PRMT BIOLOGY DURING SKELETAL MUSCLE DISUSE
PROTEIN ARGinine METHYLTRANSFerASE

EXPRESSION, LOCALIZATION, AND ACTIVITY

DURING DISUSE-INDUCED SKELETAL MUSCLE PLASTICITY

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the
Requirements for the Degree Master of Science

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Lay Abstract

Skeletal muscle is a plastic tissue that is capable of adapting to various physiological demands. Previous work suggests that protein arginine methyltransferases (PRMTs) are important players in the regulation of skeletal muscle remodelling. However, their role in disuse-induced muscle plasticity is unknown. Therefore, the purpose of this study was to investigate the role of PRMTs within the context of early, upstream signaling pathways that mediate disuse-evoked muscle remodelling. We found differential responses of the PRMTs to muscle denervation, suggesting a unique sensitivity to, or regulation by, potential upstream signaling pathways. AMP-activated protein kinase (AMPK) was among the molecules that experienced a rapid change in activity following disuse. These alterations in AMPK predicted many of the modifications in PRMT biology during inactivity, suggesting that PRMTs factor into the molecular mechanisms that precede neurogenic muscle atrophy. This study expands our understanding of the role of PRMTs in regulating skeletal muscle plasticity.
Abstract

Protein arginine methyltransferase 1 (PRMT1), PRMT4 (also known as co-activator-associated arginine methyltransferase 1; CARM1), and PRMT5 are critical components of a diverse set of intracellular functions. Despite the limited number of studies in skeletal muscle, evidence strongly suggests that these enzymes are important players in the regulation of phenotypic plasticity. However, their role in disuse-induced muscle remodelling is unknown. Thus, we sought to determine whether denervation-induced muscle disuse alters PRMT expression and activity in skeletal muscle within the context of early signaling events that precede muscle atrophy. Mice were subjected to 6, 12, 24, 72, or 168 hours of unilateral hindlimb denervation. The contralateral limb served as an internal control. Muscle mass decreased by ~30% following 168 hours of disuse. Prior to atrophy, the expression of muscle RING finger 1 and muscle atrophy F-box were significantly elevated. The expression and activities of PRMT1, CARM1, and PRMT5 displayed differential responses to muscle disuse. Peroxisome proliferator-activated receptor-γ coactivator-1α, AMP-activated protein kinase (AMPK), and p38 mitogen-activated protein kinase expression and activation were altered as early as 6 hours after denervation, suggesting that adaptations in these molecules are among the earliest signals that precede atrophy. AMPK activation also predicted changes in PRMT expression and function following disuse. Our study indicates that PRMTs are important for the mechanisms that precede, and initiate muscle remodelling in response to neurogenic disuse.
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# TABLE OF CONTENTS

Acknowledgements........................................................................................................... v
List of Figures .................................................................................................................... vii
List of Abbreviations ......................................................................................................... ix
Declaration of Academic Achievement .............................................................................. xi

**Review of the Literature** ............................................................................................. 1

1. Introduction to Skeletal Muscle .................................................................................. 2
2. Skeletal Muscle Plasticity ......................................................................................... 5
3. The Muscle Atrophy Program .................................................................................. 6
4. Protein Arginine Methyltransferases ..................................................................... 18
5. Protein Arginine Methyltransferase Biology in Skeletal Muscle ....................... 25
6. Study Objectives .................................................................................................... 29
7. References .............................................................................................................. 30

**Manuscript** ................................................................................................................... 60

1. Abstract .................................................................................................................... 61
2. Introduction ............................................................................................................... 62
3. Methods .................................................................................................................... 65
4. Results ....................................................................................................................... 71
5. Discussion ............................................................................................................... 76
6. References ............................................................................................................... 86
7. Figure Legends ......................................................................................................... 100
List of Figures

Figures from Review of the Literature

Figure 1. Overview of Skeletal Muscle Plasticity.......................................................... 7
Figure 2. Hypothetical pathway for skeletal muscle atrophy........................................... 10
Figure 3. Reactions that mediate the synthesis of methylarginine species. ................... 21
Figure 4. Putative PRMT targets that regulate skeletal muscle plasticity....................... 26

Figures from Manuscript

Figure 1. Denervation induces muscle atrophy after 168 hours................................. 105
Figure 2. Phenotype modifying protein expression and activation states are altered with muscle disuse ......................................................................................................................... 106
Figure 3. Protein arginine methyltransferase 1 (PRMT1), coactivator-associated methyltransferase 1 (CARM1), and PRMT5 expression during denervation. .............. 107
Figure 4. PGC-1α, PRMT1, CARM1, and PRMT5 cellular localization varies with disuse................................................................................................................................. 108
Figure 5. Disuse-evoked monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) content in skeletal muscle ...................................................................................................................... 109
Figure 6. Relationship between AMPK activation status and PRMT expression and function in response to neurogenic skeletal muscle disuse. .......................... 110
Figure 7. Relationship between p38 activation status and PRMT localization and activity during denervation-induced muscle remodelling.................................... 111
Figure 8. Summary of cellular events associated with disuse-evoked skeletal muscle remodelling.

.............................. ......................................................................................... 112
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>ADMA</td>
<td>asymmetric dimethylarginine</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazol-4-carboximde ribonucleoside</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>BTG1</td>
<td>B-Cell Translocation Gene 1</td>
</tr>
<tr>
<td>CARM1</td>
<td>co-activator-associated arginine methyltransferase 1</td>
</tr>
<tr>
<td>CN</td>
<td>calcineurin</td>
</tr>
<tr>
<td>CSA</td>
<td>cross-sectional area</td>
</tr>
<tr>
<td>DM1</td>
<td>Myotonic dystrophy type 1</td>
</tr>
<tr>
<td>DM2</td>
<td>Myotonic dystrophy type 2</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
</tr>
<tr>
<td>E2F1</td>
<td>E2F transcription factor 1</td>
</tr>
<tr>
<td>EDL</td>
<td>extensor digitorum longus</td>
</tr>
<tr>
<td>FOX</td>
<td>Forkhead box</td>
</tr>
<tr>
<td>GAR</td>
<td>glycine- and arginine-rich</td>
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<tr>
<td>GAST</td>
<td>gastrocnemius</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>MAFbx</td>
<td>muscle atrophy F-box</td>
</tr>
<tr>
<td>MAT</td>
<td>methionine adenosyltransferase</td>
</tr>
<tr>
<td>MEF2C</td>
<td>myocyte enhancer factor-2C</td>
</tr>
<tr>
<td>MMA</td>
<td>monomethylarginine</td>
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<tr>
<td>MuRF1</td>
<td>muscle RING finger 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MuSC</td>
<td>muscle stem cells</td>
</tr>
<tr>
<td>Myf</td>
<td>myogenic regulatory factor</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mammalian Target Of Rapamycin Complex 1</td>
</tr>
<tr>
<td>MPB</td>
<td>muscle protein breakdown</td>
</tr>
<tr>
<td>MPS</td>
<td>muscle protein synthesis</td>
</tr>
<tr>
<td>NCoR1</td>
<td>nuclear receptor corepressor 1</td>
</tr>
<tr>
<td>OGT</td>
<td>N-acetylg glucosamine transferase</td>
</tr>
<tr>
<td>p38</td>
<td>p38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>(PPARγ) coactivator-1α</td>
</tr>
<tr>
<td>PGM</td>
<td>proline-, glycine-, and methionine</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor-γ</td>
</tr>
<tr>
<td>PRMT</td>
<td>protein arginine methyltransferase</td>
</tr>
<tr>
<td>RIP140</td>
<td>receptor interacting protein 140</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>SDMA</td>
<td>symmetric dimethylarginine</td>
</tr>
<tr>
<td>SIRT1</td>
<td>silent mating type information regulator 2 homologue 1</td>
</tr>
<tr>
<td>SOL</td>
<td>soleus</td>
</tr>
<tr>
<td>TA</td>
<td>tibialis anterior</td>
</tr>
<tr>
<td>ULK1</td>
<td>UNC-51-like kinase 1</td>
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</tbody>
</table>
Declaration of Academic Achievement

DWS was the principal contributor. AM performed cell fractionation procedures and some Western blotting. VL assisted with conceiving and designing the study, as well as with writing the manuscript.
Review of the Literature
1. Introduction to Skeletal Muscle

Skeletal muscle constitutes about 40% of total body mass in mammals and is primarily responsible for locomotion, posture, and other body movements (1,2,3). All skeletal muscles are composed of multinucleated cells, called skeletal muscle fibers, which extend the entire length of the muscle. The multiple nuclei in a skeletal muscle fiber are located at the periphery of the cell just beneath the sarcolemma, and result from the fusion of myoblast precursor cells. The sarcolemma, also known as the plasma membrane, encloses each skeletal muscle fiber. Each muscle fiber contains several bundles of myofibrils, composed of actin and myosin filaments that form sarcomeres, which extend the length of the muscle fibers. In addition to sarcomeres, organelles such as mitochondria and glycogen granules also reside in the cell cytoplasm, which in muscles is called the sarcoplasm. Along the surface of the sarcolemma are transverse tubules characterized by invaginations that allow action potentials to quickly penetrate to the interior of the cell. The sarcoplasmic reticulum surrounds each myofibril and regulates the calcium ion concentration in the sarcoplasm. According to the sliding filament theory, sarcomeres shorten when actin and myosin filaments slide past one another following neuronal stimulation and calcium release into the cytosolic space (4,5). Sarcomere shortening causes muscle fibers to contract. Thus, the reduction of sarcomere length is responsible for skeletal muscle contraction and ultimately locomotion, posture, and other body movements.
Based on the particular myosin heavy chain isoform that they express, skeletal muscle fibers can be classified either as slow twitch (type 1) or fast twitch (type 2). Fast-twitch fibers can be further classified into types 2A and 2X in humans, while a sub-classification of type 2A, 2X and 2B exists in rodents (2,6,13,15). In addition to myosin heavy chain isoforms, many other components contribute to a fiber’s physiological characteristics. Type 1 fibers have the smallest cross-sectional area (CSA) and contain the greatest capillaries per fiber, mitochondrial density, satellite cell count, and neuromuscular junction size (2,13). Furthermore, type 1 muscle fibers are predominantly red in appearance since they have the greatest abundance of myoglobin and mitochondria. With these characteristics, type 1 fibers are highly oxidative and possess the greatest endurance capacity. In contrast, type 2B muscle fibers are white in appearance and contain the largest CSA along with the fewest capillaries per fiber, mitochondrial density, satellite cell count, and neuromuscular junction size (2,13). These myocytes also possess the greatest force generation and contraction velocity. In addition, 2B fibers are the most glycolytic and fatigue the quickest. Type 2A, along with hybrid myofibers, display morphological, molecular, biochemical, and functional characteristics somewhere in the spectrum between type 1 and 2B fibers.

Skeletal muscles possess varying proportions of different fiber types. For example, the distribution of type 1, 2A, 2X, and 2B myofibers, respectively, in murine tibialis anterior (TA), extensor digitorum longus (EDL), soleus (SOL) and
gastrocnemius (GAST) muscles are as follows: TA = ~0%, ~29%, ~26%, ~45%; EDL = ~4%, ~17%, ~27%, ~52%; SOL = ~42%, ~49%, ~7%, ~2%; GAST = 4%, ~18%, ~49%, ~29% (15,35). It is important to note that these proportions are plastic since muscle fibers have the ability to remodel their phenotype in response to various stimuli (2). A discussion of the skeletal muscle remodelling process appears below in section 2.

Skeletal muscle fiber type can influence the susceptibility and resistance to certain neuromuscular diseases and other muscle wasting disorders. For instance, type 2 fibers in Duchenne muscular dystrophy (DMD) patients are the first myofibers to degenerate and die, whereas type 1 fibers are more resistant to the dystrophic pathology (7,8). Similarly, myotonic dystrophy type 2 (DM2) patients demonstrate preferential type 2 fiber atrophy (9). Likewise, the loss of skeletal muscle mass and strength due to aging is characterized by greater atrophy of type 2 muscle fibers, as compared to type 1 myofibers (10). In contrast, myotonic dystrophy type 1 (DM1) patients display greater type 1 myofiber atrophy in comparison to type 2 muscle fibers (9). In addition, muscle disuse due to bed rest and spinal cord injury has been reported to cause more pronounced muscle wasting in type 1 fibers relative to type 2 (11,12). It remains obscure as to why certain fiber types are preferentially affected in these, and other conditions. However, factors such as the differences in sarcolemmal protein composition, intracellular calcium dynamics, as well as the contractile apparatus may play a role in muscle fiber type resistance or susceptibility to various muscle diseases.
(14). Furthermore, oxygen transport and utilization capabilities, along with molecular signalling infrastructure, could all contribute to this intriguing and important physiologically relevant phenomenon (14). Thus, targeting muscle fiber type plasticity in order to remodel myofibers to be more pathologically resistant may be a feasible strategy for treating various neuromuscular diseases and other muscle wasting disorders.

2. Skeletal Muscle Plasticity

Skeletal muscle is a highly dynamic tissue that continuously remodels in response to various environmental demands. For instance, a phenotypic shift from a fast, glycolytic muscle type toward one with slower, more oxidative characteristics can be initiated with chronic muscle use (16,30). On the other hand, induction of the fast, glycolytic myogenic program, as well as muscle atrophy, can be triggered via chronic muscle disuse (17). These adaptations depend on the chronic activation or inhibition of various intracellular signaling pathways, which result in altered skeletal muscle gene expression (2,31). Intracellular signaling molecules such as peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α), calcineurin (CN), p38 mitogen-activated protein kinase (p38), AMP-activated protein kinase (AMPK), silent mating type information regulator 2 homologue 1 (SIRT1), PPARβ and tumor suppressor protein p53 play a role in remodelling skeletal muscle toward a slower, more oxidative phenotype (16,18,19,20,21,22,23,24,25). In contrast, receptor interacting protein 140 (RIP140), E2F transcription factor 1 (E2F1), nuclear receptor corepressor 1
(NCoR1), and Baf60c have been shown to promote faster, more glycolytic characteristics (26,27,28,29,32). Thus, numerous molecules participate in the coordinated expression of morphological, biochemical and functional characteristics that define, and remodel, skeletal muscle phenotype (Fig. 1).

3. The Muscle Atrophy Program

3.1 Skeletal muscle adaptations to disuse

Various models of chronic muscle disuse elicit prolonged reductions in muscle activity, thereby triggering various adaptations in the muscle tissue itself, as well as neuromuscular junctions and microvasculature. In addition to reduced muscle mass, skeletal muscle atrophy is characterized by decreased muscle fiber CSA and contractile protein content (85,89,91). Other changes include endothelial degradation, increased capillary density, decreased mitochondrial quantity, higher levels of intermuscular adipose tissue and connective tissue, as well as increased expression of type 2 muscle fiber types (85,86,87,89,90,91). Chronic muscle disuse also results in increased insulin resistance, decreased calcium ion concentration, degeneration of neuromuscular junctions, nerve terminal disruption, plus decreased maximal voluntary force production and lower fatigue resistance (85,88,89,91,92,93). Therefore, adaptations associated with chronic muscle disuse are structural, physiological, and functional.
Figure 1. Overview of skeletal muscle plasticity. Skeletal muscle is a highly malleable tissue, capable of remodelling its morphological and functional characteristics, such as capillary density, mitochondrial content, fatigue resistance, contraction velocity, and pathological resistance. Receptor interacting protein 140 (RIP140), E2F transcription factor 1 (E2F1), nuclear receptor corepressor 1 (NCoR1), and Baf60c and chronic muscle disuse (e.g., denervation, hind limb unloading) promote a faster, more glycolytic phenotype (26,27,28,29). In contrast, chronic activation of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), calcineurin (CN), p38 mitogen-activated protein kinase (p38), AMP-activated protein kinase (AMPK), silent mating type information regulator 2 homologue 1 (SIRT1), PPARβ and tumor suppressor protein p53 by way of transgenic, physiological (e.g., endurance exercise), or pharmacologic (e.g., AICAR) mechanisms foster slower, more oxidative characteristics (16,18,22,24,25). PGC-1α plays a major role in remodelling skeletal muscle due to its ability to translocate to the nucleus and co-activate several transcription factors that bind to promoters of genes indicative of the slow, oxidative myogenic program (16,22,24). CN indirectly and p38 directly activates upstream transcription factors of PGC-1α (16,18). Both p38 and AMPK directly stimulate PGC-1α through phosphorylation, while SIRT1 directly activates PGC-1α via deacetylation, and RIP140 suppresses PGC-1α (16,32). In addition to PGC-1α, both p38 and AMPK promote mitochondrial biogenesis by activating p53 via phosphorylation (24).
3.2 Molecular mechanisms of skeletal muscle atrophy

Skeletal muscle mass is regulated by the balance between the rate of muscle protein synthesis (MPS) and muscle protein breakdown (MPB). Muscle atrophy occurs when MPB chronically exceeds MPS, and may be induced in a variety of conditions. In both human and rodent models, it is generally agreed that MPS rates decline immediately following disuse and remain suppressed for the duration of disuse (31,33). Notably, the cellular mechanisms responsible for this decline in MPS are poorly understood. It has been hypothesized that under conditions of chronic muscle disuse, decreases in MPS are the consequence of mammalian target of rapamycin complex 1 (mTORC1) inhibition (31). Studies of several different rodent models of muscle wasting have indicated that MPB also contributes toward muscle atrophy (31,34). More specifically, accelerated proteolysis via the ubiquitin-proteasome pathway is primarily responsible for MPB (34). The second protein degradation pathway, termed the autophagy-lysosome system, is also activated during muscle atrophy (96). Our understanding regarding molecules and cellular pathways that drive the skeletal muscle atrophy program is limited.

Muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx) are ubiquitin-protein ligases that selectively bind and mark substrates for ubiquitination and subsequent degradation by the 26S proteasome (17). Although only a limited number of specific cellular targets of MuRF1 and MAFbx have been identified, the evidence clearly demonstrates that these enzymes drive the
muscle atrophy program (17,83). The class O-type forkhead transcription factors (FOXO1, FOXO3a) transcriptionally upregulate MuRF1 and MAFbx during atrophy-inducing conditions (17). These FOXO enzymes require dephosphorylation in order to translocate to, and enter, the myonuclei where they perform their transcriptional activities. AKT-dependent phosphorylation of FOXO1 at Ser256 prevents its entry into the nucleus (78,117), whereas phosphorylation by AKT on residues Thr32 and Ser253 also suppresses FOXO3a activity by cytosolic retention (44). In response to atrophy-evoking conditions, AKT-induced FOXO phosphorylation is reduced (94). Thus, elevated ubiquitinated protein content, MuRF1 and MAFbx expression, along with nuclear localization of FOXO1 and FOXO3a, all mark a shift in protein balance from net synthesis to net degradation. Recent evidence strongly suggests that PGC-1α, p38 and AMPK play also important roles in the atrophy signaling cascade, and thus in the muscle atrophy process (Fig. 2).

PGC-1α appears to play a protective role against skeletal muscle atrophy. Transgenic PGC-1α overexpression can impede muscle wasting (42,43,49). For example, Sandri et al. and Hindi et al. showed that mice overexpressing PGC-1α have suppressed FOXO3a action following denervation, and demonstrate an attenuated muscle wasting response, as compared to wild-type animals (43,52). In a similar fashion, Cannavino et al. showed that overexpression of PGC-1α in skeletal muscle of transgenic mice significantly blunted the up-regulation of MuRF1 and MAFbx genes after hind limb unloading in both slow and fast
Figure 2. Hypothetical pathway for skeletal muscle atrophy. Under conditions of chronic muscle disuse, p38 mitogen-activated protein kinase (p38) and AMP-activated protein kinase (AMPK) are activated (53,54,55,56,57,62,66,67,82). However, interventions of this nature reduce peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) expression (37,43). The interaction between p38, PGC-1α, and AMPK remains to be elucidated. Furthermore, downstream events are currently unclear with respect to p38. Several studies have found that activation of p38 induces muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx) (57,60,61,64,65,69) expression, while others have shown the opposite outcome (70,71). In response to disuse, the inhibitory effect of PGC-1α and AKT on forkhead box O3a (FOXO3a) is attenuated (43,94), while augmented AMPK activation leads to increased FOXO3a transcriptional activity (76,80). In addition, AMPK activation leads to the inhibition of mammalian target of rapamycin complex 1 (mTORC1), plus activation of UNC-51-like kinase 1 (ULK1) (76). ULK1 is a key initiator of autophagy that is negatively regulated by mTORC1 (75). MuRF1 and MAFbx are upregulated via FOXO3a during atrophy-inducing conditions (17). Increased expression of MuRF1 and MAFbx leads to the attachment of ubiquitin cofactors to proteins, thus rendering them targets for the proteasome degradation pathway (17). Therefore, activation of p38 and AMPK, plus downregulation of PGC-1α leads to increased muscle protein breakdown, and ultimately muscle atrophy.
muscles (50, 51). When exposed to constitutively active FoxO3a, Brault et al. reported that PGC-1α overexpression in myotubes blocked the catabolic actions of FOXO3a (49). A downregulation of PGC-1α has been observed during chronic muscle disuse (37,43). This disuse-evoked decline in PGC-1α is associated with enhanced FOXO-dependent loss of muscle mass (43). Thus, PGC-1α expression is negatively regulated during muscle disuse, while its presence can attenuate atrophy under these conditions.

Unlike PGC-1α, the influence of p38 on the muscle atrophy program is relatively unclear. Several studies have reported increased p38 activity in response to immobilization, hind limb suspension and denervation (53,54,55,56,57,62,66,67,69). In support of the notion that elevation of p38 activity promotes protein degradation, numerous papers have showed that induction of MuRF1 can be blocked by p38 inhibitors and that constitutive activation of p38 induce MuRF1 gene expression in vivo and in vitro (57,63,65). Similarly, other studies have demonstrated that activation of p38 is necessary and sufficient for the up-regulation of MAFbx and that inhibitors of p38 block the up-regulation of MAFbx and avert muscle wasting in vivo and in vitro (60,61,64,69). In contrast, Hunter et al. found no change in skeletal muscle p38 activity after 7 days of hind limb unloading (58). Shi et al. did not detect a significant effect of p38 inhibition on MuRF1 and MAFbx expression in vitro, yet reported that inactivation of p38 in vivo actually induced profound muscle atrophy (59). In agreement with Shi et al., studies have shown that inhibition of p38 triggers the
nuclear localization of FOXO3a and subsequent activation of its target genes (70, 71). Furthermore, activation of p38 decreased the expression of MAFbx in vitro (70). Due to the conflicting data regarding the regulation of MuRF1 and MAFbx by p38, it remains uncertain whether p38 promotes, or protects against, protein degradation and atrophy. It is unlikely that these discrepancies are due to differential subcellular compartmentalization of p38, since it has been shown that p38 resides mainly in the cytosol in both innervated and denervated muscle (68). Instead, the conflicting data may be due to different isoforms of p38 playing distinct functions in response to atrophy or the possibility that p38 function varies at different times during the muscle atrophy process. Additional research is clearly necessary to fully define the precise functional role of p38 in response to catabolic stimuli.

In addition to PGC-1α and p38, AMPK plays a role in mediating atrophy in skeletal muscle. Several studies have shown that 5-aminoimidazol-4-carboximide ribonucleoside (AICAR), a pharmacological agent commonly used to artificially activate AMPK, increases MuRF1, MAFbx, FOXO1 and FOXO3a gene and protein expression in vitro and in vivo (73, 74, 76). In support of this, inhibition of AMPK in vitro has been reported to prevent increased atrogate expression in response to serum deprivation, as well as AICAR and dexamethasone treatments (73). Studies investigating various models of atrophy in vitro, and in vivo further suggest that AMPK mediates muscle protein degradation (51, 79, 82, 97). For example, Guo et al. showed that AMPKα
phosphorylation levels increased in atrophic muscles after 7 days of denervation, suggesting AMPKα was activated in response to disuse (82). Additionally, deletion of AMPKα2 blunted MuRF1 and MAFbx expression, as well as preserved phosphorylation levels of FoxO3a at Ser253, thus inhibiting FOXO3a (82). Interestingly, Barthel et al. showed that AMPK activation via AICAR led to the inhibition of FOXO1, suggesting that FOXO1 and FOXO3a may be differentially regulated by AMPK (80,81). In addition to the ubiquitin-proteasome pathway, studies have shown that AMPK can upregulate MPB by activating the autophagy system. For example, evidence suggests that AMPK directly activates UNC-51-like kinase 1 (ULK1), the master regulator of autophagy, through phosphorylation (75,76,77). Furthermore, AMPK activation leads to the inhibition of mTOR and subsequently lifts the breaking effect of mTOR on the ULK1 autophagic complex (1,75,76). Evidently, AMPK fosters protein degradation via the autophagy system and the ubiquitin-proteasome pathway. Thus, PGC-1α, p38, and AMPK appear to be important players in regulating the skeletal muscle atrophy program.

3.3 Experimental models of muscle disuse

Beyond the repertoire of cellular and molecular pathways, the rate and amount of muscle atrophy is governed, in part, by 1) the model of disuse, 2) muscle physiological function, and 3) muscle fiber type composition (31).

Various models of disuse in humans and rodents include immobilization, hind limb suspension, bed rest, microgravity, spinal cord injury, spinal cord
isolation, and denervation (31). Comparison of disuse studies in humans and rodents reveal similar adaptations, including reductions in muscle mass. However, a clear difference exists in the rate at which atrophy occurs, with loss of muscle mass in rodents being considerably faster than that observed in humans (33). Muscle atrophy under conditions of disuse varies significantly depending on the level of contractile activity (31). For example, the amount of muscle loss is often greater under conditions of joint immobilization than bed rest and hind limb suspension (85). It is noteworthy that muscle atrophy is the most pronounced after denervation, as compared to other models of muscle disuse (3,48). Indeed, Sacheck et al. reported a more profound loss of muscle mass in response to denervation compared to spinal cord isolation-induced disuse in rodents (37). This may be due to the fact that denervation not only prevents muscle contraction, but also leads to the loss of trophic factors released by innervating motor neurons (3). However, the consensus in the literature indicates that the lack of trophic, non-motor factors do not play a significant role in denervation-induced adaptations (84). This conclusion is based on a wealth of data that demonstrates that all denervation-evoked changes (e.g., atrophy, fibrillation, acetylcholine sensitivity, sarcolemmal electrical properties) can be counteracted by chronic electrical stimulation-induced contractile activity of the denervated muscle, as well as evidence indicating that complete conduction block in the muscle nerve can cause denervation-like effects (84). Therefore, the extent of muscle atrophy depends on the model of disuse.
3.4 Physiological function of skeletal muscle

Muscles differing with respect to their function also affect the degree of disuse-related atrophy observed in humans and rodents (31,33). Generally, extensor muscles of the ankle (i.e. SOL and GAST) atrophy far more rapidly than the flexor muscles (i.e. TA and EDL) (31,33,36,41). Despite higher levels of protective PGC-1α, the degree of muscle atrophy is generally more pronounced in slower, more oxidative muscles during unloading or denervation (31,33,36,48). For example, Roy et al. reported greater atrophy-induced effects in the GAST than the TA following spinal cord isolation in rodents (38). Furthermore, de Boer et al. showed a larger impact of atrophy in the GAST versus the TA after 5 weeks of bed rest in humans (39). Since the ankle extensors are primary postural muscles that exhibit chronic activation, as compared to the more phasically active ankle flexors responsible for locomotion (95), the dissimilar response of SOL/GAST and TA/EDL muscles to disuse likely reflects differences in loading under normal conditions. Thus, the physiological function of skeletal muscle influences atrophy.

3.5 Muscle fiber type composition

Muscle wasting does not occur similarly in all types of muscle fibers. During fasting (45), sepsis (46), and cancer cachexia (47), type 2 muscle fibers display greater atrophy than type 1 myofibers. On the other hand, within a particular muscle, the reduction of fiber CSA due to disuse is first observed in type 1, followed by type 2A, 2X and 2B myofibers (31). For instance, Allen et al. observed that fiber CSA was significantly reduced in type 1, but not type 2,
muscle fibers of rat soleus muscles after 14 days of spaceflight (40). Unlike rodents, human muscle exhibits smaller differences between fiber types in their degree of disuse atrophy (33). For example, no differences were observed in the degree of fiber atrophy among type 1, 2A, and 2X myofibers following 14 (122) or 21 days (123) of immobilization in humans. These lack of fiber-specific differences in human muscle may be due to the possibility that muscle biopsy samples are too small to adequately detect differences (33). Unlike the muscle wasting caused by some disease states, disuse atrophy is initiated by a reduction in muscle contractile activity. Therefore, there is a general understanding that the degree of atrophy is dependent on the fiber type composition of the affected muscles.

3.6 Acute molecular responses to muscle disuse

A common limitation of both human and rodent studies is that few data are available on the intracellular signalling and gene expression events that may precede any disuse-induced alteration in phenotype (e.g., loss of muscle mass). Indeed, the majority of studies that investigate the mechanisms of disuse-evoked muscle remodelling examine the muscles as early as 3-7 days after the introduction of the disuse stimulus when muscle atrophy is first observed (50,51,98,100,101,102,106,107,108,109,110,111,112,114,120,121). To increase our understanding of the upstream cellular events that drive the plasticity of muscle under these conditions, it is necessary to study muscle biology during the initial stages of disuse. Notably, Ferreira et al. investigated cell proliferation in
mice skeletal muscle after 6 hours of hindlimb suspension (72). To our knowledge, this study marks the earliest published time point examined in rodents after disuse. The authors found increased proliferation of satellite cells as early as 6 hours after disuse.

Multiple studies in rodents have demonstrated that MuRF1 and MAFbx gene expression levels are elevated prior to muscle atrophy (37, 103,104,105,116). Sacheck et al. found that MuRF1 and MAFbx gene expression were significantly increased at as early as 1 day of denervation-induced muscle disuse, while muscle atrophy was observed 2 days later (37). Recent work from Tang et al. revealed that histone deacetylase (HDAC) 4, HDAC5, and myogenin were also activated prior to denervation-induced muscle atrophy, suggesting that HDAC4, HDAC5, and myogenin may act upstream of, or in concert with, MuRF1 and MAFbx (103). AMPK has been shown to regulate myogenin expression through phosphorylation of HDAC5 (118), indicating that AMPK may help mediate the early upstream cellular events that drive skeletal muscle plasticity after disuse. Furthermore, PGC-1α mRNA expression has been shown to decrease by ~80% after 1 day of denervation (37), indicating that PGC-1α may also play an important role in muscle remodelling immediately following disuse. Thus, in response to disuse, there appear to be various signaling and transcriptional events occurring within the muscle that precede phenotypic remodelling. It is reasonable to assume that at least some of these processes are required in order for muscle remodelling to take
place. Further studies are required to elucidate the critical upstream molecules and events that drive inactivity-induced muscle plasticity.

In studies investigating the early mechanisms of disuse-evoked muscle remodelling in humans, increased skeletal muscle MAFbx gene expression following 4-11 days of cast immobilization has been observed, while augmented MAFbx mRNA following 2 days of unloading has also been noted (99,113). Furthermore, decreased phosphorylation of AKT has been observed after 2 days of immobilization (115). Interestingly, work by Gustafsson et al. revealed that the mRNA levels of MuRF1 and MAFbx were elevated following 3 days of unilateral lower limb suspension in human participants despite no alterations in phosphorylated AKT, FOXO1, FOXO3a, and p38 levels (119). These models of limb immobilization and unloading generally result in a ~10% reduction in CSA after approximately 21 days of disuse (33). These data suggest that increased MuRF1 and MAFbx gene expression are among the first indicators of muscle wasting in humans. Collectively, studies in rodent models and humans point to numerous molecules that are activated prior to the initiation of muscle atrophy, which likely play important roles in driving the remodelling process.

4. Protein Arginine Methyltransferases

4.1 Characteristics of Protein Arginine Methyltransferases

Protein arginine methyltransferases (PRMTs) are a family of enzymes that catalyze the addition of one or two methyl groups to the guanidine nitrogen atoms of arginine residues on target proteins, thereby altering the stability, localization
and activity of the marked molecules (124,125,126,127,131,139,158). This post-
translational modification of histones, transcription factors, and other proteins
enables PRMTs to regulate many diverse cellular processes such as gene
transcription, mRNA splicing, DNA repair, signal transduction, protein
subcellular localization, and cell cycle progression (124,127,128,131). PRMTs
are generally ubiquitously expressed and deregulation of these enzymes has been
implicated in the pathogenesis of several different diseases such as cancer (124).

The PRMT family consists of numerous members, all of which use S-
adenosyl-L-methionine (SAM) as a methyl donor (125,126,131,158). SAM is
generated by the enzyme methionine adenosyltransferase (MAT) using the
substrates methionine and ATP (186). All PRMTs utilize SAM and L-arginine to
catalyze the formation of the monomethylarginine (MMA) mark onto target
molecules, which also results in the product S-adenosylhomocysteine (SAH)
(138). Proteins that contain glycine- and arginine-rich (GAR) motifs and/or
proline-, glycine-, and methionine (PGM)-rich regions are major targets for
arginine methylation (124).

Although PRMTs share many common features, they also have their own
unique attributes. Among these, type I PRMTs, including PRMT1, PRMT2,
PRMT3, PRMT4 [also called co-activator-associated arginine methyltransferase 1
(CARM1)], PRMT6, and PRMT8 catalyze the deposition of methyl groups on
target arginine residues and produce the asymmetric dimethylarginine (ADMA)
mark, whereas type II PRMTs, including PRMT5, PRMT7, and PRMT9 generate
the formation of the symmetric dimethylarginine (SDMA) mark (124,125,126,127,128). The ADMA and SDMA reactions require MMA and L-arginine. Of all the PRMTs, PRMT1 and PRMT5 are the enzymes primarily responsible for generating ADMAs and SDMAs, respectively (124,128). Notably, PRMT1 is the predominant PRMT in mammalian cells, and carries out ~85% of total PRMT activity (125,127,128). The complete loss of either PRMT1 or PRMT5 are embryonic lethal, while CARM1 knockout mice die shortly after birth, indicating that these enzymes are critical for survival (129,130,132,133,136) (Fig. 3).

4.2 Regulation of PRMT expression and activity

PRMT expression levels are mediated, in part, at the transcriptional level. For example, studies in vitro and in vivo have shown that signal transducer and activator of transcription-6, nuclear factor kappa-light-chain-enhancer of activated B cells, and PPARγ are among the factors that control PRMT1 transcriptional activation (157,159). Furthermore, early growth response-1 and nuclear transcription factor Y have been identified as transcription factors that bind to promoters of CARM1 and PRMT5, respectively (160,161). Further studies are required in order to gain a better understanding of the transcriptional control of PRMT promoters, as well as whether PRMT expression is regulated at the post-transcriptional level, for example by spliceosomal processing, nuclear mRNA export, and/or mRNA stability.
Figure 3. Reactions that mediate the synthesis of methylarginine species.

A) Methionine adenosyltransferase (MAT) is the enzyme responsible for the synthesis of S-adenosyl-L-methionine (SAM), inorganic phosphate (P_i), and pyrophosphate (PP_i) using methionine, ATP, and H_2O. Notably, SAM is the donor substrate for methyl group transfer and is produced in the cytosol (186). Furthermore, the formation of monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) in mammalian cells is carried out by a family of enzymes known as protein arginine methyltransferases (PRMTs) (124,127,138).

B) All PRMTs generate MMA by using arginine and SAM substrates. After methyl groups are added to compounds, SAM is converted to S-adenosylhomocysteine (SAH) (138,186).

C, D) The subsequent generation of ADMA and SDMA are catalyzed by type I and type II PRMTs, respectively (124,127).
Regulation of PRMT activity can be achieved through post-translational modification of the enzymes. Post-translational modifications of PRMTs include automethylation, phosphorylation and glycosylation. Although automethylation activity has been reported for PRMT1 and CARM1, the functional impact of this specific reaction has yet to be fully elucidated (163,164). Recent work has demonstrated that phosphorylation by as yet unidentified kinases impairs the methyltransferase activity of PRMT1 and CARM1 (165,167). Furthermore, PRMT5 catalytic activity has been shown to be disrupted when phosphorylated by the kinase JAK2 (162). Interestingly, overexpression of N-acetylglucosamine transferase increases O-GlcNAcylation of CARM1 and prevents phosphorylation of the enzyme (166). These results suggest that post-translational modifications via phosphorylation generally inhibit PRMT-mediated arginine methylation, while glycosylation may provide the opposite effect. In any case, there is currently a gap in the literature regarding the identity of upstream intracellular signaling molecules that conduct post-translational modifications of PRMTs. Clearly, more work is needed in this critical area of PRMT biology.

A variety of PRMT-binding proteins can also regulate methyltransferase activity by inhibition, activation, or even through changing PRMT substrate specificity. For instance, B-Cell Translocation Gene 1 (BTG1) and BTG2 stimulate PRMT1 activity towards selected substrates (168). The mechanism by which this enzymatic activation occurs is not yet completely understood. In contrast, orphan nuclear receptor TR3, as well as protein phosphatase 2A bind to
the catalytic domain of PRMT1 to inhibit PRMT1 methyltransferase activity (169,170), presumably via dephosphorylation in the latter case. In addition, both the nucleosomal methylation activator complex and the hSWI/SNF complex further enhance the ability of CARM1 and PRMT5, respectively, to methylate histones (142,171).

In addition to post-translational modifications and protein-protein interactions affecting PRMT activity, PRMT function is also regulated by its localization within the cell. In various cell types, it is generally accepted that PRMT1 and PRMT5 are predominantly localized in the nucleus and cytosol, respectively (125). CARM1 however, has been found primarily in nuclei where it serves as a methylation-mediated transcriptional co-activator (125). Similar to CARM1, PRMT1 can also function as a transcriptional coactivator, whereas in contrast, PRMT5 has been identified as a transcriptional repressor (127). Furthermore, to some extent, all PRMTs have an epigenetic function in nuclei, which is histone methylation. This epigenetic role of PRMT1, CARM1, and PRMT5 methyltransferase activities further distinguishes each enzyme from its PRMT family counterparts.

PRMT1, CARM1, and PRMT5 carry out differential post-translational modifications. PRMT1 catalyzes the asymmetric dimethylation of histone 4 arginine 3 (H4R3), whereas PRMT5 carries out symmetric dimethylation of H4R3 and H3R8 (142,143). Importantly, asymmetric dimethylation via PRMT1 activates H4R3, whereas symmetric dimethylation by way of PRMT5 results in
H4R3 repression (127). CARM1 modifies H3R17 by depositing the ADMA mark (140, 141). For example, in human cancer cells, arginine methylation of H3R17 by CARM1 occurs coincident with the transcriptional activation of the E2F1 promoter (141). E2F1 has also been found to undergo residue-specific methylation by PRMT1 and PRMT5 (152), making E2F1 an example of a molecule that is regulating by methylation at multiple stages of the gene expression pathway. Interestingly, E2F1 is among several proteins targeted by PRMTs that function to mediate skeletal muscle plasticity (27,18). A limited number of in vitro studies in muscle cells, as well as numerous other in vitro and in vivo investigations in non-muscle tissues have shown that PRMT1, CARM1, and PRMT5 can stimulate or suppress molecules important for muscle remodelling by way of their specific methyltransferase activities. For example, p53 activity is significantly altered when arginine methylated by PRMT1, CARM1, or PRMT5 (149,150,151). RIP140 transcriptional corepressor activity is suppressed by PRMT1 methylation at any one of three specific arginine residues, including R240, R650, or R948 (145). PRMT1 has also been shown to potentiate PGC-1α coactivator activity (146,147,148) by methylating arginine residues in the C-terminal Glu-rich E region of PGC-1α, which plays a critical role in the coactivator function of the enzyme (146). Furthermore, PRMT1 and PRMT5 mediate the activation of p38, while CARM1 has been reported to regulate AMPK (153,154,155,156). Therefore, in vitro studies in muscle cells, as well as cell culture and in vivo investigations in non-muscle tissues examining PRMT targets
and activity, suggest that PRMT1, CARM1, and PRMT5 have the potential to robustly regulate skeletal muscle plasticity in vivo (Fig. 4).

5. Protein Arginine Methyltransferase Biology in Skeletal Muscle

5.1 The role of protein arginine methyltransferases in skeletal muscle

The first investigation of PRMT biology in skeletal muscle was performed by George Muscat’s laboratory in 2002 (175). The authors detailed the expression and function of CARM1 during myogenesis of C2C12 mouse skeletal muscle cells in vitro. The next hallmark paper was by Iwasaki and Yada in 2007, who examined the role of PRMT1 in insulin-stimulated glucose metabolism in L6 myotubes in culture (173). The majority of current research in skeletal muscle PRMT biology has focused on PRMT1, CARM1, PRMT5, and PRMT7, with more recent studies being performed in vivo in rodent models. Relative to other PRMTs, CARM1 mRNA transcripts are expressed to the highest degree in skeletal muscle, followed by PRMT5 and PRMT1 (174). Relative protein expression and enzyme activity levels between PRMTs in skeletal muscle have not yet been determined.

PRMT1, CARM1, and PRMT5 participate in the regulation of muscle development. During myoblast fusion, PRMT1 exhibits the highest methyltransferase activity (177), while CARM1 is required for expressing genes necessary for the later stages of skeletal muscle differentiation (134,175). Indeed, inhibition of CARM1 has been shown to abrogate the expression of transcription factors important for initiating differentiation such as myogenin and myocyte
Figure 4. Putative PRMT targets that regulate skeletal muscle plasticity.

In vitro studies in muscle cells, as well as several other in vitro and in vivo investigations in non-muscle tissues have shown that protein arginine methyltransferase 1 (PRMT1), co-activator-associated arginine methyltransferase 1 (CARM1), and PRMT5 target proteins that mediate skeletal muscle remodelling. For example, PRMT1 has been shown to inhibit receptor interacting protein 140 (RIP140) and promote forkhead box O1 (FOXO1), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), tumor suppressor protein p53, p38 mitogen-activated protein kinase (p38), and E2F transcription factor 1 (E2F1) activities (144,145,146,147,148,149,152,154,155,156). CARM1 has also been reported to promote AMP-activated protein kinase (AMPK) and p53 activities (149,153). Furthermore, PRMT5 has been found to stimulate p53, p38, plus inhibit E2F1 activity (150,151,152,156). Thus, PRMT1, CARM1, and PRMT5 have the potential to play key roles in skeletal muscle plasticity.
enhancer factor-2C (MEF2C) (175,176). In contrast, PRMT5 is required for early gene expression, suggesting that distinct PRMTs are preferentially active at different times throughout myogenesis (134,176,180). PRMT5 has also been reported to regulate myogenin, MEF2C, MyoD, and myogenic regulatory factor 5 (Myf5) expression (137,176,178,181). In addition, CARM1 and PRMT5 can be recruited to the promoter of the myogenin gene to further enhance its transcriptional activation during cell differentiation (183). Notably, these studies describing the expression and function of PRMTs in muscle development were all performed in vitro, utilizing rodent muscle cell culture models. Recent investigations demonstrate that CARM1 and PRMT5 are also crucial for muscle regeneration in vivo. Following injury to skeletal muscle, CARM1 methylates Pax7 and is required for the induction of Myf5 transcription during regeneration (135). Work by Zhang and colleagues (182) showed that PRMT5 is required for proliferation and differentiation of MuSC during recovery from cardiotoxin-induced muscle damage (182). Thus, PRMT1, CARM1, and PRMT5 are important players in the muscle development and regeneration programs.

PRMT1 and CARM1 may also be involved in the adaptive response to exercise. For example, the first in vivo description of PRMT biology in mammalian skeletal muscle demonstrated the differential induction of PRMT1 and CARM1 transcript levels after a single bout of exercise (185). The exercise-evoked increase in PRMT expression was influenced by prior AMPK stimulation. Additionally, PRMT1 and CARM1 serve as important regulators of skeletal
muscle glucose metabolism (173,174), which is profoundly affected during exercise. Taken together, these studies highlight a potential role of PRMTs in the regulation of skeletal muscle plasticity.

5.2 Protein Arginine Methyltransferases as potential therapeutic targets for neuromuscular disorders

PRMTs may be pharmacologic and/or physiological targets for various neuromuscular diseases, including acquired and inherited neurogenic disorders, muscular dystrophies, motoneuron disorders, as well as the sarcopenia of aging. Among the many characteristics that these conditions share, the progressive skeletal muscle wasting may be among those affected by PRMT expression and function. For example, in human embryonic kidney cells, PRMT1-dependent FOXO methylation inhibits its phosphorylation by AKT, thereby enabling FOXO nuclear localization (144). Recall that FOXO is a powerful driver of the muscle atrophy program via its promotion of the ubiquitin-proteasome and autophagy systems. Furthermore, recent studies have demonstrated that PRMT1 and CARM1 have direct roles in the autophagic signalling pathway (172,179). Although these studies were not performed in skeletal muscle, the data suggest that PRMTs may be important mediators of muscle protein metabolism. Interestingly, spinal muscular atrophy is characterized by abnormally upregulated CARM1 in motoneurons (184), and PRMT1 and CARM1 expression content is higher in dystrophic versus healthy muscle (185). Therefore, interventions that alter the expression and activity of PRMTs may therefore offer an effective strategy for
blunting the atrophy associated with various neuromuscular conditions. Further studies are required in order to define the role of PRMTs in mediating skeletal muscle plasticity.

6. Study Objectives

6.2 Objectives

Despite the limited number of studies investigating PRMT biology in skeletal muscle, the evidence strongly suggests that this family of enzymes are important players in the regulation of skeletal muscle plasticity. However, their role within the context of disuse-induced muscle remodelling is unknown. Thus, the objectives of this thesis were to 1) determine whether denervation-induced muscle disuse alters PRMT expression, localization, and activity in skeletal muscle, and 2) to characterize the early disuse-evoked signaling events that precede muscle atrophy. We hypothesized that important metrics of PRMT biology, including expression, localization, and activity would be altered during disuse-induced muscle remodelling. Furthermore, we anticipated that these changes would occur early in the denervation time course within the context of signaling pathways that mediate muscle plasticity.
References


over-expression in fast muscle atrophy following hindlimb unloading. *J Physiol.*, 593(8),1981-95.


starvation and AICAR, an activator of AMP-activated protein kinase.

*Endocrinology*, 143(8), 3183-6.


reveals alterations in mRNA and protein for extracellular matrix components.  


   (2004). Human SWI/SNF-associated PRMT5 methylates histone H3 arginine
   8 and negatively regulates expression of ST7 and NM23 tumor suppressor

   Low levels of miR-92b/96 induce PRMT5 translation and H3R8/H4R3
   methylation in mantle cell lymphoma. EMBO J. 26(15), 3558-69.

144. Yamagata, K., Daitoku, H., Takahashi, Y., Namiki, K., Hisatake, K., Kako,
   FOXO transcription factors inhibits their phosphorylation by Akt. Mol Cell.,
   32(2), 221-31.

145. Mostaql Huq, M.D., Gupta, P., Tsai, N.P., White, R., Parker, M.G. & Wei,
   activity by protein arginine methylation. EMBO J, 25(21), 5094-104.

   Activation of nuclear receptor coactivator PGC-1alpha by arginine

   Jeannesson, E., Maskali, F., Gueguen, N., Marie, P.Y., Lacolley, P.,
   Herrmann, M., Juillière, Y., Malthiery, Y. & Guéant, J.L. (2011). Methyl
   donor deficiency induces cardiomyopathy through altered


Protein Arginine Methyltransferase Expression, Localization, and Activity During Disuse-induced Skeletal Muscle Plasticity

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Abstract

Protein arginine methyltransferase 1 (PRMT1), PRMT4 (also known as co-activator-associated arginine methyltransferase 1; CARM1), and PRMT5 catalyze the methylation of arginine residues on target proteins. Previous work suggests that these enzymes regulate skeletal muscle plasticity. However, the function of PRMTs during disuse-induced muscle remodelling is unknown. Thus, the purpose of our study was to determine whether denervation-induced muscle disuse alters PRMT expression and activity in skeletal muscle and to contextualize PRMT biology within the early disuse-evoked events that precede muscle atrophy. Mice were subjected to 6, 12, 24, 72, or 168 hours of unilateral hindlimb denervation. The contralateral limb served as an internal control. Muscle mass decreased by ~30% after 168 hours of neurogenic muscle disuse. The expression levels of muscle RING finger 1 and muscle atrophy F-box were elevated 72 hours post-denervation, occurring during the atrophy anticipation phase prior to the appearance of muscle loss. The expression, localization, and activities of PRMT1, CARM1, and PRMT5 were also modified, exhibiting changes in gene expression and activity that were PRMT-specific. Alterations in peroxisome proliferator-activated receptor-γ coactivator-1α, AMP-activated protein kinase (AMPK), and p38 mitogen-activated protein kinase were observed as early as 6 hours after denervation, which indicate that changes in the expression and activity of these enzymes are among the earliest signals during atrophy anticipation. These disuse-evoked alterations in AMPK activation
predicted changes in PRMT expression and function. Our data demonstrate that PRMT biology is integral to the mechanisms that precede, and initiate skeletal muscle plasticity during conditions of neurogenic disuse.

Introduction

Skeletal muscle is a plastic tissue that is capable of adapting to various physiological demands. For example, chronic muscle disuse can elicit muscle atrophy, as well as induce a shift toward a faster, more glycolytic phenotype (1). Conversely, various modes of chronic muscle use, for example exercise training, can trigger the opposite effect (2). An abundance of intracellular signaling molecules have been shown to regulate skeletal muscle plasticity. For instance, stimulation of AMP-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (p38) and the downstream, transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) promote a shift toward slower, more oxidative characteristics (2,3,4,5,6). In contrast, activation of receptor interacting protein 140 (RIP140) in skeletal muscle drives phenotypic remodelling in the opposite direction (7). Further elucidation of the molecular mechanisms that govern the maintenance and remodelling of muscle phenotype will increase our understanding of skeletal muscle biology, and will facilitate the discovery of therapeutic approaches for myopathies and neuromuscular disorders.

Protein arginine methyltransferases (PRMTs) also have the potential to mediate skeletal muscle plasticity. PRMTs are a family of enzymes that catalyze
the methylation of arginine residues on target proteins (8,9). In turn, the stability, localization and activity of these marked proteins are altered. Thus, this post-translational modification enables PRMTs to regulate cellular processes such as signal transduction, DNA repair, gene transcription and mRNA splicing (9,10).

All PRMT enzymes, whether classified as type I or type II, deposit the monomethylarginine (MMA) mark on their target molecules. PRMT1 and PRMT4 (also known as co-activator-associated arginine methyltransferase 1; CARM1) are type I PRMTs, which form asymmetric dimethylarginines (ADMA) by transferring two monomethyl groups from S-adenosyl-L-methionine to the same nitrogen atom of arginine residues on target proteins (10,11). PRMT5 is a type II PRMT that generates symmetric dimethylarginine (SDMA) marks on target substrates (10,11). PRMT1, CARM1, and PRMT5 account for over 90% of cellular methyltransferase activity (12). The complete loss of PRMT1 or PRMT5 is embryonic lethal, and newborn CARM1 knockout mice die shortly after birth, which indicates that these enzymes are indispensable for survival (9,10,13).

Studies in non-muscle tissues have shown that PRMTs suppress and stimulate RIP140 and PGC-1α transcriptional coregulator activities, respectively (14,15), as well as activate p53 and E2F1 (16,17,18). Previous investigations have demonstrated the ability of these transcription factors and coregulators to govern skeletal muscle remodelling (2,6,7,19).

There are few studies that have examined PRMT biology in skeletal muscle. However, this literature, although limited, supports the assertion that
PRMTs are important regulators of skeletal muscle phenotype. For example, PRMT1 has been shown to influence myogenic and insulin-signaling pathways in cell culture studies (20,21,22), CARM1 appears to be critical for muscle development, regeneration, and glycogen metabolism (23,24,25,26,27,28,29,30), while recent studies have demonstrated the requirement of PRMT5 for myogenesis and regeneration (21,23,25,29,31,32,33,34). Many of the investigations of PRMTs in skeletal muscle have been conducted in vitro employing myogenic and/or muscle cell cultures. The dearth of in vivo studies currently limits our understanding about the physiological impact of PRMTs. Ljubicic and colleagues were the first to demonstrate PRMT transcript and protein levels in mammalian muscle during conditions of phenotypic remodelling in vivo (35). The authors found differential PRMT1 and CARM1 protein expression between healthy and dystrophic muscle, as well as the induction of PRMT1 and CARM1 transcripts in response to a single bout of exercise. The expression of PRMTs during conditions of skeletal muscle plasticity in vivo is otherwise largely unknown. Thus, the purpose of this study was to characterize PRMT1, CARM1, and PRMT5 expression and activity during an acute time course of denervation-evoked skeletal muscle disuse. Moreover, we sought to examine PRMT biology within the context of early, disuse-induced signaling events that precede muscle atrophy. We hypothesized that important metrics of PRMT biology, including expression and activity, would be altered during disuse-induced muscle remodelling. Furthermore, we anticipated that these changes would occur early in
the denervation time course within the context of signaling pathways that mediate muscle plasticity.

Methods

*Animal surgery.* Male C57BL/6J mice (n = 41; ~30 g body weight; 17-weeks-old) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in an environmentally controlled room (23°C, 12 hour light/12 hour dark cycle) and provided food and water ad libitum. The animals were anesthetized by inhalation of isoflurane before surgery and received a subcutaneous injection of anafen (2mg/kg) for post-operative analgesia. A 1-2 cm skin incision was made in the posterior thigh musculature and blunt dissection was employed to expose the sciatic nerve. Unilateral denervation of the lower limb was induced by excising a ~0.5 cm section of the sciatic nerve in the right hind limb. The overlying musculature was sutured with silk, and the skin was secured using veterinary staples. Mice were subjected to 6, 12, 24, 72, or 168 hours of unilateral denervation (n=8-9/group) followed by euthanasia via cervical dislocation. We selected this model of acquired neurogenic muscle disuse because it elicits rapid, robust, and reproducible remodelling of skeletal muscle, while also allowing for the use of the contralateral, innervated limb to serve as an intra-animal control (36, 37, 38). At each experimental time point, the tibialis anterior (TA), extensor digitorum longus (EDL), and gastrocnemius (GAST) muscles from both hind limbs were rapidly excised, weighed, and frozen in liquid nitrogen. All
muscles were then stored at -80°C until subsequent analyses. All protocols were approved by the Animal Research Ethics Board at McMaster University and were carried out according to the regulations of the Canadian Council on Animal Care.

**RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)**. Total RNA was isolated from the EDL muscles as described previously (35). All samples were homogenized using 1 mL of Trizol reagent (H00007, Invitrogen) in Lysing D Matrix tubes (N00420, MP Biomedicals) with the FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals) at a speed of 6.0 m/s for 40 seconds. Homogenized samples were then mixed with 200 µL of chloroform (I00007, Fisher), agitated vigorously for 15 seconds and centrifuged at 12,000 x g for 10 minutes. The upper aqueous (RNA) phase was purified using the Total RNA Omega Bio-Tek kit (S00153, VWR) as per the instructions provided by the manufacturer. RNA concentration and purity was determined using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). RNA samples were then reverse-transcribed into cDNA using a high-capacity cDNA reverse transcription kit (V00086, Fisher Scientific) according to the manufacturer instructions. All individual qRT-PCRs were run in duplicate 25 µL reactions containing RT SYBR Green qPCR Master Mix (V00067, Qiagen). Data was analyzed using the comparative $C_T$ method (39). 18S ribosomal RNA (18S) was used as the internal control gene, as the $C_T$ values for this gene did not change between non-denervated and denervated muscles within, as well as between, time points (data not shown). This control $C_T$ value was
subtracted from the $C_T$ value of the gene of interest [$\Delta C_T = C_T (\text{target gene}) - C_T (\text{endogenous control})$]. The $\Delta C_T$ value of the non-denervated muscle was then subtracted from the $\Delta C_T$ value of the denervated muscle [$\Delta \Delta C_T = \Delta C_T (\text{denervated}) - C_T (\text{non-denervated})$]. Results are reported as fold changes using the $\Delta \Delta CT$ method, calculated as $2^{-\Delta \Delta CT}$. The primers used were: PGC-$\alpha_1$ forward (F)-AGTGGTGTAGCGACCAATCG, reverse (R)-GGGCAATCCGTCTTCATCCA; muscle RING finger 1 (MuRF1) F-CACGTGTGAGTGCCTACTTT, R- CACCAGCATGGAGATGCAGT; muscle atrophy F-box (MAFbx) F- TGAGCGACCTCAGCAGTTAC, R-ATGGCGCTCCTTCTGACTTC; PRMT1 F-CACCTTGCTAATGGGATGAG, R-GTGAAACATGGAGTTGCGGT; CARM1 F-CAACAGCGTCCTCATCCAGT, R-GTCCGCTCACTGAACACAGA; PRMT5 F-TCTCCCCACCAGCATTTTCC, R-TGGAGGGCGATTGCTTTGA; 18S F-AGTTAGCATGCGCAGTGCTC, R-TGCATGGGCCGTTCTTAGTTG.

Whole muscle protein extract preparation. Muscle samples were processed as described previously (35). Briefly, frozen TA muscle was ground to a powder using a porcelain mortar and pestle on liquid nitrogen. Muscle samples were suspended in RIPA buffer (P00178, Sigma), supplemented with eComplete Mini Protease Inhibitor Cocktail (G00065, Sigma-Roche Applied Science) and PhosSTOP Phosphatase Inhibitor Cocktail (G00064, Sigma-Roche Applied Science). All samples were homogenized in Lysing D Matrix tubes (N00420, MP Biomedicals) with the FastPrep-24 Tissue and Cell Homogenizer (MP
Biomedicals) at a speed of 4.0 m/s for 6 X 20 seconds with 5 minutes between each bout. The lysate was mixed by end-over-end inversion for 60 min at 4 °C followed by centrifugation at 14,000 x g for 10 min. The supernatant was snap-frozen in liquid nitrogen, and stored at -80 °C for further analysis. The protein concentrations of the supernates were determined using the BCA protein assay (V00072, Fisher).

**Isolation of nuclear and cytosolic compartments.** Nuclear and cytosolic fractions were isolated from denervated and control GAST muscles according to procedures described previously (40), with modifications. GAST muscles were ground to a fine powder using a Cell Crusher tissue pulveriser (CellCrusher). Next, STM buffer was added to each sample prior to being homogenized on ice using sonication (Fisher Scientific) at 100% power for 10 X 2 seconds, with 30 seconds between each bout. Additional STM buffer was added prior to spinning samples. After 3 centrifuge steps, the supernatant and pellet were separated, plus each pellet was re-suspended in STM buffer. Following the third centrifuge step, NET buffer was added, which was vortexed and then placed on ice for 30 minutes. Next, the sample was sonicated at 100% power for 10 X 2 seconds, with 30 seconds between each bout. After another series of centrifuge steps, nuclear and cytosolic fractions were identified, snap-frozen in liquid nitrogen, and stored at -80°C for further analysis. The protein concentrations of nuclear and cytosolic fractions were determined by BCA protein assay (V00072, Fisher).
Western Blot analyses. Proteins extracted from muscle samples were resolved on 10-12.5% SDS-PAGE gels and were subsequently transferred onto nitrocellulose membranes. After transfer, membranes were stained with Ponceau S solution (G00040, Sigma) in order to serve as a loading control. Membranes were washed with 1 x TBST and blocked with 5% milk-TBST or 5% BSA-TBST for one hour before being incubated in a primary antibody overnight at 4 °C with gentle rocking. Antibodies against PRMT1 (07-404, EMD Millipore), CARM1 (A300-421A, Bethyl Laboratories), PRMT5 (07-405, EMD Millipore), MMA (8015S, Cell Signaling), ADMA (13522, Cell Signaling), SDMA (13222, Cell Signaling), histone 4 arginine 3 (H4R3; 39705, Active Motif), H3R17 (ab8284, Abcam), and H3R8 (ab130740, Abcam) were employed to examine PRMT expression and function during skeletal muscle plasticity. Antibodies against ubiquitin (3933S, Cell Signaling), MuRF1 (AF5366, R&D Systems), and MAFbx (AP2041, ECM Biosciences) were used to identify important markers of skeletal muscle atrophy (1,41,42). Antibodies against PGC-1α (AB3242, EMD Millipore), AMPK (2532S, Cell Signaling), phosphorylated AMPK (2535S, Cell Signaling), p38 (9212S, Cell Signaling) and phosphorylated p38 (9215S, Cell Signaling) were used because these proteins are critical upstream signaling molecules that mediate various forms of muscle remodelling (2,3,4,5,6). Histone 2B (H2B; 8135S, Cell Signaling) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ab9483, Abcam) served as markers of purity for nuclear and cytosolic fractions, respectively. After overnight incubation in primary antibody, blots were washed
with 1 x TBST and incubated in the appropriate secondary antibody coupled to horseradish peroxidase with gentle rocking at room temperature for one hour. Blots were then washed again with 1 x TBST, followed by visualization with enhanced chemiluminescence (G00069, GE Healthcare Bio-Sciences). Blots were then developed and analyzed using ImageJ.

Statistical analyses. Differences in expression levels over time were compared by a 1-way ANOVA and Tukey’s post hoc analysis. Differences between denervated and contralateral control hind limbs at each time point were assessed with a Student’s paired t-test. Statistical differences were considered significant if $p < 0.05$. Data are expressed as mean ± SEM. All statistical measures were performed on the raw data sets prior to conversion to the –fold difference values displayed in the graphical summaries. Simple linear regression was also used to determine the relationships between denervation-induced changes in AMPK and p38 activation status with changes in inactivity-evoked alterations in nuclear PRMT1, CARM1, and PRMT5 protein expression, cytosolic PRMT1, CARM1, and PRMT5 content, as well as cellular MMA, ADMA and SDMA levels. Pearson correlation coefficient ($r$) effect sizes were classified as small ($r = ± 0.1$), medium ($r = ± 0.3$), or large ($r = ± 0.5$).
Results

The effects of short-term denervation on the muscle atrophy program. We first wished to examine the effects of the muscle disuse paradigm utilized in this study on the skeletal muscle atrophy program. A period of acute denervation-induced muscle disuse from 6-72 hours had no effect on TA, EDL or GAST muscle mass (Fig. 1A). However, when compared to the contralateral, non-denervated control (CON) limb, TA, EDL and GAST muscle mass were significantly reduced by 32%, 29% and 24%, respectively, in the contralateral denervated (DEN) limb following 168 hours of disuse. Next, we assessed ubiquitinated protein levels, as well as the expression of MuRF1 and MAFbx in order to characterize muscle atrophy signaling. Following 168 hours of denervation, ubiquitinated proteins in the DEN TA muscle were significantly elevated by 2.4-fold, relative to the CON TA muscle (Fig. 1B, C). MuRF1 and MAFbx mRNA content in the DEN EDL muscle increased 2.6-2.8-fold (p < 0.05), as compared to the CON muscle after 72 hours of denervation (Fig. 1D). MuRF1 and MAFbx protein expression increased 5.1- and 2-fold fold (p < 0.05) in the DEN TA muscle compared to the CON TA muscle after 72 hours of denervation, respectively, and 4.4- and 1.9-fold (p < 0.05) after 168 hours of denervation, respectively (Fig. 1B, E). These denervation-induced increases were greater (p < 0.05) for MuRF1 compared to MAFbx.

Inactivity-induced changes in the expression and activation states of phenotype modifying signaling proteins. We were particularly interested in
investigating the impact of an acute denervation timecourse on the expression and activation of signaling molecules known to be involved in the regulation of skeletal muscle plasticity. Thus, we examined the levels of PGC-1α, as well as the expression and activation of AMPK and p38, proteins that are critically important for muscle remodelling (43). PGC-1α mRNA content significantly decreased by 77% after 72 and 168 hours of denervation, while PGC-1α protein levels were reduced by 46% and 52% at 6 and 168 hours, respectively, in the DEN EDL muscle relative to the CON muscle (Fig. 2A, B). AMPK phosphorylation levels were decreased by 45% and 32% (p < 0.05) after 6 and 12 hours, respectively, and elevated by 1.9-fold at 168 hours in the DEN compared to the CON muscle (Fig. 2A, C). In contrast, p38 phosphorylation levels were significantly increased early in the disuse timecourse at 6 and 12 hours by ~2-fold. Total AMPK and p38 protein expression did not change during the experimental time course (Fig. 2A, D). As such, the activation status of these enzymes (i.e., the phosphorylated form of the protein relative to the total amount of the enzyme) were similar to the levels of protein phosphorylation. AMPK activation status was significantly decreased by approximately 40-54% throughout the first 12 hours and was elevated by 1.7-fold (p < 0.05) after 168 hours of denervation in the DEN compared to the CON muscle (Fig. 2E). Following 6 and 12 hours of denervation, the activation status of p38 increased by 2.0- and 1.9-fold (p < 0.05), respectively, in DEN versus CON muscle.
Impact of denervation-induced muscle disuse on PRMT gene expression.

For our initial analysis of PRMT biology in muscle, we investigated the mRNA and protein expression of PRMTs across the acute timecourse of neurogenic muscle disuse. PRMT1, CARM1, and PRMT5 mRNA expression were significantly elevated by 4.2-, 1.9-, and 2.6-fold in the DEN EDL versus the contralateral CON muscle after 72, 6, and 72 hours, respectively (Fig. 3A). Notably, PRMT1 (p = 0.07) and CARM1 (p = 0.09) mRNA levels approached statistical significance with denervation-evoked increases of ~2-fold after 6 and 72 hours, respectively. Following 72 and 168 hours of denervation, PRMT1 protein content significantly increased 3.6-5-fold, CARM1 protein levels were augmented by 1.6-2.1-fold (p < 0.05), and PRMT5 protein expression was significantly elevated by 1.8-2.9-fold in the DEN TA muscle, as compared to the CON TA muscle (Fig. 3B, C). These denervation-induced increases were greater (p < 0.05) for PRMT1, as compared to CARM1 and PRMT5.

Cellular localization of PGC-1α and PRMTs during muscle disuse.

Nuclear and cytosolic levels of PGC-1α and PRMTs were measured in order to further characterize their expression during acute skeletal muscle disuse. Here, the GAST muscle was utilized to fractionate myocytes into highly purified nuclear and cytosolic compartments, as demonstrated in Figure 4A. Generally, in both the CON and DEN GAST muscles, we found that nuclear PRMT protein expression was ~85% lower (p < 0.05) compared to the cytosolic PRMT content (data not shown). Nuclear PGC-1α protein content decreased 33% and 46% (p < 0.05)
following 6 and 168 hours of denervation, respectively, but were significantly elevated by 1.6-1.9-fold following 12 and 24 hours of disuse in the DEN versus CON GAST muscle (Fig. 4B, C). Nuclear PRMT1 expression significantly decreased by 40% at 6 hours prior to increasing by ~2.8-fold \( (p < 0.05) \) after 72 and 168 hours of denervation. This disuse-induced augmentation in PRMT1 was greater \( (p < 0.05) \) versus nuclear PGC-1\( \alpha \) and PRMT5 at 72 hours, as well as compared to PGC-1\( \alpha \), CARM1 and PRMT5 following 168 hours. CARM1 levels in myonuclei were significantly augmented by 92% at 72 hours, before decreasing by 32% \( (p < 0.05) \) following 168 hours in the DEN relative to the CON GAST muscle. After 6 and 12 hours, nuclear PRMT5 protein content increased 2.5-fold \( (p = 0.12) \) and 2.8-fold \( (p < 0.05) \), respectively, in the DEN relative to the CON GAST muscle, but was significantly reduced by 43% and 63% following 72 and 168 hours of denervation, respectively. After 6 hours, the denervation-induced elevation was greater \( (p < 0.05) \) for nuclear PRMT5, as compared to nuclear PGC-1\( \alpha \), PRMT1, and CARM1. Furthermore, the elevation in nuclear PRMT5 was significantly greater versus the other PRMTs at 12 hours.

Cytosolic PGC-1\( \alpha \) protein content significantly increased 55-60% after 6, 72, and 168 hours in the DEN versus CON GAST muscle (Fig. 4B, D). Cytosolic PRMT1 protein expression increased by 2.4- and 4.1-fold \( (p < 0.05) \) following 72 and 168 hours of denervation, respectively. These denervation-induced increases were greater \( (p < 0.05) \) for cytosolic PRMT1 versus the levels of other PRMTs at 72 hours, as well as compared to cytosolic PGC-1\( \alpha \), CARM1, and PRMT5.
content after 168 hours. CARM1 and PRMT5 protein expression in the cytosol significantly increased by ~80% after 168 hours in the DEN muscle versus CON muscle. Summaries of the relative denervation-evoked changes in PGC-1α, PRMT1, CARM1, and PRMT5 at the whole muscle level (from data in Figures 2A, B and 3B, C), as well as in the nuclear an cytosolic compartments (from Figure 4B-D) are displayed in Figure 4E-H.

Effects of neurogenic muscle disuse on skeletal muscle methylarginine levels. We next examined the contents of MMA, ADMA, and SDMA in skeletal muscle. These methylarginine marks were utilized as global myocellular indicators of PRMT, Type I PRMT, and Type II PRMT activities, respectively. MMA and ADMA content in the DEN TA muscle significantly increased 1.8-2.2-fold following 72 and 168 hours of denervation relative to the CON muscle (Fig. 5A, B). SDMA content was augmented by 1.3-1.9-fold (p < 0.05) following 24-168 hours of denervation in the DEN TA versus CON TA muscle.

Relationship between upstream intracellular signalling and PRMT localization and function. We performed simple linear regression analyses to determine if disuse-induced alterations in AMPK and p38 activation predicted changes in metrics of PRMT expression and function. While AMPK activation status did not predict nuclear CARM1 content (Fig. 6B), AMPK activation levels accounted for 20% and 40% of the variance (p < 0.05) in nuclear PRMT5 and PRMT1 content, respectively, indicating medium (r = ± 0.3) to large (r = ± 0.5) effect sizes (Fig. 6A, C). AMPK activation status predicted cytosolic PRMT
content with large effect, accounting for 42-63% of the variance. Furthermore, AMPK activation explained 32-39% of the variance ($p < 0.05$) in cellular methylarginine levels, revealing a large effect size (Fig. 6G-I).

Denervation-evoked changes in p38 activation status did not predict alterations in nuclear or cytosolic PRMT levels (Fig. 7A-F), however a medium to large effect size was demonstrated between p38 activation and myocellular methylarginine content, which accounted for 21-29% of the variance ($p < 0.05$; Fig. 7G-I).

**Discussion**

We investigated PRMT biology within the context of early upstream signalling pathways that mediate disuse-induced muscle remodelling. The data revealed that denervation-induced muscle disuse evoked rapid changes in AMPK and p38 activation status, as well as PGC-1α expression, and suggest that alterations in the activity, content and/or localization of these molecules may be among the earliest signals that mediate adaptations to inactivity. Indeed, changes in AMPK activation status predicted alterations in numerous metrics of PRMT expression and function. We found that PRMT1, CARM1 and PRMT5 content and activity exhibited differential responses to denervation, suggesting unique sensitivity to, or regulation by, upstream signalling and transcriptional pathways. This study supports the notion that alterations in PRMT biology help characterize an initial period of atrophy anticipation, which includes cellular events that
precede the muscle atrophy indicative of disuse-induced phenotypic remodelling (Fig. 8).

*Atrogene induction during the period of atrophy anticipation.* We first sought to further characterize the temporal profile of the muscle atrophy program elicited by muscle denervation. In agreement with other studies, we reported a ~30% decline in muscle mass following 1 week of denervation (44,45,46). The muscle atrophy program, comprised primarily of the ubiquitin-proteasome system (47), is largely driven by the E3 ubiquitin ligase atrogenes MuRF1 and MAFbx (1). We found increased MuRF1 and MAFbx levels at 72 hours post-denervation, which was followed by downstream elevations in ubiquitinated protein content. Previous studies have also shown that MuRF1 and MAFbx mRNA induction precedes muscle remodelling (37,45,48,49). Utilizing an alternative model of acquired muscle atrophy, Gomes et al. observed increased MAFbx transcripts as early as 16 hours after food removal (50). Therefore, augmented expression and function of atrogenes precede, and may be required for, the initiation of skeletal muscle wasting. Along these lines, inhibiting MuRF1 and MAFbx expression and activity in fasting and disuse models significantly attenuates muscle loss (51,52,53,54,55), while MuRF1 and MAFbx muscle-specific knockout mice are resistant to denervation-induced atrophy (56). Collectively, the data therefore suggest that elevations in MuRF1 and MAFbx during atrophy anticipation are required for the initiation of muscle plasticity.
PGC-1α, AMPK, and p38 are rapidly altered with muscle disuse. PGC-1α, AMPK, and p38 expression and/or activity are affected during skeletal muscle atrophy (37,57,58,59,60). Moreover, these powerful phenotype-bending molecules also participate in mediating disuse-induced remodelling (61,62,63,64,65). We examined these proteins in an effort to enhance their temporal resolution during the early stages of muscle disuse. Similar to Sacheck et al. (37), we found that reduced PGC-1α mRNA expression preceded the appearance of muscle atrophy. Our data showed that PGC-1α protein content was significantly decreased after only 6 hours of neurogenic muscle disuse, independent of changes in PGC-1α mRNA. This suggests that the expression of the coactivator early in the disuse timecourse is governed, in part, at the post-transcriptional level. This hypothesis is supported by recent studies demonstrating that PGC-1α mRNA stability is affected by both muscle phenotype and activity levels (66,67). Furthermore, rapid changes in PGC-1α protein content within the context of acute skeletal muscle remodelling have been observed. For example, whole muscle PGC-1α protein expression was induced ~2.5-fold 3 hours after a single bout of exercise (68). These data suggest that rapid alterations in PGC-1α levels are among some of the earliest signals associated with various modes of skeletal muscle plasticity.

The rapid changes in PGC-1α expression were accompanied by alterations in the activation status of upstream signaling molecules AMPK and p38. The initial reduction in AMPK activation during the onset of the atrophy program is in
agreement with previous studies since inhibition of AMPK has been reported to prevent increased atro gene expression (57,69). Indeed, MuRF1 and MAFbx expression levels were not elevated until 72 hours after denervation, which occurred coincident with a return of AMPK activation status to basal levels. It is reasonable to speculate that the steady rise in AMPK activation from immediately following inactivity up to 168 hours post-denervation was due, in part, to altered levels of AMPK-regulating molecules, such as AMP and ADP, as well as the activity of upstream kinases and phosphatases (70). In contrast, p38 activation status was initially elevated at 6 and 12 hours of denervation, and was subsequently reduced. This behaviour of the kinase was likely mediated by the progressive change in the cellular milieu, including alterations in mechanical, metabolic, and oxidative stress (2). Previous studies have also reported increased p38 activation 1-10 days after disuse in immobilization and hindlimb suspension models before declining thereafter (58,60,71,72). Since both AMPK and p38 regulate PGC-1α via direct phosphorylation (2), additional work is required to more clearly elucidate the mechanisms that drive the rapid changes in expression and function of these proteins during conditions of muscle remodelling.

Muscle disuse elicits alterations in PRMT content, localization and function. PRMT1, CARM1, and PRMT5 are emerging players in skeletal muscle biology (20,23,24,25,31). We found that PRMT gene expression at the mRNA and protein levels was increased before and during skeletal muscle atrophy. The magnitude and timing of PRMT induction exhibited a measure of PRMT-
specificity, which suggests a differential sensitivity to regulatory mechanisms. This was not surprising since PRMTs possess muscle-specific mRNA levels (28), and that PRMT1 and CARM1 respond differently to alternative cues for skeletal muscle remodelling (35). It is interesting to note that the temporal signature of PRMT expression during denervation was very similar to that of the muscle atrophy program (i.e., MuRF1, MAFbx, and protein ubiquitination). This underscores the possibility that a common pathway controls PRMT expression and the cellular apparatus required for muscle atrophy. In support of this, PRMT1 has been shown to stimulate FOXO1, which upregulates MuRF1 and MAFbx transcription during atrophy-inducing conditions (73). Furthermore, protein degradation is impaired when PRMT1 activity is reduced (22), while CARM1 expression is increased in hepatic cells undergoing atrophy (74). Collectively, the evidence suggests that PRMTs participate in the advancement from the atrophy anticipation phase through to atrophy induction. Further studies are necessary to identify the upstream mechanisms that govern PRMT expression during atrophy anticipation and initiation.

PRMT1, CARM1, PRMT5 and PGC-1α intracellular localization is dynamic during conditions of cellular remodelling (20,24,75,76). We observed that cytosolic PRMT content generally recapitulated PRMT expression at the whole muscle level, which reflects the fact that the majority (~85%) of PRMT protein was found within the cytosolic compartment. In contrast, the timing and magnitude of nuclear PRMT accumulation was unique between PRMT1, CARM1,
and PRMT5. Nuclear PRMT1 followed its cytosolic and whole muscle pattern throughout the experimental timecourse, whereas nuclear CARM1 rose at 72 hours during atrophy anticipation and fell to below control levels during atrophy. PRMT5 accumulated in the myonuclei very early in the atrophy anticipation phase, then was significantly reduced coincident with muscle atrophy. This differential localization profile is likely related to the disparate functions of each PRMT. For example, while all PRMTs display epigenetic targets in the nucleus, primarily histones (77), PRMT1 has been shown to mark sarcolemmal proteins (20), while CARM1 targets p53 (16), a protein found in the nuclear, cytosolic, and mitochondrial compartments in skeletal muscle (78). Moreover, Zhang et al. (33) recently demonstrated that PRMT5 controls proliferation of muscle stem cells by direct transcriptional repression of the cell cycle inhibitor p21. It is logical to speculate then that the early nuclear retention of PRMT5 played a role in atrophy anticipation, as satellite cell proliferation occurs rapidly in response to muscle disuse (79).

Our data show that nuclear and cytosolic PGC-1α localization diverged throughout the denervation timecourse. Similarly, the activity of AMPK and p38, which stimulate PGC-1α via phosphorylation (80,81), also displayed inverse localization patterns. Notably, both AMPK activation status and nuclear PGC-1α content were reduced at 6 hours post-denervation, which is in line with the idea that AMPK-mediated phosphorylation of PGC-1α is required for enhanced transcriptional coactivator activity of the enzyme (80). Our data suggest that the
net sum of PGC-1α post-translational modifications by AMPK, p38, and potentially SIRT1 (82) and others (6,43), combine to determine PGC-1α localization and activity during denervation-induced muscle remodelling. Although PGC-1α was shown to be a target for arginine methylation by PRMT1 in other cell types (83,84,85), there was little enzyme colocalization in either myonuclear or cytosolic fractions during the disuse timecourse. More refined protein-protein interaction analyses are necessary to shed light on the potential physical connection between PGC-1α and PRMT1 during conditions of muscle plasticity.

We observed increased MMA, ADMA, and SDMA content after denervation, collectively indicating an upregulation of global PRMT activity. All PRMTs catalyze the formation of MMA, while type I PRMTs add an additional methyl group to produce ADMA, and type II PRMTs synthesize the SDMA mark. As such, we employed MMA content as a global marker of all PRMT activity, ADMA levels as an indicator of PRMT1 and CARM1 activities, and SDMA content as a marker of PRMT5 activity. PRMT1 is the primary methyltransferase that produces ADMA and is responsible for approximately 85% of total cellular arginine methylation activity (10), whereas PRMT5 is the major type II PRMT (9). The utilization of these methylarginine marks as metrics for PRMT activity should also be considered with the particular tissue context. Specifically, in skeletal muscle, PRMT1, CARM1, and PRMT5 are the most abundantly expressed PRMTs (28). We report significant increases in MMA, ADMA, and
SDMA content after 72 and 168 hours of denervation, indicating that PRMT activity, along with PRMT expression, was augmented at the later stages of the experimental time course. SDMA content increased earlier, after 24 hours of denervation, suggesting that PRMT5 activity is more sensitive to the signals associated with muscle disuse. Considering that the occurrence of arginine methylation is comparable to other widespread modifications, such as phosphorylation (86), it is essential to continue investigating the impact that this mark has on skeletal muscle phenotypic maintenance and remodelling.

*PRMT biology is related to AMPK and p38 activation status during neurogenic muscle disuse.* AMPK and p38 are upstream signalling molecules that are critically involved in driving skeletal muscle remodelling (2,6). These kinases exhibited significant, early differences in their activation patterns in response to muscle disuse, as well as subsequent, steady inverse trends throughout atrophy anticipation and during the onset of muscle wasting. These findings compelled us to investigate the potential relationship between AMPK and p38 activation with PRMT expression and function to ascertain if denervation-induced alterations in these phenotype-modifying enzymes could predict changes in PRMT biology. With the exception of nuclear CARM1 content, the level of AMPK activation accounted for ~20-65% of the variance in PRMT localization and activity, with medium to large effect sizes. Interestingly, AMPK had a particularly strong influence on PRMT1 expression and activity. Although tyrosine phosphorylation of PRMT1 reduces its activity (87), our data in skeletal muscle introduce the
possibility that AMPK-mediated phosphorylation of the enzyme may enhance its methylating activity. Unlike AMPK, p38 activation status demonstrated limited predictive power, with only PRMT activity showing a significant negative correlation. It will be important for future studies to examine the potential interactions between PRMTs and AMPK and p38, as well as whether PRMTs are in fact phosphorylation targets of these enzymes.

In summary, our data reveal that alterations in PGC-1α, AMPK, and p38 expression and activation are among the earliest signals associated with skeletal muscle plasticity following acquired neurogenic muscle disuse. Furthermore, PRMT1, CARM1, and PRMT5 expression, localization, and activity were altered following denervation-induced inactivity. We also found that AMPK activation status, and to a lesser extent p38 activation, predict PRMT expression and function in skeletal muscle. This study provides evidence that alterations in PRMT biology are important for muscle remodelling throughout the atrophy anticipation phase, as well as during muscle atrophy elicited by denervation. Continued investigation of PRMTs in the regulation of skeletal muscle plasticity is warranted.
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Author contributions

DWS and VL conceived and designed the study. DWS performed all experiments and analyses. AM performed cell fractionation procedures and some Western blotting. DWS and VL wrote the manuscript.
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References


52. Lee, D. & Goldberg, A.L. (2015). Muscle Wasting in Fasting Requires Activation of NF-κB and Inhibition of AKT/Mechanistic Target of
Rapamycin (mTOR) by the Protein Acetylase, GCN5. *J Biol Chem.*, 290(51), 30269-79.


Activation of nuclear receptor coactivator PGC-1alpha by arginine

Jeannesson, E., Maskali, F., Gueguen, N., Marie, P.Y., Lacolley, P.,
Herrmann, M., Juillière, Y., Malthiery, Y. & Guéant, J.L. (2011). Methyl
donor deficiency induces cardiomyopathy through altered
methylation/acetylation of PGC-1α by PRMT1 and SIRT1. *J Pathol.*, 225(3),
324-35.

interacting protein mediates hepatic lipogenesis and inflammation via
PRMT1 and PGC-1α regulation in vitro and in vivo. *J Hepatol.*, 61(5),
1151-7.

86. Larsen, S.C., Sylvestersen, K.B., Mund, A., Lyon, D., Mullari, M., Madsen,
analysis of arginine monomethylation reveals widespread occurrence in

87. Rust, H.L., Subramanian, V., West, G.M., Young, D.D., Schultz, P.G. &
Thompson, P.R. (2014). Using unnatural amino acid mutagenesis to probe the
Figure Legends

Figure 1. Denervation induces muscle atrophy after 168 hours. A. Tibialis anterior (TA), extensor digitorum longus (EDL), and gastrocnemius (GAST) muscle mass from denervated (D) hindlimb compared to the TA, EDL, and GAST muscle from the contralateral, non-denervated control (C) hindlimb after 6, 12, 24, 72, and 168 hours of unilateral denervation (n = 6-8). * p < 0.05 vs. contralateral, non-denervated limb. B. Representative blots of ubiquitinated protein content, along with muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx) expression in denervated (D) and contralateral, non-denervated control (C) TA muscles 6, 12, 24, 72, and 168 hours after unilateral denervation with typical Ponceau S stain. C. Graphical summary of ubiquitinated proteins. D. MuRF1 and MAFbx mRNA content in the D EDL muscle compared to the C muscle after denervation across the experimental time course. E. MuRF1 and MAFbx protein expression. n= 6-8; * p < 0.05 D vs. C; # p < 0.05 vs. MAFbx at same timepoint.

Figure 2. Phenotype modifying protein expression and activation states are altered with muscle disuse. A. Representative Western blots of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), phosphorylated AMP-activated protein kinase (AMPK), total AMPK, phosphorylated p38, and total p38 protein content, plus representative Ponceau S, for C and D TA muscles across the experimental time course. B. PGC-1α mRNA and protein content in EDL and TA muscles, respectively. C. Phosphorylated AMPK and phosphorylated p38 content.
D. Total AMPK and p38 content. E. AMPK and p38 activation status (phosphorylated form of the enzyme versus the total amount of the protein) in the D TA muscle compared to the C muscle after denervation. n= 5-8; * p < 0.05 D vs. C.

Figure 3. Protein arginine methyltransferase 1 (PRMT1), coactivator-associated methyltransferase 1 (CARM1), and PRMT5 expression during denervation. A. PRMT1, CARM1, and PRMT5 mRNA content in the D EDL muscle compared to the C muscle after 6, 12, 24, 72, and 168 hours of unilateral denervation. B. Representative Western blots of PRMT1, CARM1, and PRMT5 protein expression with a typical Ponceau S, for C and D TA muscles after 6, 12, 24, 72, and 168 hours of unilateral denervation. C. Graphical summary of PRMT1, CARM1, and PRMT5 protein content. n= 6-8; * p < 0.05 D vs. C; # p < 0.05 vs. other proteins at same timepoint.

Figure 4. PGC-1α, PRMT1, CARM1, and PRMT5 cellular localization varies with disuse. A. Representative Western blots of histone 2B (H2B) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) demonstrating purity of nuclear and cytosolic fractions for 6, 12, 24, 72, and 168 hours of unilateral denervation. B. Representative Western blots for nuclear and cytosolic PGC-1α, PRMT1, CARM1, PRMT5 protein content from C and D GAST muscles across the experimental time course. Ponceau S stains for nuclear and cytosolic fractions
are also included. C. Graphical summary of nuclear PGC-1α, PRMT1, CARM1, and PRMT5 protein expression in the D GAST muscle versus the C muscle after 6, 12, 24, 72, and 168 hours of unilateral denervation. D. Graphical summary of cytosolic PGC-1α, PRMT1, CARM1, and PRMT5 protein expression. Summaries of nuclear, cytosolic, and whole muscle PGC-1α (E), PRMT1 (F), CARM1 (G), and PRMT5 (H) protein levels across the denervation timecourse. Whole muscle data is redrawn from Figures 2 and 3. n= 5-8; * p < 0.05 D vs. C, # p < 0.05 vs. other proteins at same timepoint, ¶ p = 0.05 vs. other PRMTs at same timepoint, § = p < 0.05 vs. PGC-1α and PRMT5 at same timepoint.

Figure 5. Disuse-evoked monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) content in skeletal muscle. A. Representative Western blots of MMA, ADMA, and SDMA content, plus a representative Ponceau S, for C and D TA muscles across the experimental time course. B. Graphical summary of MMA, ADMA, and SDMA content after 6, 12, 24, 72, and 168 hours of unilateral denervation. n= 5-8; * p < 0.05 D vs. C.

Figure 6. Relationship between AMPK activation status and PRMT expression and function in response to neurogenic skeletal muscle disuse. Linear regression plots of (A) nuclear PRMT1 protein content, (B) nuclear CARM1 protein levels, (C) nuclear PRMT5 protein expression, (D) cytosolic
PRMT1 protein, (E), cytosolic CARM1 levels, (F) cytosolic PRMT5 protein content, (G) MMA content, (H) ADMA levels, and (I) SDMA content. n= 23-29.

**Figure 7. Relationship between p38 activation status and PRMT localization and activity during denervation-induced muscle remodelling.** Linear regression plots of (A) nuclear PRMT1 protein content, (B) nuclear CARM1 protein levels, (C) nuclear PRMT5 protein expression, (D) cytosolic PRMT1 protein, (E), cytosolic CARM1 levels, (F) cytosolic PRMT5 protein content, (G) MMA content, (H) ADMA levels, and (I) SDMA content. n= 23-29.

**Figure 8. Summary of cellular events associated with disuse-evoked skeletal muscle remodelling.** Denervation-induced alterations in skeletal muscle are shown along the disuse timecourse with each timepoint marked to scale. Muscle mass (light grey solid line) denotes the average mass of the TA, EDL, and GAST muscles. The atrophy program (black solid line) is comprised of the combination of ubiquitinated protein levels, as well as MuRF1 and MAFbx protein content at each experimental timepoint. The dark grey solid and dashed lines indicate AMPK and p38 activation status, respectively. PRMT expression and activity (black dashed line) includes the average values for all whole muscle PRMT mRNA and protein content (including PRMT1, CARM1, and PRMT5), as well as cellular arginine methylation status (including MMA, ADMA, and SDMA). The events occurring prior to 72 hours of neurogenic muscle disuse precede muscle...
atrophy, and therefore anticipate the subsequent remodelling. Significant skeletal muscle atrophy takes place sometime between 72-168 hours of denervation-evoked disuse (shaded area).
Figure 1

A

Muscle mass (% D / C)

Denervation (hours)

Tibialis anterior
Extensor digitorum longus
Gastrocnemius

B

Ubiquitinated Protein

MuRF1
MAFbx
Ponceau S

C

Ubiquitinated protein content (D / C)

Denervation (hours)

D

mRNA content (D / C)

Denervation (hours)

E

Protein content (D / C)

Denervation (hours)
Figure 2

A

![Western Blot Images]

B

![Bar Chart]

C

![Bar Chart]

D

![Bar Chart]

E

![Bar Chart]
Figure 3

A

mRNA expression (D / C)

Denervation (hours)

PRMT1  
CARM1  
PRMT5

B

PRMT1  
CARM1  
PRMT5  
Ponceau S

C

Protein content (D / C)

Denervation (hours)
Figure 4

A  

B  

C  

D  

E  

F  

G  

H  

PRMT1 protein content

Nuclear  
Cytosolic

PGC-1α protein content

Denervation (hours)

PRMT5 protein content

Denervation (hours)

PGC-1α  
PRMT1  
CARM1  
PRMT5

Denervation (hours)

PRMT5 protein content

Denervation (hours)

PGC-1α  
PRMT1  
CARM1  
PRMT5

Denervation (hours)

PGC-1α  
PRMT1  
CARM1  
PRMT5

Denervation (hours)

PGC-1α  
PRMT1  
CARM1  
PRMT5

Denervation (hours)

PGC-1α  
PRMT1  
CARM1  
PRMT5

Denervation (hours)
Figure 5

A

MMA

ADMA

SDMA

Ponceau S

B

Methylarginine content (D / C)

0 1 2 3

MMA  ADMA  SDMA

Denervation (hours)

6 12 24 72 168
Figure 6

A. \( r = 0.64, R^2 = 0.40 \)
\[ p = 0.0002 \]

B. \( r = 0.15, R^2 = 0.02 \)
\[ p = 0.476 \]

C. \( r = -0.45, R^2 = 0.20 \)
\[ p = 0.031 \]

D. \( r = 0.80, R^2 = 0.63 \)
\[ p < 0.0001 \]

E. \( r = 0.65, R^2 = 0.42 \)
\[ p = 0.0003 \]

F. \( r = 0.78, R^2 = 0.61 \)
\[ p < 0.0001 \]

G. \( r = 0.62, R^2 = 0.39 \)
\[ p = 0.0003 \]

H. \( r = 0.57, R^2 = 0.32 \)
\[ p = 0.002 \]

I. \( r = 0.56, R^2 = 0.32 \)
\[ p = 0.002 \]
Figure 7

A \[ r = -0.23, R^2 = 0.05 \]
\[ p = 0.277 \]

B \[ r = -0.14, R^2 = 0.02 \]
\[ p = 0.532 \]

C \[ r = 0.28, R^2 = 0.08 \]
\[ p = 0.192 \]

D \[ r = -0.25, R^2 = 0.06 \]
\[ p = 0.232 \]

E \[ r = -0.13, R^2 = 0.02 \]
\[ p = 0.547 \]

F \[ r = 0.13, R^2 = 0.02 \]
\[ p = 0.525 \]

G \[ r = -0.54, R^2 = 0.29 \]
\[ p = 0.005 \]

H \[ r = -0.46, R^2 = 0.21 \]
\[ p = 0.014 \]

I \[ r = -0.48, R^2 = 0.23 \]
\[ p = 0.008 \]
Figure 8

The figure illustrates the effect of neurogenic muscle disuse (D/C) on various factors such as muscle mass, Atrophy program, PRMT expression & activity, AMPK activation, and p38 activation. The graph shows changes over time (denervation hours), with specific markers indicating Atrophy Anticipation and Muscle Atrophy.