Stimuli and sensors that initiate skeletal muscle hypertrophy following resistance exercise

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Abstract

One of the most striking adaptations to exercise is the skeletal muscle hypertrophy that occurs in response to resistance exercise. A large body of work shows that a mTORC1-mediated increase of muscle protein synthesis is the key, but not sole, mechanism by which resistance exercise causes muscle hypertrophy. Whilst much of the hypertrophy signaling cascade has been identified, the initiating, resistance exercise-induced and hypertrophy-stimulating stimuli have remained elusive. For the purpose of this review, we define an initiating, resistance exercise-induced and hypertrophy-stimulating signal as "**hypertrophy stimulus**", and the sensor of such a signal as "**hypertrophy sensor**". In this review we discuss our current knowledge of specific mechanical stimuli, damage/injury-associated and metabolic stress-associated triggers as potential hypertrophy stimuli. Mechanical signals are the prime hypertrophy stimuli candidates and a Filamin-C-BAG3-dependent regulation of mTORC1, Hippo and autophagy signalling is a plausible albeit still incompletely characterised hypertrophy sensor. Other candidate mechanosensing mechanisms are nuclear deformation-initiated signalling or several mechanisms related to costameres, which are the functional equivalents of focal adhesions in other cells. Whilst exercise-induced muscle damage is probably not essential for hypertrophy, it is still unclear whether and how such muscle damage could augment a hypertrophic response. Interventions that combine blood flow restriction and especially low load resistance exercise suggest that resistance exercise-regulated metabolites could be hypertrophy stimuli but this is based on indirect evidence and metabolite candidates are poorly characterised.

Introduction

Adequate muscle mass and strength are not only important for sporting performance but these attributes also are associated with good health and longevity (25, 162). For example, a recent analysis of the data of half a million people demonstrated that low grip strength is associated with a higher all-cause and disease-specific mortality as well as disease incidence for several major diseases (20). The key intervention to induce muscular hypertrophy and to make us stronger is resistance exercise in combination with nutrition. The current recommendation is for individuals to train with ≈40-80% of their 1 repetition maximum (1RM, i.e. the maximal weight that we can lift once) for hypertrophy, with loads >60% to increase maximal strength (135). Additionally, exercisers should perform multiple sets, rest for >2 min in-between sets and consume a diet that contains at least 1.6 g of protein per kg body weight per day (101).

With respect to the muscle protein synthesis and the hypertrophic response to resistance exercise, the mechanistic target of rapamycin (the key mTOR containing complex is abbreviated as mTORC1) is a downstream hypertrophy signaling "hub" that controls protein synthesis (15, 94, 117). This is supported by extensive experimental evidence including research showing that mTORC1 blockade with rapamycin prevents or reduces the increase of muscle protein synthesis and/or muscle size after resistance exercise in humans (33) and in rodents (83) or when muscle is overloaded through synergist ablation (15, 53). Other signaling pathways and genes (94, 155) also regulate muscle size but their specific contribution to resistance exercise-induced muscle hypertrophy is incompletely understood.

Whilst many studies have identified molecules and molecular mechanisms that regulate muscle mass, one key question has remained largely unanswered. It is: "What are the initiating **hypertrophy stimuli** that trigger hypertrophic signal transduction and skeletal muscle fiber hypertrophy in response to resistance exercise and what are their **sensors**?" Here, we define "**hypertrophy stimulus**" as a "first-in-line", initiating stimulus that is of a sufficient magnitude and duration to trigger a skeletal muscle hypertrophic response to resistance exercise. Additionally, we define "**hypertrophy sensor**" as a sensor that senses hypertrophy stimuli. This definition means that hypertrophy regulators such as IGF-1 or its MGF splice variant are not hypertrophy stimuli because their expression change after resistance exercise (56) must be preceded by signaling events that alter their expression. Therefore, hypertrophy regulators such as IGF-1 are not "first-in-line", initiating hypertrophy stimuli. So why are hypertrophy stimuli important? No matter how we vary resistance exercise variables such as load, repetitions or sets, it is the hypertrophy stimuli that will induce hypertrophic signal transduction and the resultant hypertrophy. Thus if we would

know the actual hypertrophy stimuli then we could measure them with the goal of identifying interventions that maximally induce these signals.

The aim of this review is to summarize our current understanding of candidate hypertrophy stimuli and sensors in three sections. First, we will discuss evidence that mechanical signals can act as hypertrophy stimuli after resistance exercise. In the second and third sections we will review evidence that exercise-induced muscle damage and metabolic signals, respectively, can trigger or augment a muscle hypertrophic response to resistance exercise. We aim to reconcile differences wherever possible and we will end with a statement of research directions.

Is mechanical load a hypertrophy stimulus?

Several reviews already discuss how mechanical stimuli could trigger a skeletal muscle hypertrophic response (18, 67, 127). Here we provide an update with a focus on mechanical stimuli of muscle hypertrophy and their sensors. Mechanical signals are arguably the most intuitive hypertrophy stimuli. This is based on three lines of indirect evidence. First, muscles atrophy when mechanical load is reduced through limb immobilization (e.g. (122), reviewed by (6)]. This suggests that a “normal” mechanical loading pattern is essential for baseline muscle mass. Second, Alfred Goldberg (51) and others have mechanically overloaded muscles such as the plantaris in rodents through the ablation of plantar flexor synergists, or cast-induced stretch. Because the overloaded muscles hypertrophied in a range of experimental conditions, the researchers concluded that mechanical overload is sufficient for skeletal muscle hypertrophy (reviewed in (57)). The issue with these studies is that the models used do not only alter mechanical load but additionally a host of other, potentially confounding variables such as metabolism, or cause damage. Third, mechanical load is also the key candidate hypertrophy stimulus that links human resistance exercise to skeletal muscle hypertrophy. This is because high forces distinguish hypertrophy-inducing resistance exercise from low load endurance exercise that triggers little or no hypertrophy. However, as we will address later, mechanical loading does not need to be excessive for muscle hypertrophy stimulation. Loads as low as ≈30% of the 1RM seem sufficient to trigger a near maximal hypertrophic response (2).

The importance of mechanical load for muscle growth was demonstrated in a study where either young (24 ± 6 years) or older (70 ± 5 years) males completed similar work (i.e. the force x time-under-tension product) of leg extensor exercise at 20-90% of the 1RM. This study showed greater muscle protein synthesis (labelled the fractional synthetic rate, FSR) at higher loads peaking between 60 and 90% of the 1RM (84). A caveat to these findings is that,

in an effort to equate workload, participants did not exercise to failure, especially when using lighter loads. To study the effect of different loads on muscle hypertrophy whilst training to failure, Lasevicius et al exercised subjects for 12 weeks using leg extension and elbow extension with one leg or arm at 20% 1RM and then either 40%, 60% or 80% with the opposite leg or arm (86). This study showed that resistance training of at least 40% of the 1RM to failure caused a similar amount of hypertrophy as the higher load conditions. This finding is in line with a meta-analysis that concluded that lower load (≤60% 1RM) resistance training causes a similar degree of hypertrophy as higher load (>60%) resistance training (135). In untrained individuals even submaximal aerobic training (i.e. low mechanical load exercise) (77), or very low loads (16% of the 1RM) can increase muscle protein synthesis somewhat (4). In summary, a large amount of mainly indirect evidence suggests that mechanical load is a key hypertrophy stimulus associated with resistance exercise. However, the actual loads do not need to be excessive as loads of ≈30% of 1RM seem sufficient to trigger near maximal hypertrophic gains.

Candidate molecular sensors that are capable of sensing mechanical load in skeletal muscle. Life on Earth evolved in an environment where a gravity of 9.8 $m \cdot s^2$ mechanically loaded organisms. It is therefore no wonder that living beings and their cells have not only evolved mechanical structures such as the muscles, the skeleton and cytoskeleton to withstand or overcome the pull of gravity, but also a plethora of sensors that detect mechanical stimuli. Such mechanosensors not only help cells to adapt to the direct force of a muscle fiber contraction, but also to adapt to more indirect mechanical signals such as shear stress, deformation, compression, and the stiffness of the extracellular matrix that surrounds each cell (18, 49, 145). In this section we discuss several types of candidate mechanosensors that allow muscle fibers to sense mechanical signals during and after resistance exercise, and trigger hypertrophic signaling and skeletal muscle hypertrophy.

Mechanosensors within the skeletal muscle force transduction system. Skeletal muscle fibers are unique because they generate much higher forces than non-muscle cells. Single, skinned human type I and IIa muscle fibers have been reported to generate forces of 532±208 and 549±262 μN, respectively (81), with each myosin contributing ≈6 pN (120). Non-muscle cells can also produce force through their actin-cytoskeleton but the forces are lower. For example, fibroblasts have been reported to produce forces of 16±7 μN per cell (79). Whilst these force values are just examples, they demonstrate that striated muscle fibers are unique in their high force-generating ability.

The forces generated by the sarcomeres of a muscle fiber are transmitted to tendons and bones via two force-transducing systems:

1) Forces are transmitted longitudinally from one end of a muscle fiber to the other end.
2) Forces are additionally transmitted laterally from the sarcomere through the muscle fiber membrane (sarcolemma) to the extracellular matrix (141) via costameres (73) which are the focal adhesion equivalent in muscle fibers.

There are several candidate mechanosensors in the skeletal muscle force transduction systems. For a true hypertrophy-triggering mechanosensor, a mechanism must exist by which force modifies the mechanosensor to trigger an early signaling response that then initiates hypertrophic signaling and muscle hypertrophy. Here we discuss costameres, titin and filamin-C-Bag3 signaling as potential mechanosensors in the force transmission systems of muscle fibers.

Costamere-related mechanosensors. Historically, mechanical stimuli became a research focus when researchers discovered in the 1950s that cancer cells can grow on soft agar without anchorage whereas most non-cancer cells cannot. Researchers then discovered from the 1970s onwards that cells anchor the extracellular matrix through focal adhesion complexes that include proteins such as vinculin, talin and integrins as well as kinases including focal adhesion kinase or integrin-linked kinase (Ilk). Focal adhesions not only anchor cells on a substrate but also connect the exterior mechanically to the cytoskeleton and can sense and trigger adaptations to mechanical stimuli (72, 145).

Costameres are the functional equivalent of focal adhesions in skeletal muscle. They are Z-disc associated structures of muscle fibers that are related to focal adhesions of other cells. Costameres connect the cytoskeleton to the extracellular matrix and also transmit force laterally from the sarcomere to the extracellular matrix. There are two costamere complexes which are the dystrophin-glycoprotein complex and the vinculin-talin-integrin complex. Costameres are clearly essential for normal muscle function as the mutation of costamere genes such as the dystrophin-encoding *DMD* gene often result in severe muscle diseases such as Duchenne muscular dystrophy (73). Given that these complexes function to anchor muscle fibers on the extracellular matrix to transmit force laterally, can they potentially function as sensors that sense mechanical stimuli? Is there evidence that costamere-associated proteins are hypertrophy sensors?

In skeletal muscle, focal adhesion kinase (the protein is abbreviated as FAK and encoded by the gene *PTK2*) is a nonreceptor tyrosine kinase that moves to focal adhesions upon the

adhesion of a cell to a substrate (54). In cultured C2C12 myotubes, IGF1 can increase FAK Tyr397 autophosphorylation and FAK is required for IGF1-induced hypertrophy and Tsc2, mTOR and S6K1 signalling (28). However, it is unclear whether and how FAK itself is activated by mechanical load during resistance exercise and whether there is a mechanical hypertrophy sensor that can activate FAK. At the moment there is no compelling evidence that FAK is directly activated by mechanical load during resistance exercise because unlike filamin-C or titin, FAK does not appear to have a mechano-activated protein domain. Moreover, 4 sets of 10 repetitions of resistance exercise did not affect activity-related FAK Tyr576/577 phosphorylation 6 h after exercise in fasted and fed individuals (50). However, phosphorylated FAK Tyr397 was increased 60-90 minutes post eccentric exercise when compared to concentric bout exclusively at the distal site of the vastus lateralis muscle (43). Generally, whilst FAK might help to regulate muscle size, there is no evidence yet that FAK is linked to a mechanosensor that senses mechanical load as a hypertrophy stimulus during resistance exercise.

Focal adhesions are associated with phosphatidic acid-generating enzymes, such as phospholipases. Recently, it has been shown that mechanical stimuli in the form of attachment to either a soft or stiff substrate promote the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidic acid. This synthesis of phosphatidic acid was catalyzed by phospholipase Cγ1 (PLCγ1) and activated the Hippo pathway effectors Yap (Yes-associated protein 1, gene *Yap1*) and its paralogue Taz (gene *Wwtr1*) (98). Yap and Taz are mechanosensitive (34) transcriptional co-factors that regulate gene expression mainly by co-activating Tead1-4 transcription factors. Yap and Taz regulate muscle differentiation, satellite cell function (157), are affected by many exercise-associated stimuli (47) and increased Yap activity in muscle fibers can cause hypertrophy (52, 159). Whilst these papers suggest no link to mTORC1 and even demonstrate that Yap can cause hypertrophy with rapamycin treatment (52), there are known links between Yap and mTORC1. Yap has been reported to suppress the mTORC1 inhibitor Pten (151) and to induce the expression of *Slc7a5* and *Slc3a2* that encode the Lat1 amino acid transporter (58). Whilst Pten expression does not decrease in the vastus lateralis 2.5 h and 5 h after human resistance exercise (156) and in synergist-ablated, hypertrophying plantaris muscle (21), the expression of the Lat1-encoding genes *Slc7a5* and *Slc3a2* as well as of other Yap targets such as *Ankrd1* increases in both situations. Collectively this suggests a scenario where mechanical load, via an as yet unknown sensor, increases phosphatidic acid to activate Yap and Taz. Yap and Taz then increase the abundance of Lat1 which would sensitize the mechanically loaded muscle to leucine stimulation of mTORC1. However, phosphatidic acid not only modulates Hippo signalling but importantly for muscle, it can also activate mTORC1

(68), which is the primary regulator of muscle protein synthesis. Indeed, hypertrophy-inducing eccentric contractions increased the concentration of phosphatidic acid for up to 60 minutes in tibialis anterior muscles (109). Moreover, inhibition of phosphatidic acid synthesis by butanol prevents the phosphorylation of mTORC1 activity markers, suggesting that phosphatidic acid is a mediator of eccentric exercise-induced hypertrophic signalling (109). Whilst the Hornberger group first identified Z-disc-linked phospholipase D (Pld) as a phosphatidic acid-synthesizing enzyme (i.e. phosphatidic generating-enzymes are not only located in focal adhesions), they later identified a reaction catalyzed by diacylglycerol kinase ξ (Dgkξ) as another source of phosphatidic acid in mechanically loaded muscle (164). Collectively, these studies suggest that mechanical stimuli can activate phospholipases to synthesize phosphatidic acid which in turn can activate mTORC1 and the Hippo effectors Yap and Taz. However, whilst these studies elucidate key signalling mechanisms in-between the mechanical stimulus and hypertrophy-mediating pathways, neither study identifies the actual mechanosensor. To identify the actual, phosphatidic acid synthesis-stimulating mechanosensor is a key task for future research in this area.

Integrins are another protein group that are part of costameres. Specifically, the $\alpha_7\beta_1$-integrin isoform (encoded by the gene *Itga7*) has been linked to muscle size as $\alpha_7\beta_1$-integrin overexpressing mice have larger muscle fibers and increase muscle fiber size after eccentric exercise training when compared to wild-type mice. Also mTOR and its downstream target p70S6k are more phosphorylated at activity-related residues at rest and after eccentric exercise in $\alpha_7\beta_1$-integrin overexpressing mice (167), suggesting that $\alpha_7\beta_1$-integrin might help to activate mTORC1 signaling in response to exercise. However, it is unknown whether and how $\alpha_7\beta_1$-integrin is activated by a mechanical hypertrophy stimulus during resistance exercise, and how $\alpha_7\beta_1$-integrin then activates mTORC1 and other signaling proteins that cause the muscle fiber to hypertrophy.

Costamere-based mechanosensors may also sense two additional types of mechanical stimuli that have been discussed as hypertrophic triggers in the more applied literature. The first stimulus is muscle fiber swelling, which is known as the “pump” by exercisers. The second potential mechanical stimulus is a change in the stiffness of the extracellular matrix as a result of resistance exercise. We will briefly discuss these two potential mechanical stimuli here. Resistance exercise results in a temporary perception frequently described as a “pump,” which is interpreted as muscle fiber swelling (134). Moreover, exercise-induced muscle damage (EIMD) can also lead to muscle swelling (116), although the associated edema from EIMD can last far longer than the “pump”. Whilst little definite evidence exists for actual muscle fiber swelling (i.e. a swelling of the muscle fiber and not of the interstitium)

after resistance exercise, at least the whole muscle can swell as a result of single-bout of resistance exercise (39). In primary rat myotubes, swelling brought about by culture in a hypoosmotic culture medium increases glutamine uptake by 71% when compared to isotonic culture medium. This is dependent on integrins and the cytoskeleton, as integrin or cytoskeleton inhibitors prevent this effect (90). Together these data suggest that differentiated muscle can respond to cell swelling with increased glutamine uptake and that this depends on integrin or cytoskeletal loading. Such glutamine intake is potentially important, as it is a requirement for the uptake of protein synthesis-stimulating essential amino acids such as leucine (105). However, it is unknown whether the duration and extent of swelling is sufficient to load the cytoskeleton and that such cytoskeletal loading does not only induce glutamine uptake but also protein synthesis for up to three days post resistance exercise (99). Muscle swelling also occurs up to several days after exercise-induced muscle damage (166) at a time when muscle protein synthesis should have returned to baseline (99). Given that costameres are the sites where the cytoskeleton connects to the extracellular matrix and where mechanical signals can be sensed, it seems likely that any fiber swelling exerts a strain on costameres which then could trigger the hypertrophy response.

Titin (gene: *Ttn*). Titin is a giant protein that is essential for muscle function and human health as mutations in the titin-encoding *Ttn* gene cause various human genetic diseases including myopathies (130). Titin spans half a sarcomere, from the Z-disc at the end of a sarcomere to the M-line in the middle (82). The I-band-spanning portion of titin is elastic and contributes to the elasticity of a passively stretched muscle. The M-line portion of titin contains a stretch-activated kinase. The kinase within the titin protein is activated when a stretch pulls several amino acids out of a so-called ATP-binding pocket, allowing ATP to bind. ATP binding then causes titin to tyrosine-phosphorylate itself, which in turn activates the kinase within the titin protein (124). Because of its stretch-activated kinase and association with numerous other proteins, titin has been proposed to be an exercise-related mechanosensor (82). Using our terminology, mechanical load would be the hypertrophy stimulus and titin the hypertrophy sensor.

So what is the evidence for titin being a mechanical hypertrophy sensor? There are two points to consider. First, titin lies parallel to the force-generating actin-myosin proteins. This means if myosin and actin generate force and shorten a muscle fiber then titin will go slack. Consequently, the forces within a titin molecule should actually decrease rather than increase during a concentric contraction. Thus, titin cannot be a true force sensor in this situation. However, at longer muscle lengths titin forces increase and titin unfolds (62) and so

this might activate titin kinase and trigger downstream signaling events. Related to this, resistance training at longer muscle lengths may cause a greater hypertrophy when compared to resistance training with shorter muscle length (96, 107).

Second, whilst many signaling interactions have been reported for titin (82) there is not yet a convincing link between titin and mTORC1 signaling, which is the primary mediator of the muscle hypertrophy response to resistance exercise (see above). However, some titin signaling interactions are related to protein turnover through Murf1/2-proteasome and autophagy signalling and thus could regulate some aspects of muscle hypertrophy (82). In conclusion, whilst titin is a mechanosensitive skeletal muscle protein with a kinase domain it seems unlikely that it is the major mechanical hypertrophy sensor during standard resistance exercise, except perhaps at long muscle lengths.

Filamin-C Bag3 (genes: *Flnc* and *Bag3*). Bag3 and filamin-C are proteins important for muscle function as mutations of these proteins cause severe myofibrillar myopathies (137). Filamin-C and Bag3 localize to the Z-disc in human muscle (153). Here, we discuss evidence that filamin-C and Bag3 form mechanosensor complex that is capable of activating mTORC1, the Hippo effector YAP1 and autophagy (see **Figure 1**). Filamins are mechanosensitive, actin-crosslinking molecules. In skeletal muscle, filamin-C is the major filamin located at the Z-disc (137). Filamins form V-shaped homodimers and forces of ≈5-20 pN deform the so-called domain pair 20-21 (128). One myosin head generates a force of 6 pN (120) and thus actin-linked filamins should deform if sufficient myosin heads pull on the actin to which the filamins are attached.

In addition, filamins bind multiple proteins including the androgen receptor (112), which influences muscle size (71), and the Z-disc linked protein Bag3 (153) which has been proposed to sense the mechanical loading of filamin (152). However, how a mechanically loaded filamin dimer activates Bag3 is still unclear. Assuming that mechanically loaded filamin-C can activate Bag3, how could Bag3 trigger a hypertrophic signaling response? BAG3 connects through its WW domain (WW stands for the two tryptophanes that are separated by ≈20 amino acids (142)) to proline-rich motifs (e.g. PPxY motifs) of other proteins to potentially regulate three muscle hypertrophy-associated functions:

1) **mTORC1 signaling**. The WW domain of Bag3 binds the proline-rich motif of the mTORC1 inhibitor tuberous sclerosis 1 (TSC1). So the hypertrophy-inducing mechanism might be that Bag3 sequesters TSC1 away from mTORC1, resulting in mTORC1 activation and increased protein synthesis in response to mechanical loading (75).

2) **Hippo signaling**. Bag3 sequesters through its WW domain proteins such as LATS1 and AMOTL1 which normally inhibit the Hippo effector YAP (152). As a consequence, YAP will be more active in mechanically loaded muscle, which is relevant for muscle size because increased YAP activity in muscle fibers can elicit muscle fiber hypertrophy (52, 160).
3) **Autophagy**. Bag3 binds synaptopodin-2 (Synpo2) to regulate chaperone-assisted selective autophagy (CASA) of damaged Z-disc proteins (7, 154). This might contribute to the increased autophagy (61) and rate of protein breakdown seen after resistance exercise (149), a process that may be important in full and functional muscle hypertrophy.

Phosphoproteomic studies have shown that both Filamin-C and Bag3 change their phosphorylation after high intensity exercise in human muscle (65) and after maximal intensity stimulation of mouse skeletal muscle (121). This suggests that Filamin-C and Bag3 are additionally targeted by currently unknown kinases and phosphatases that might further help to regulate Bag3 activity in a contracting skeletal muscle.

The aforementioned Bag3-focussed hypertrophy stimulus-sensing mechanisms are illustrated in **Figure 1**.

Please insert Figure 1 here.

In summary, a filamin-Bag3-mTORC1/YAP/autophagy signaling cascade is a plausible but far from completely characterised mechanism by which mechanical loading during resistance exercise could stimulate hypertrophic signaling and skeletal muscle hypertrophy. However, whilst physiological, mechanical forces will probably deform a filamin homodimer, it is unclear how this then activates Bag3 and other hypertrophic signaling. Also the kinases and phosphatases that phosphorylate and dephosphorylate Filamin-C and Bag3 during exercise are currently unknown and it remains unclear as to how such phosphorylation affects Filamin-C and Bag3 function and muscle size. This is clearly another important area for future research.

Nuclear deformation and signal transduction. In muscle fibers, myonuclei are surrounded by thick tubulin filaments (17) and by intermediate desmin filaments (126). These filaments not only anchor myonuclei to the cytoskeleton but also expose them to forces when the cytoskeleton is loaded (8) either by a passive stretch, by an active contraction or by muscle fiber swelling. For example, when muscle fibers are passively stretched, myonuclei

subsequently deform (113). Intriguingly, such nuclear deformation has recently been identified as a mechanism by which mechanical load causes the Hippo effector Yap and potentially other proteins to translocate from the cytosol to the nucleus (37). Given that increased YAP activity can induce muscle fiber hypertrophy (52, 160), this might be a mechanism by which mechanical loading could contribute to skeletal muscle growth. Together the above filamin-Bag3-YAP and nuclear deforming-YAP-mTORC1 signaling cascades are plausible mechanisms by which a mechanical hypertrophy stimulus could be sensed and trigger hypertrophy signaling. However, there are two caveats to this hypothesis. First, YAP-induced muscular hypertrophy is comparatively small and seems to be independent of mTORC1 as it can occur when mTORC1 is blocked with rapamycin (52). Second, myonuclear deformation has so far only been demonstrated for passive stretch (113), and not for an active, shortening contraction. Nevertheless, proteins that sense nuclei deformation to activate Hippo signaling should be characterized in the future.

Another type of mechanosensor are stretch-activated ion channels encoded by the genes *PIEZO1* and *PIEZO2*. Spangenburg and McBride demonstrated that broad, non-specific inhibition of stretch-activated ion channels in rats *in vivo* with streptomycin or gadolinium could attenuate the load-induced activation of mTORC1 (140). However, the expression of *PIEZO1/2* stretch-activated ion channels is among the lowest in human skeletal muscle when compared to other tissues (www.gtexportal.org, see (97)) and so the effect might not depend on the inhibition of PIEZO1/2 channels in skeletal muscle. For that reason we do not discuss stretch-activated ion channels further.

In summary, there are several plausible but far from completely characterised mechanisms by which mechanical hypertrophy stimuli could activate mechanical hypertrophy sensors after a bout of resistance exercise. It may well be that there are several mechanical hypertrophy stimuli (e.g. the contraction force, loading of the cytoskeleton and the mechanical properties of the extracellular matrix) and sensors as has previously been proposed by the Hornberger group (45). To date no mechanism is fully characterised as either the mechanosensing mechanism or the link to mTORC1 or other hypertrophy-regulating signaling proteins is incompletely described in skeletal muscle. Research into such mechanisms is further hampered by the fact that the knockout of putative mechanosensors such as Bag3 not only abolishes a potential hypertrophy response to resistance exercise, but often leads to severe myopathies and dystrophies. This means that researchers can in many cases not use global knock out animal models to test whether these proteins are essential for the hypertrophy response to exercise.

Is exercise-induced muscle damage a hypertrophy stimulus?

The possible role of exercise-induced muscle damage (EIMD) as a hypertrophy stimulus has been discussed and studied since it was proposed in the 1990's (29, 38, 132). EIMD is damage that is triggered when individuals engage in new types of exercise, especially lengthening or eccentric contractions conducted with a large range of motion (115, 132). However, there is usually little EIMD when already resistance-trained individuals lift weights due to the "repeated bout effect". EIMD is associated with microscopic, structural changes such as Z-line streaming in skeletal muscle myofibrils. This is then usually followed by a local inflammatory response, disturbed Ca^{2+} regulation, activation of protein breakdown and increased levels of proteins such as creatine kinase in the blood that escape or are secreted from damaged muscle fibers (23, 76, 115). In their review, Hyldahl and Hubal (2014) propose a continuum of skeletal muscle fiber damage after eccentric exercise that spans possible adaptive cell signaling responses to pervasive membrane damage and tissue necrosis as the most severe form of EIMD (69).

Evidence from human studies for EIMD as a hypertrophy stimulus. Although some authors have endeavored to test whether EIMD contributes to muscle hypertrophy, the results of these interventions are difficult to interpret. This is because the manipulation of resistance training parameters to alter EIMD can also directly affect muscle mass, not just EIMD. Therefore it is difficult to separate the effect of EIMD on muscle hypertrophy from the effect of the confounding factors. For instance, training at long muscle lengths (i.e. the stretched position) is not only associated with a greater magnitude of EIMD (11, 115) but also possibly with increased muscle hypertrophy when compared to exercising with short muscle lengths, at least in some muscles (14, 107) . However, this may not be due to EIMD but due to the larger force production at longer fascicle lengths (40). Similarly, eccentric muscle actions not only increase EIMD but also cause a slightly larger hypertrophic response than concentric muscle action (32, 136). Again, it is unclear whether this is due to a higher dose of an EIMD-associated hypertrophy stimulus after eccentric exercise (100) or simply due to a confounding factor such as increased training load (36, 100). Collectively some studies suggest a connection between EIMD and muscle hypertrophy but this could be due to confounding factors.

In contrast, other studies show that the extent of muscle damage does not correlate with muscle protein synthesis (48) or the magnitude of hypertrophy. Severe EIMD does not give any further benefit on hypertrophy but rather attenuates it (42). Flann et al compared muscle hypertrophy of naïve and pre-trained group with the same cumulative workload. The pre-trained group did not experience EIMD as judged by plasma creatine kinase levels and

muscle soreness but increased muscle strength and volume at the same magnitude as the naïve group suggesting that EIMD is not essential for hypertrophy (41). However, in an effort to reduce EIMD, the pre-trained group performed an additional three weeks of resistance training, which may have confounded results.

A final argument against EIMD as a hypertrophy factor is that EIMD also occurs after exercise that does not typically induce hypertrophy. For example EIMD occurs after endurance exercise with an eccentric component such as marathon running (63) but damage in these situations alone does not seem to cause muscle hypertrophy. If anything, marathon running decreases muscle fiber size (150). However, these data are again difficult to interpret as endurance athletes may have a low trainability for muscle hypertrophy and their long-duration exercise, combined with low energy availability, may excessively activate AMPK and thereby inhibit mTORC1 (70) for long periods. In summary, it is difficult to conclude based on indirect human studies whether and how EIMD contributes to muscle hypertrophy. The key reason for this is that it is virtually impossible to separate direct EIMD stimuli from confounding stimuli that co-occur with EIMD.

Muscle damage or increased regeneration alone may induce muscle hypertrophy. The regeneration of skeletal muscle after injury is a (re-) growth process but can injury per se promote muscle fiber hypertrophy? In mice, severe injury of mouse tibialis anterior muscles e.g. through cardiotoxin injection results in larger, but fewer muscle fibers when compared to uninjured fibers (59), suggesting that injury alone is sufficient to trigger the hypertrophy of some muscle fibers. A caveat is that we do not know whether the larger fibers are hypertrophied, regenerated fibers or whether these are new but muscle fibers that are larger than the previous muscle fibers. There is some evidence that injured muscle fibers and their satellite cells can contribute to hypertrophy as transplanting muscle fiber-associated satellite cells into a recipient muscle whilst inducing injury results in a near-lifelong muscle hypertrophy (55). Together, these data suggest that injury alone and the combination of injury and more satellite cells can lead to the development of larger muscle fibers or induce muscle fiber hypertrophy.

Satellite cells, EIMD and muscle hypertrophy. Satellite cells are the resident stem cells of skeletal muscle (131) and add nuclei to adult muscle fibers after resistance training (24). Although non-damaging exercise can activate satellite cells to proliferate (27), satellite cell activation and proliferation is larger after exercise that induces EIMD (26). In humans, individuals that responded with greater hypertrophy to a resistance training programme also added more myonuclei, presumably derived mainly from satellite cells, than individuals that

responded less with less hypertrophy to the same training programme (118). This suggests that the ability of satellite cells to proliferate and to add new myonuclei to muscle fibers might limit muscle hypertrophy. However, satellite cells may expand especially in response to EIMD to have a role in muscle repair and less so to increase myonuclei when muscle actually hypertrophies, at least in the early stages of muscle growth (30).

The causal role of satellite cells on muscle hypertrophy has been investigated in mice. It seems that the initial hypertrophy in response to mechanical overload can occur in wildtype and satellite cell-depleted muscles (95, 103). However, the initial hypertrophy cannot be maintained for months when satellite cells are depleted (46). Other research suggests that satellite cells are also required for the initial hypertrophy at the muscle fiber level (35). Collectively these studies show that satellite cells are essential for full skeletal muscle hypertrophy over time and that satellite cell numbers and myonuclei increase after resistance training. It is not, however known whether EIMD is essential in the long run to induce satellite cells to proliferate and in turn trigger a muscle hypertrophic response to resistance training.

However, our main question in the present review is not whether satellite cells are essential for hypertrophy but: How do hypertrophy stimuli activate satellite cells in the first step and how do activated satellite cells cause muscle fiber hypertrophy in a second step? According to our definition, the EIMD-related hypertrophy stimulus would be the repeated mechanical load that causes muscle damage in a susceptible muscle. A damage-associated stimulus would then activate satellite cells in the first step. There are too many possible stimuli activating satellite cells to be effectively covered in this review. Currently the strongest candidate pathway to activate quiescent satellite cells to proliferate following injury as well as after exercise or mechanical stretching is the nitric oxide-metalloproteinase-hepatocyte growth factor (HGF) pathway (147). Whether these stimuli activate satellite cells in a context of resistance exercise bout especially after EIMD is unknown.

Other potential EIMD-associated hypertrophy stimuli and their sensors. EIMD is associated with potential hypertrophy stimuli such as amino acids that result from protein breakdown or factors linked to the immune and inflammatory response to EIMD and to satellite cells. As a consequence of EIMD, inflammatory cells enter muscles and produce substances including myokines such as IL-6 that have been reported to be able to both increase (138) or decrease muscle size (10) in different contexts. The inflammatory response to EIMD is thought to also induce cyclooxygenase production, which may aid hypertrophy as non-steroidal anti-inflammatory drugs (NSAID, which target cyclooxygenase) blunt hypertrophy following regimented resistance training (88). There is also evidence that

reactive oxygen species (ROS) promote hypertrophy, as antioxidant supplementation can blunt hypertrophic signaling (114) and reduce the magnitude of exercise-induced muscle hypertrophy (12). However, even if IL-6 and ROS can influence muscle size, they are clearly middlemen in the hypertrophic process, as there must be upstream hypertrophy stimuli and sensors that increase their concentration in response to resistance exercise. Moreover, ROS are not only induced by EIMD but also by endurance running (125), which does not typically cause hypertrophy. In summary, the evidence suggesting that EIMD is associated with hypertrophy is mostly indirect, some is contradictory, and putative mechanisms and sensors are incompletely characterised.

Is metabolic stress a hypertrophy stimulus?

We have already mentioned that mechanical forces are probably the most important hypertrophy stimuli. When mechanical forces are absent or reduced, other signals typically only have small effects on muscle size. For example, when post-operative brace-immobilized knee surgery patients intermittently occluded their thighs, their muscles atrophied by ≈7% within 14 days which was significantly less than the ≈15% atrophy seen in the no occlusion controls (146). This experiment suggests that potential occlusion-related hypertrophic stimuli cannot compensate for the loss of mechanical loading but that they can limit atrophy. However, when combining vascular occlusion with dynamic muscular contractions, marked hypertrophy invariably occurs, even when employing relatively light loads or no external loads at all (1, 89). In these training regimes, the vascular occlusion increases metabolic stress as judged by the drop in phosphocreatine (PCr) and pH (143). Similarly, muscles hypertrophy more if resistance training with relatively heavy load is conducted under intermittent hypoxia versus normoxia (85, 93, 106). The fact that blood flow restriction and hypoxia affect metabolism has led some researchers to suggest that metabolic stress-associated signals such as metabolites (i.e. molecules involved in metabolism that are typically below ≈1500 Da) may have an anabolic effect and contribute to muscle hypertrophy (133). An alternative proposal is that "*metabolites simply augment muscle activation and cause the mechanotransduction cascade in a larger proportion of muscle fibers*" (31). This is another way of saying that some fibers fatigue during contraction, which is linked to changes in metabolite concentrations such as a drop of phosphocreatine or increase of lactate. As a consequence, additional fibers need to be recruited to sustain force output and these additional fibers are then additionally exposed to hypertrophy stimuli. However, recent work found that the addition of blood flow restriction training to a traditional resistance training program preferentially enhanced type 1 fiber cross sectional area in a cohort of elite powerlifters (13). This seemingly refutes the hypothesis that the hypertrophic effects of blood flow restriction training are simply a function of increased high-threshold motor unit

recruitment, and raise the possibility that the associated metabolite accumulation may induce anabolism via other mechanisms. Henceforth, we discuss the potential role of metabolites as hypertrophy stimuli.

Metabolic stress. Metabolic stress can be defined as the changes in energy metabolism and metabolites that occur during non-steady state muscle contractions. Non-steady state contractions are contractions where not all of the hydrolyzed ATP can be resynthesized by oxidative phosphorylation alone. As a consequence, the concentration of PCr will continuously decline as PCr resynthesizes ADP to ATP via the Lohmann reaction (PCr+ADP↔ATP+creatine). Moreover, the lactate concentration will rise and the pH will drop as ATP is additionally resynthesized through glycolysis. Thus, a low PCr concentration, a high lactate concentration and a low pH are biomarkers for metabolic stress. In relation to these metabolites, blood flow restriction will not change the rate of ATP hydrolysis but it will reduce oxygen delivery and oxidative ATP resynthesis, which requires greater PCr breakdown and a higher rate of glycolysis in active muscle fibers (143, 144).

Metabolic stress during resistance exercise versus other types of exercise. The higher the exercise load, the more ATP will be hydrolyzed per second and the faster PCr, lactate and the pH will change. Thus, during high intensity resistance exercise, the PCr concentration and the pH will drop more per second than during low load resistance exercise (143, 144, 158). However, as metabolic stress either causes fatigue or is associated with it (5), metabolic stress will be higher at the end of a longer duration set with low loads because we can lift a lower load with a more fatigued muscle than during a shorter duration set with high loads as we can only lift a high load if fatigue and metabolic stress are low.

The logic that a set with lower loads to exhaustion will cause more metabolic stress than a set with heavy loads is supported by experimental data. In a biopsy study, Tesch et al measured intramuscular PCr and other metabolites in the vastus lateralis before and after several sets of ≈10 repetition leg muscle contractions to failure in trained bodybuilders. Intramuscular PCr decreased from 21.3±3.7 mmol/kg pre exercise to 10.9±2.5 mmol/kg (51% of pre-exercise) after the last set of exercise (148), suggesting moderate metabolic stress. In contrast, during intermittent resistance exercise with 25% of the 1RM which is suboptimal for hypertrophy, PCr decreased to 17±12% of the pre-exercise concentration in adult women and 18±16 % of the pre-exercise concentration in adult men, respectively (74), suggesting high metabolic stress. Similarly, PCr decreased from 15.8±1.7 mmol/kg to 1.7±0.4 (11% of pre-exercise) after a 400 m run (64). Collectively this shows that metabolic stress is typically

greater during non-steady state exercise with intensities that are sub-optimal for hypertrophy (86) than during “classic” ≈10 repetition resistance training in trained individuals.

Metabolites that have anabolic signaling properties. Metabolic stress is a vague concept given that ≈2700 metabolic enzymes catalyze ≈900 metabolic reactions (129) and that ≈4000 metabolites can be detected in human serum alone (123). So given the plethora of metabolites, are there any metabolites or other metabolic stress-related factors that can act as hypertrophy stimuli? Are there any metabolites that can be considered to be hypertrophy stimuli according to our definition?

Lactate is a key biomarker for metabolic stress, used as a biomarker for performance, and one of the most studied exercise metabolites. There is some evidence that lactate may affect muscle differentiation and have some anabolic effects (104). In the most extensive study to date, lactate affected the expression of regulators of muscle differentiation in vitro. Also the authors found that a combination of a 30 min low-intensity running training program together with a dose of lactate and caffeine increased muscle mass and hypertrophic signaling in rats (111). It is not possible to conclude, however, how much of the hypertrophy was due to lactate. Other studies suggest that skeletal muscle may sense changes in extracellular lactate. For instance work form George Brooks’ lab demonstrated that when 20 mM lactate caused L6 rat myotubes to express lactate-related genes (60), but this did not show that lactate is a hypertrophy-stimulus. More recently, Ohno et al found that 20 mM lactate was able to induce anabolic signaling and hypertrophy in C2C12 cells, possibly in a GPR81 dependent manner (110). This suggests that extracellular lactate can initiate signaling events through membrane-bound receptors in skeletal muscle. Whilst these data indicate that lactate may be a modifier of muscle signaling and hypertrophy, lactate concentrations are typically highest during exercise that is suboptimal for hypertrophy such as a 400 m run.

Another anabolism-related energy metabolite is α-ketoglutarate which is not only a citrate cycle metabolite but also a nitrogen scavenger (163). Long term supplementation for 9 weeks of the drinking water with 2% α-ketoglutarate resulted in significant gastrocnemius skeletal muscle hypertrophy and increased markers of mTORC1 activity (19), suggesting that α-ketoglutarate could stimulate muscle hypertrophy. In contrast, however, L-arginine α-ketoglutarate supplementation did not increase strength measures such as the 1 RM after a resistance training program in humans when compared to placebo control (161).

Other anabolic metabolites are phosphatidic acid and lysophosphatidic acid, which can activate mTORC1 (68, 139) and Hippo (165) signaling, respectively. We have already

discussed in the mechanotransduction section that hypertrophy-inducing eccentric contractions increase the phosphatidic acid concentrations in tibialis anterior muscles (109).

Another potential source of hypertrophy-inducing metabolites is from muscle protein breakdown. The activation of skeletal muscle protein synthesis by resistance exercise seems to be correlated to the activation of skeletal muscle protein breakdown (119). Cell based experiments demonstrate that simply increasing the intracellular concentration of key amino acids like leucine by as little as 7% is sufficient for half maximal activation of mTORC1 (22). Additionally, a single bout of resistance exercise in rodents causes an approximate 25% increase in the intramuscular leucine concentration (91). It is theorized that this increase in intracellular leucine, possibly from protein breakdown, is sensed by the amino acid sensor mVPS34 leading to mTORC1 activation (91). However, feeding 40 g of protein can almost triple the intracellular leucine content in human skeletal muscle (92) and it seems unlikely that the small transient changes in intramuscular leucine as a result of resistance exercise make a major contribution to the hypertrophy response to resistance exercise

In addition to metabolites, metabolic enzymes might also be involved in hypertrophy signaling, too. Researchers found in HEK293 cells and fibroblasts that the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) binds Rheb and inhibit mTORC1 signaling. However, when glycolytic flux is high as would be at the end of a set of resistance exercise then GAPDH no longer inhibited mTORC1 and cells grow (87). In this scenario, the signals that activate glycolytic enzymes such as phosphorylase and phosphofructokinase would be the hypertrophy stimuli and the enzymes would be their sensors. This shows a plausible mechanism by which a signal related to glycolytic flux might act as a hypertrophy stimulus capable of activating mTORC1 and skeletal muscle hypertrophy. These and further links between metabolism, muscle mass and regeneration have recently been reviewed (78).

Studies that do not support energy stress being a hypertrophy stimulus. During evolution, mechanisms evolved that reduce protein synthesis and cell growth when there is metabolic stress. For example, when the metabolic stress-mimicking AMPK activator AICAR was given to rats then muscle protein synthesis reduced significantly to 55% of the protein synthesis measured in control rats (16). Soon after the Guan group demonstrated that the metabolic stress sensor AMPK inhibited mTORC1 via TSC2 (70). Consistent with this, the synergist-ablated plantaris hypertrophied more in AMPKα1 knockout than wildtype control mice suggesting that energy-stress activation of AMPK can blunt hypertrophy at least in some hypertrophy models (102). However, whilst prolonged metabolic stress might work through such mechanisms to explain reduced muscle hypertrophy during concurrent

endurance and resistance training (9), it is unclear whether these studies explain what happens during short term metabolic stress during acute resistance exercise, which might exert its effect via different metabolites and signaling molecules.

Overall summary, conclusions and directions for future research

Whilst there is a large amount of mainly indirect evidence about hypertrophy stimuli and their sensors, this evidence is often difficult to interpret and as a consequence many questions remain. Mechanical stimuli stand out as the most likely and most potent hypertrophy stimuli and several potential mechanosensing mechanisms have been partially characterised. To us, a key question is whether muscle fibers, which are the cells that produce the highest forces, have their own specific mechanosensing system in addition to the generic focal adhesions (i.e. costameres in muscle) that sense the mechanical environment of most cells. The Z-disc is a prime striated muscle-specific candidate site for muscle-specific force sensing. Z-discs are not only directly exposed to the forces generated by sarcomeres but Z-discs additionally transmit these forces longitudinally and laterally via costameres (44). Moreover, the Z-disc becomes a signalling hub when muscles contract with high intensity and generate large forces. This is supported by the results of a recent phosphoproteomic study which reported that the majority of Z-disc proteins robustly alter their phosphorylation in response to maximal intensity contractions of mouse muscles. In particular, the Z-disc localized kinases obscurin and Speg change their phosphorylation and the Z-disc localized Filamin-Bag3 complex proteins are also phosphorylated (121, 153).Thus future studies should seek to answer the question “Is it mainly the Z-disc or the costameres where mechanical hypertrophy stimuli are sensed and transduced after resistance exercise?”

Data suggesting and supporting EIMD or metabolic stress-related hypertrophy stimuli are mostly indirect and the related molecular mechanisms are poorly understood. Moreover, growth can occur in the relative absence of either of these putative signals, lending further support for the hypothesis that mechanical stimuli are the primary hypertrophy stimuli. That said, research indicates that both EIMD and metabolic stress regulate multiple factors involved in the hypertrophic process, and a sound rationale exists whereby their resistance training-induced manifestation may contribute to hypertrophic adaptations. If so, it remains to be determined whether these factors are additive to mechanically-derived signaling or perhaps redundant provided a given level of mechanical force is achieved. Moreover, if these signals are indeed additive, it remains to be determined whether an upper threshold exists beyond which no further growth-related benefits are realized. In particular, any hypertrophic effects of EIMD would almost certainly follow a hermetic curve, with benefits seen only up to a given point and they ultimately inhibit hypertrophy when EIMD is excessive. To this point, a

high degree of EIMD impairs a muscle's force-producing capacity, which in turn interferes with an individual's ability to train as well as negatively impacting recovery (80, 108). Thus, there may be a "sweet spot" whereby a combination of mechanical, metabolic, and damage-related signals interact synergistically to promote a maximal hypertrophic response.

Finally, how to proceed towards the long-term goal of identifying all major hypertrophy stimuli and their sensors? It is clear that the leading researchers must move beyond indirect association studies as there are just too many confounding variables to draw valid conclusions. Force, metabolism and EIMD are all linked and it seems impossible to vary only one of these variables during resistance exercise without varying the others and so such studies are never fully conclusive. One key experiment is to assess whether a putative hypertrophy sensor is essential for the muscle hypertrophy adaptation to resistance exercise. To test for this, the gene that encodes the sensor needs to be knocked out or inhibited pharmacologically to evaluate whether this prevents adaptation to exercise. However, the problem with this approach is that putative hypertrophy sensors such as Bag3 are essential for normal muscle function (66). Hence, their global knock out typically causes a myopathy or dystrophy, which limits the usefulness of such models for studying their role in hypertrophy signaling. Here, more sophisticated transgenic animal models are needed. Strategies could involve targeting the transgenesis to skeletal muscle only, making it inducible and modulating solely those sites of a protein that are likely mediators of the hypertrophy-sensing function. But even a highly targeted transgenesis may cause problems, as mechanosensors may already be essential for normal muscle function. This is a major challenge for researchers in this area. Another strategy to identify the hypertrophy sensor is based on the knowledge that any hypertrophy sensing protein must physically interact with the proteins that mediate hypertrophy further downstream. Here, interaction proteomic studies in resting and resistance trained skeletal muscle could provide some answers (3). For example, researchers could co-immunoprecipitate mTORC1 protein complexes in resting and resistance exercise-trained muscle to see via mass spectrometry analysis what proteins interact with mTORC1 under load or metabolic stress when compared to rest. This might reveal either the hypertrophy sensor itself or intermediate proteins that connect a hypertrophy sensor to mTORC1 and other downstream hypertrophy mediators. Whilst this sounds feasible, it will be a difficult experiment in reality as the interpretation of interaction proteomic experiments is typically hampered by many false positive results.

In summary, conclusively identifying major hypertrophy stimuli and their sensors is clearly one of the big remaining questions in exercise physiology. However, experimentally this is difficult to achieve, which explains why there is still a large amount of uncertainty despite

many studies. We hope that this review helps to update on the status quo and to stimulate future research in this area.

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Figure legends

Figure 1. Schematic overview over how Filamin-C and Bag3 might trigger muscle hypertrophy in response to resistance exercise (see text for references). 1 Filamin is a Z-disc-linked protein that binds to actin and becomes deformed in response to mechanical load. 2 Filamin is linked to Bag3 and both Filamin and Bag3 become phosphorylated by unknown kinases during intense muscle contractions. 3 Bag3 has a WW domain through which is can bind and sequester proteins with proline-rich PPXY domains including Tsc1, a mTORC1 inhibitor. 4 Bag3 can also sequester inhibitors of the Hippo effector Yap such as Lats1, Amotl1, and Amotl2. Alternatively, YAP might be important into myonuclei as a result of nuclear deformation as has been demonstrated in non-muscle cells. Such Yap activation could be relevant for hypertrophy as YAP can induce the gene that encodes the Lat1 leucine transporter. 5 Finally, Bag3 also binds to Synpo2 which regulates chaperone-assisted selective autophaghy (CASA) which regulates the degradation of damaged Z-disc proteins.