation (10) and growth (34). Currently, changes in RNA content are primarily determined by crude measures of RNA concentrations, with limited methods in place to determine dynamic rates of RNA synthesis *in vivo*. Our approach will provide insight into the workings of dynamic ribosomal biogenesis in vitro, in animal models and in humans – across cell types.

Method development and validation of *in vitro* RNA synthesis in skeletal muscle cells

Nucleotide synthesis involves complex precursor pools with variable nucleotide salvage (23) making the incorporation of stable isotope labeled compounds difficult to interpret (13). The advantage of using D_2O is that deuterium becomes incorporated into the backbone hydrogen of ribose during nucleotide synthesis, with deoxyribose from purine deoxyribonucleosides primarily synthesized via *de novo* synthesis; as such, providing a reliable input of isotope (23, 29). As deoxyribonucleotides are reduced from ribonucleotides, this further creates a viable method for measures of RNA synthesis and

for the first time we have shown a constant incorporation of deuterium across a range of media concentrations into purine ribose.

Total RNA encompasses rRNA, tRNA and mRNA that will have variable turnover rates (33). The quickest of these will be tRNA and mRNA that will contribute to early increases in detectable enrichment. However in making up <20% of total cellular RNA, these pools become quickly saturated and deuterium incorporation follows a rise to plateau in proliferating cells reflecting the required expansion of rRNA for cell division (Fig 3B) (8, 10). Further, as initial validation using established stimulators of *in vitro* myotube hypertrophy and ribosomal biogenesis (i.e. IGF-1)(7), we were able to detect simultaneous increases in both RNA content and deuterium incorporation into RNA. Therefore, deuterium incorporation into RNA was reflective of newly synthesized RNA.

To use precursor product calculations, a measure of the precursor, or a proxy thereof, is required (38). Alternatively, when using D_2O, an amplification factor can be used to represent the amount of accessible hydrogen in the precursor that can incorporate deuterium and be multiplied by the body water enrichment (4). Nucleotide precursor pools are difficult to measure, with continuous input of unlabeled substrates such that the maximal theoretical plateau is never achieved (24). To investigate the number of accessible hydrogen in ribose *in vitro*, murine C2C12 skeletal muscle cells were repeatedly passaged in a range of D_2O media enrichments, revealing a constant incorporation of ~2.1 deuterium's out of a total 6. Previously, values of ~3.1 have been

reported for deoxyribose out of a total 7 (26, 29), expectedly higher due to the additional hydrogen that exchanges with ribonucleotide reductase.

Validation of *in vivo* RNA synthesis in an animal model

Compared to many tissues, skeletal muscle has a relatively slow habitual protein renewal rates, with little to no active DNA synthesis (11); in contrast actual RNA synthesis rates are practically unknown and will vary considerably across tissues. As D_2O can be simply administered by oral consumption and easily maintained, D_2O can be used to capture a vast range of synthesis rates. Recently, D_2O has been used to measure ribosome renewal in mouse liver, although in using GC-MS this requires high levels of enrichment (5% APE) and fast rates of turnover (~10%) that can be burdensome and limit applications(25). Applying our validated *in vitro* methods to rodents, to our knowledge, we made the first long term measures of RNA synthesis in skeletal muscle. In doing so, we demonstrated there is active renewal of RNA pools of ~1%.d^{-1}. Furthermore, using an exercise stimulus to activate ribosomal biogenesis (39) we validated the existence of a significant increase in deuterium incorporation into RNA, demonstrating increased RNA synthesis. Intriguingly, RNA synthesis rates were correlated with MPS, which we speculate is due to a co-ordinate regulation in response to exercise.

RNA Synthesis in Human Muscle and the Effect of Resistance Exercise

Presumably most tissues will have a constant level of rRNA synthesis to maintain functional ribosomes for cellular protein synthesis. That said, since ribosome biogenesis consumes considerable energy and will therefore likely be maintained at minimal

requirements. Using the methods described here, to our knowledge we report the first measures of RNA synthesis in human muscle, showing a constant synthesis rate of ~0.8 $\%.d^{-1}$ during "habitual activity". Further, to asses precursor enrichment, we measured the plateau enrichment of a population of cells 100% replenished (PBMCS) over the labeling period (29). This accounts for individual variability in the number of accessible hydrogens and further we showed on average 2.6 deuterium's were incorporated, similar to our *in vitro* measures.

Skeletal muscle RNA content is highly responsive to functional overload (28, 37) and here we have shown that in response to RET, RNA synthesis was significantly increased after 3 and 6 weeks of exercise training in humans. Once again, RNA synthesis was correlated with MPS which further validates that in muscle, ribosome and protein metabolism are likely to be inextricably related (likely via mTORc1 (18)). Similarly, although there is little other data for us to compare our results to, whole body rRNA turnover determined by breakdown products in urine have been estimated ~2.5% (36). Further this showed a strong relationship between whole body protein degradation rates of r^2=0.7 supporting that these are closely linked process in muscle homeostasis.

Further Application of Methods for RNA synthesis

Previously, measures of RNA synthesis have been made in humans using 6,6$^2H_2$-Glucose, however this requires large amounts of tracer to be consumed (1g/Kg) and is generally limited to fast turnover cells (8). Further, achieving high levels of enrichment to perform GC-MS analysis in humans is costly, requiring high levels of D_2O consumption that is burdensome on the individual and may potentially cause adverse

effects such as nausea and vertigo (19). Further, rates of RNA synthesis will vary considerably across tissues, making the detection of slow turnover pools such as muscle using GC-MS techniques difficult. Here, by combining sensitive GC-MS/MS techniques (detection limits of ≥0.02% MPE) and the ability to administer D_2O from days to weeks, this method creates opportunities to measure RNA synthesis over a range of rates and tissues. Such measures can be employed through simple D_2O administration and access to tissue samples or blood- with some prior expectation of synthesis helpful. For instance, human body water enrichment can be simply maintained ~ 0.15-0.2% APE using an initial bolus of 150 ml D_2O, followed by weekly doses of 50 ml (3). In this situation, sampling from a tissue after 5 days with an RNA turnover rate of ~10 $\%.d^{-1}$ would result in an easily detectable product enrichment using GC-MS/MS of ~ 0.075-0.1%, whereas an RNA turnover rate of ~1 $\%.d^{-1}$ would result in an undetectable enrichment of ~ 0.0075%-0.01%. This is not to say these measure can't be made by other means. Raising body water enrichment will increase end point enrichment and body water enrichments as high as 2% would make GC-MS techniques an option. However D_2O consumption of such high levels is costly and burdensome on subjects. As such the methods used here can be readily applied to many situations.

Conclusion

In summary we have developed and validated the use of D_2O in measurement of RNA synthesis both *in vitro* and *in vivo.* With many RNA synthesis rates unknown, these new methods will have a significant impact in being able to measure a wide range of RNA turnover rates in varied tissues. Further, ribosomal biogenesis has been the interest of recent publications in muscle adaptive mechanisms (12, 20, 34, 35) and will likely play

445 a significant role elucidating muscle metabolism at rest and in response to

446 hypertrophic/atrophic conditions.

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Disclosures

No conflicting interests

Author contributions

All experiments were performed at i) the Clinical, Metabolic and Molecular Physiology laboratories, Royal Derby Hospital, University of Nottingham (humans and cells), ii) The Biology of Physical Activity laboratories, Neuromuscular Research Centre, Faculty of Sport and Health Sciences, University of Jyvaskyla, Jyvaskyla, Finland (animal experiments) and iii) the department of Anaesthesiology Laboratories, University of Michigan, Ann Arbor, Michigan, USA (animal propagation). D.J.W., K.S., P.L.G., N.J.S., L.G.K., S.L.B., H.K. and P.J.A. conducted the study conception and design; M.S.B., W.K.M., B.E.P., S.L. and D.J.W. performed the experiments; M.S.B., B.E.P., D.J.W., K.S. and P.J.A. analyzed the data; M.S.B., B.E.P., D.J.W., K.S. and P.J.A. interpreted the results; M.S.B., B.E.P., W.K.M., D.J.W., K.S., N.J.S., J.N.L., P.L.G., H.K., S.L., L.G.K., S.L.B., and P.J.A. drafted the manuscript; M.S.B., B.E.P., W.K.M., D.J.W., K.S., N.J.S., J.N.L., P.L.G., H.K., S.L., L.G.K., S.L.B., and P.J.A. edited and revised the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Figure Legends

F**igure 1. A)** Structure and mass of the ribose BHTA derivative. **B)** Typical GC-MS/MS chromatogram of the ribose derivative on a DB-17 column for the SRM transitions of (273.1-111.1). Blue line represents the M and red the M+1 **C)** Standard curve of 1-^{13}C-Ribose 0.02, 0.05, 0.1, 0.5 **D)** measurement of the +5 isotopomer of RNA bound ribose from C2C12 myotubes incubated in U-^{13}C glucose enriched media

Figure 2. A) MPE of RNA from maximally labeled C2C12's vs. D_2O media MPE and **B)** the amplification of deuterium into PBMC'S from human subjects. *** Significantly different from baseline P<0.001

Figure 3. Time course of **A)** cell number **B)** MPE of RNA in proliferating C2C12's. Time course in the concentration of **C)** RNA and **D)** MPE of RNA in non-treated condition (Control) and in responses to IGF1. Dotted line represents plateau enrichment.* Significantly different from baseline P<0.05, *** P<0.001, **** P<0.0001. § significantly different from control at that time point P<0.01

Figure 4. A) MPE of RNA bound ribose from control and exercised rat quadriceps **B)** FSR of RNA from control and exercised rat quadriceps **C)** correlation between quadriceps MPS%.d^{-1} and RNA FSR%.d^{-1}. Dotted line represents plateau enrichment *** Significantly different than control P<0.001

Figure 5. A) Time course of body water enrichment measured through saliva samples over the 6 weeks of labeling **B)** MPE of RNA bound ribose from human *VL* in rest and

RET legs. Dotted line represents the average plateau PBMC enrichment **C)** FSR of RNA bound ribose from human *VL* in rest and RET legs **D)** correlation between *VL* MPS%.d^{-1} and RNA FSR %.d^{-1}. *** Significantly different from baseline P<0.001 § Significantly different from control at that time point P<0.05

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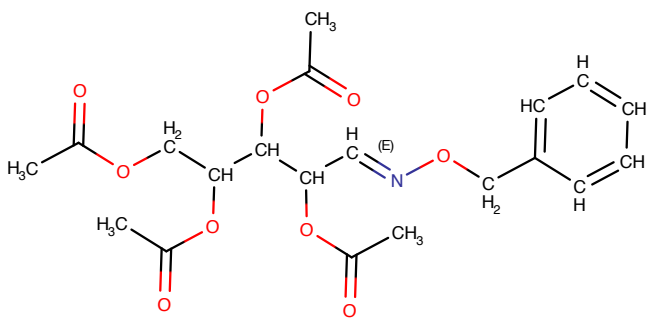
Figure 1

A



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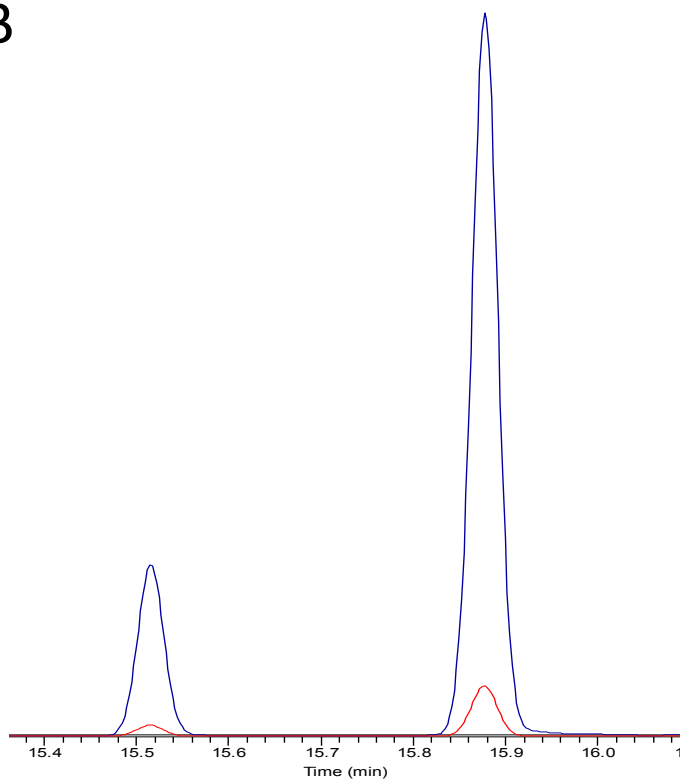

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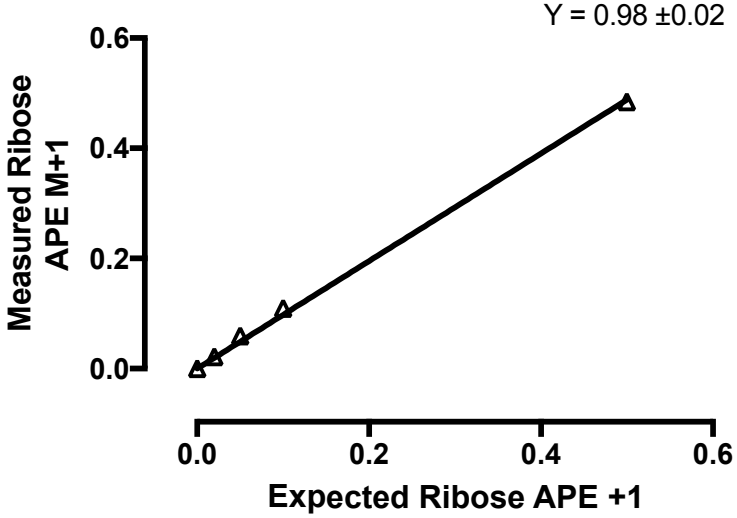

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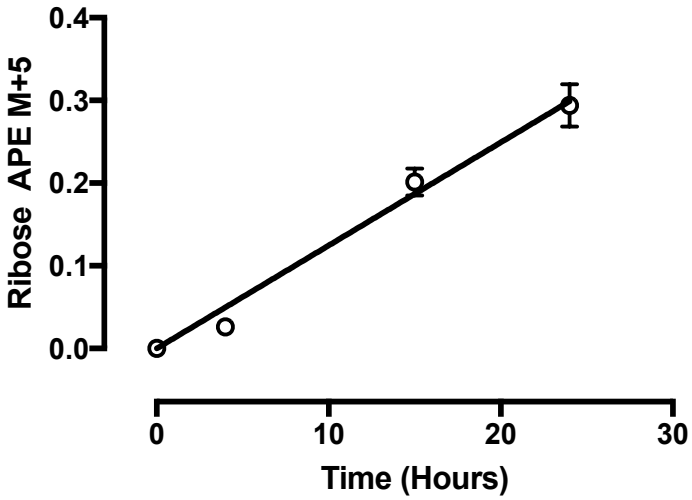
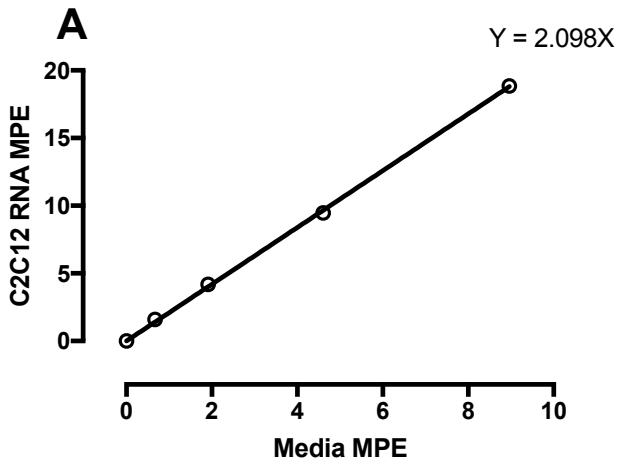


Figure 2


A
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C2C12 RNA MPE
20
15
10
5
0
0
2
4
6
8
10
Media MPE

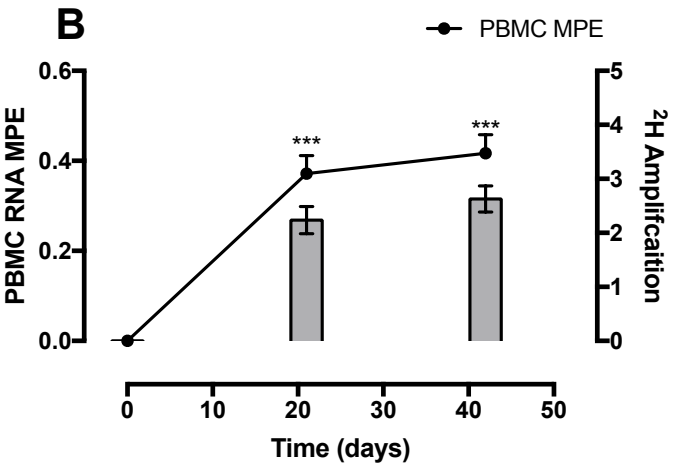

B
PBMC MPE
PBMC RNA MPE
0.6
0.4
0.2
0.0
***
***
^{2}H Amplifcaition
5
4
3
2
1
0
0
10
20
30
40
50
Time (days)

Figure 3

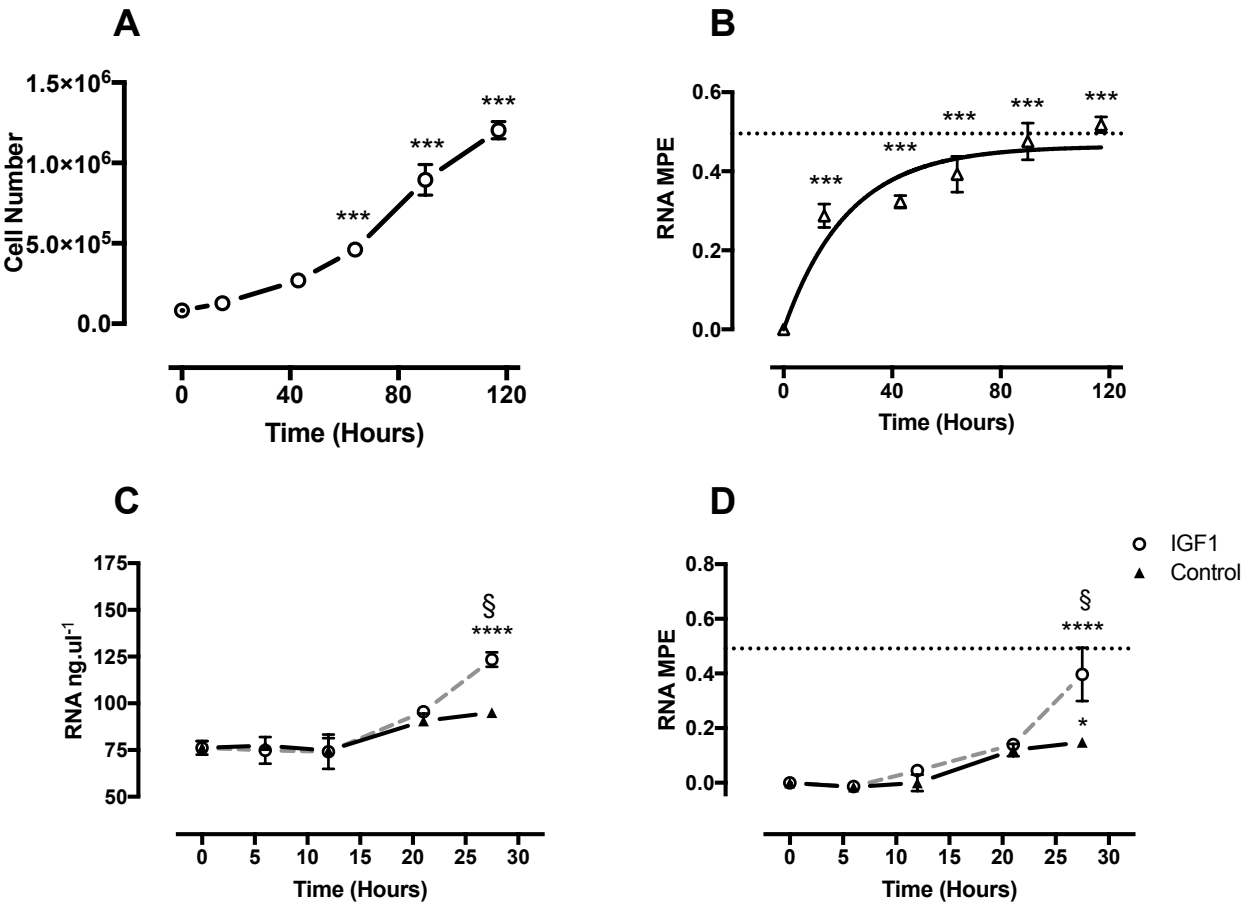

A
1.5×10^6
1.0×10^6
5.0×10^5
0.0
Cell Number
***
***
***
0
40
80
120
Time (Hours)
B
0.6
0.4
0.2
0.0
RNA MPE
***
***
***
***
***
0
40
80
120
Time (Hours)
C
175
150
125
100
75
50
RNA ng.ul-1
§
****
0
5
10
15
20
25
30
Time (Hours)
D
0.8
0.6
0.4
0.2
0.0
RNA MPE
§
****
*
0
5
10
15
20
25
30
Time (Hours)
IGF1
Control

Figure 4

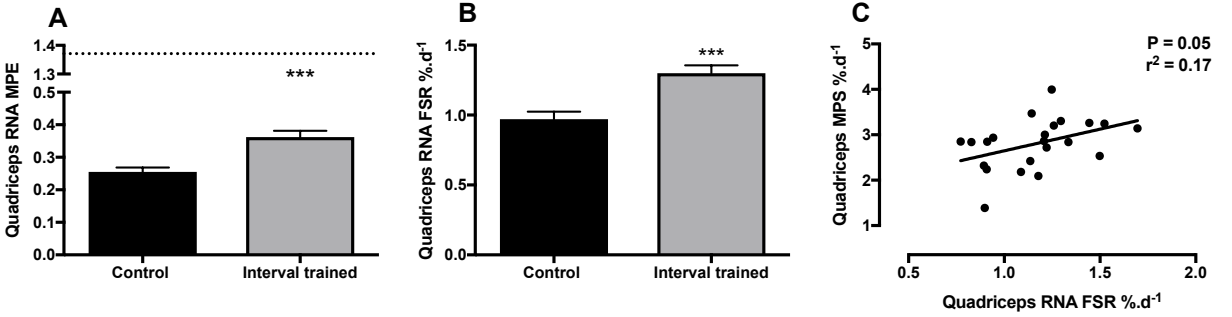
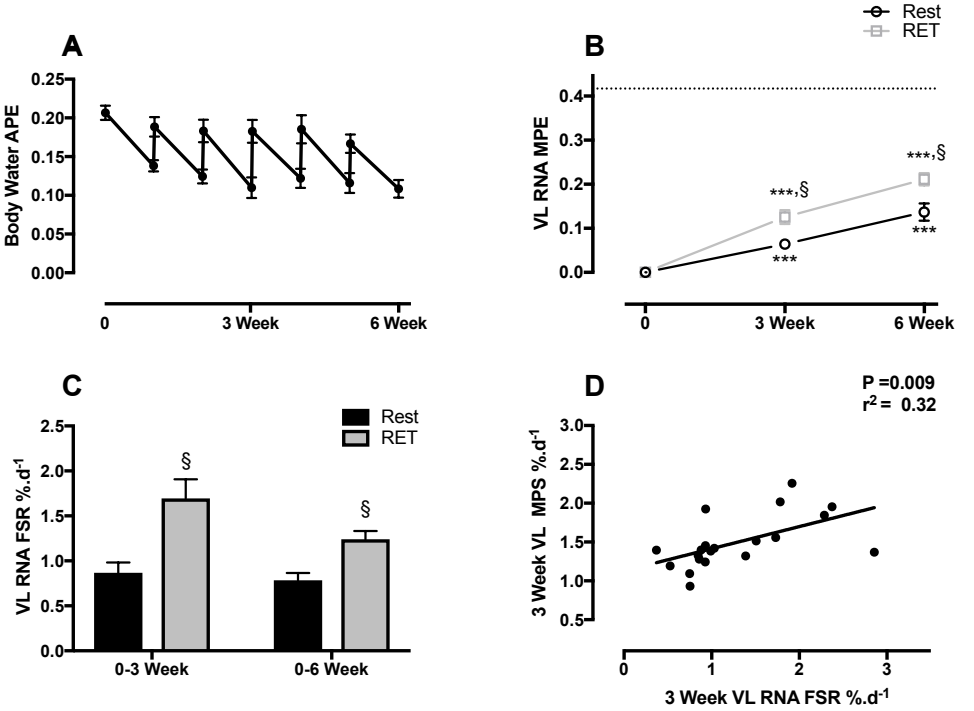

A
Quadriceps RNA MPE
1.4
1.3
0.5
0.4
0.3
0.2
0.1
0.0
***
Control
Interval trained
B
Quadriceps RNA FSR %.d-1
1.5
1.0
0.5
0.0
***
Control
Interval trained
C
Quadriceps MPS %.d-1
5
4
3
2
1
P = 0.05
r2 = 0.17
0.5
1.0
1.5
2.0
Quadriceps RNA FSR %.d-1

Figure 5


A
Body Water APE
0.25
0.20
0.15
0.10
0.05
0.00
0
3 Week
6 Week
B
Rest
RET
VL RNA MPE
0.4
0.3
0.2
0.1
0.0
***,§
***,§
***
***
0
3 Week
6 Week
C
Rest
RET
VL RNA FSR %.d-1
2.5
2.0
1.5
1.0
0.5
0.0
§
§
0-3 Week
0-6 Week
D
P =0.009
r2 = 0.32
3 Week VL MPS %.d-1
3.0
2.5
2.0
1.5
1.0
0.5
0
1
2
3
3 Week VL RNA FSR %.d-1