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Plasticity of TOM complex assembly in skeletal muscle mitochondria in response to chronic contractile activity

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ABSTRACT

We investigated the assembly of the TOM complex within skeletal muscle under conditions of chronic contractile activity-induced mitochondrial biogenesis. Tom40 import into mitochondria was increased by chronic contractile activity, as was its time-dependent assembly into the TOM complex. These changes coincided with contractile activity-induced augmentations in the expression of key protein import machinery components Tim17, Tim23, and Tom22, as well as the cytosolic chaperone Hsp90. These data indicate the adaptability of the TOM protein import complex and suggest a regulatory role for the assembly of this complex in exercise-induced mitochondrial biogenesis.

1. Introduction

The assembly of the mitochondrion is an intricate process that requires the incorporation of hundreds of proteins into an expanding reticulum. The capacity of the mitochondrial genome to synthesize these precursor proteins is limited, and therefore the majority of mitochondrial proteins must be encoded in the nucleus and translocated into the organelle via the protein import machinery (Baker et al., 2007; Bohnert et al., 2007; Bolender et al., 2008). This machinery primarily consists of two multisubunit complexes, referred to as the translocase of the outer membrane (TOM complex) and the translocase of the inner membrane (TIM complex). The TOM complex is composed of the receptor proteins Tom20, Tom22, and Tom70 which contain cytosolic transmembrane domains responsible for recognizing newly synthesized precursor proteins at the outer membrane (Bohnert et al., 2007; Bolender et al., 2008). Precursor proteins traverse the outer mitochondrial membrane via a general insertion pore of ~400 kDa, consisting of the core pore-forming protein Tom40, Tom22, and the smaller TOM subunits, Tom5, Tom6 and Tom7 (Becker et al., 2008; Bolender et al., 2008). While the mitochondrial protein import pathway has been well studied in yeast (Bohnert et al., 2007; Bolender et al., 2008; Gabriel and Pfanner, 2007; Mokranjac and Neupert, 2007), our understanding of this process in mammalian cells has remained less well defined, particularly during the induction of mitochondrial biogenesis.

Skeletal muscle serves as an excellent model of organelle biogenesis because of its remarkable adaptability to conditions of chronic muscle use and disuse (Hood, 2001). Earlier studies have documented that the protein import pathways are inducible under conditions of chronic contractile activity or thyroid hormone treatment, both of which result in an increase in mitochondrial content within the cell (Craig and Hood, 1997; Gordon et al., 2001; Takahashi and Hood, 1996; Takahashi et al., 1998). Following 7 days of chronic contractile activity, the expression of several protein import machinery components, including Tom20, mtHsp70, and Hsp60 were upregulated in both subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria (Gordon et al., 2001; Takahashi et al., 1998). These changes coincided with an accelerated rate in the import of matrix precursor proteins, including the mitochondrial transcription factor A (Tfam) and malate dehydrogenase (MDH) in both mitochondrial subfractions (Gordon et al., 2001; Takahashi et al., 1998). Therefore, these data suggest that modifications in the protein import pathway are an essential event in the response of skeletal muscle to chronic muscle use. Recently, we have also documented that protein import into skeletal muscle mitochondria is reduced by chronic muscle disuse (Singh and Hood, 2011), but is not affected by aging, despite a reduction in organelle content (Huang et al., 2010).

The dynamic nature of the protein import machinery complexes has been exemplified with the characterization of the import and assembly pathway of Tom40 into the TOM complex (Model et al., 2001; Rapaport and Neupert, 1999). Similar to other nuclear-encoded proteins, components of the TOM complex must be imported and

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targeted to the outer mitochondrial membrane via the protein import machinery. Initial studies conducted in *Saccharomyces cerevisiae* and *Neurospora crassa* (Dekker et al., 1998; Model et al., 2001; Rapaport and Neupert, 1999) demonstrated that Tom40 follows a unique pathway of assembly into the outer mitochondrial membrane. Specifically, the incorporation and assembly of the Tom40 precursor protein into the final ~400 kDa TOM complex is preceded by its incorporation into a ~250 kDa intermediate I complex first, followed by a ~120 kDa intermediate II complex. The progression of the Tom40 precursor protein into intermediates I and II is dependent on preexisting TOM subunits, small TIM proteins, and a separate sorting and assembly machinery (SAM) complex (Hoppins and Nargang, 2004; Humphries et al., 2005; Model et al., 2001; Wiedemann et al., 2003; Wiedemann et al., 2004). Interestingly, this assembly pathway is common among proteins that share a complex beta-barrel topology such as Tom40 and porin (Wiedemann et al., 2003).

To date, only a few studies have examined Tom40 import and assembly kinetics in mammalian cells (Humphries et al., 2005; Johnston et al., 2002). In HT1080 human cells, the TOM complex was shown to be assembled via a pattern which differed in comparison to yeast cells (Humphries et al., 2005; Model et al., 2001). Tom40 was first incorporated into a ~500 kDa intermediate complex I before its import into a ~120 kDa intermediate II complex and the TOM complex. These early studies suggest that species-specific differences exist in the TOM complex assembly pathway, leading us to hypothesize that distinct patterns of Tom40 import and assembly kinetics may also be present in skeletal muscle mitochondria.

Thus, given the importance of the protein import pathway for mitochondrial biogenesis, this study was undertaken to characterize the assembly of the TOM complex in mammalian skeletal muscle mitochondria, with an emphasis on gaining insight into the mechanisms governing the plasticity of the protein import and assembly pathway during chronic contractile activity-induced mitochondrial biogenesis.

2. Materials and methods

2.1. Animals and surgery

Male Sprague–Dawley rats (Charles River, St. Constant, PQ, Canada) weighing 250–300 g were housed individually and given food and water ad libitum. The procedure as outlined previously (Takahashi and Hood, 1993) was followed for the implantation of electrodes and stimulation of animals. Briefly, animals were anesthetized with a ketamine/xylazine mix. Two stimulating electrodes (Medwire, Leico Industries, New York, NY) were passed subcutaneously from the left thigh to the back of the neck. At the thigh, the electrodes were sutured on either side of the peroneal nerve and the overlying tissues were sutured closed. To avoid infection, sterile ampicillin (Penbritin, Ayerst, Montreal, PQ, Canada) was applied to the incision site. A portable stimulator unit (Takahashi and Hood, 1993) was placed in a plastic housing and attached to the exteriorized wires at the neck. The stimulation unit was then secured to the animals back with adhesive tape. All animal experimentation was approved by the York University Animal Care Committee in accordance with the guidelines provided by the Canadian Council on Animal Care.

2.2. Stimulation protocol

Animals were allowed one week to recover from surgery and were stimulated (10 Hz, 0.1-ms duration) for 3 h/day for 7 days, as done previously (Takahashi et al., 1993, 1998). After the indicated number of days of stimulation, the animals were anesthetized and the tibialis anterior and the extensor digitorum longus muscles were removed from the stimulated and the contralateral limb that served as a control. Muscles were immediately placed in cold buffer for mitochondrial isolation.

2.3. Mitochondrial isolations

Muscle subsarcolemmal mitochondria were isolated by differential centrifugation from whole rat tibialis anterior and extensor digitorum longus muscle, as described previously (Cogswell et al., 1993; Takahashi and Hood, 1996). Mitochondria were resuspended in a buffer containing 10 mM HEPES, 0.25 M sucrose, 2.5 mM potassium phosphate dibasic, 10 mM succinate, 0.21 mM ADP, and 1 mM dithiothreitol (pH 7.4). Protease inhibitors (2 μl/ml Leupeptin, 1 μl/ml Pepstatin A, 1 μl/ml Aprotinin, 1 mM DTT, 1 mM PMSF, 1 mM Sodium Orthovanadate) were also added to the buffer and protein concentrations were measured using a Bradford assay (Bradford, 1976).

2.3.1. Isolation of cytosolic fraction

Following the final centrifugation to isolate the subsarcolemmal mitochondria, the supernate was removed and retained. The supernatant fractions from each of the control and stimulated muscles were then centrifuged at 100,000×*g* for 1 h at 4 °C. These fractions were concentrated in an ultrafiltration cell (Amicon, Beverly, MA) to a volume of <1 ml, and were used as cytosolic fractions.

2.4. In vitro synthesis of precursor proteins

Full length cDNAs encoding Tom40 (pGEM4Z/hTom40, from Dr. M. T. Ryan, La Trobe University, Melbourne, Australia) and ornithine carbamoyltransferase (rat OCT, from Dr. G. C. Shore, McGill University, Montreal, Canada), were isolated using an alkaline lysis DNA preparation method and linearized using *Bam*HI and *Sac*I, respectively. Both linearized plasmids were resuspended in Tris-EDTA (pH 7.8) to a final concentration of 0.8 μg/μl. Transcription reactions were performed with SP6 RNA polymerase (Promega, Fisher Canada) for 90 min at 40 °C (Takahashi and Hood, 1996). Both Tom40 and OCT mRNAs were isolated by phenol extraction followed by ethanol precipitation, and final concentrations were adjusted to 2.8 μg/μl. In vitro translation was performed at 30 °C for 30 min using cell free rabbit reticulocyte lysate (Promega, Fisher Canada) in the presence of [^{35}S] methionine (Perkin Elmer, Canada).

2.4.1. Import of precursor proteins into isolated mitochondria

Isolated mitochondria and lysate containing the translated radiolabeled precursor were initially allowed to equilibrate separately at 30 °C for 10 min. The two samples were then combined and further incubated at 30 °C for various time points. For standard import reactions using SDS-PAGE, 25 μg of mitochondria and 12 μl of reticulocyte lysate were used. In contrast, for determining Tom40 assembly by BN-PAGE, 65 μg and 31.2 μl of mitochondria and lysate were used, respectively. To determine the role of ATP in Tom40 import and assembly, apyrase (2U/reaction; Sigma Aldrich) was added to the lysate and mitochondria and incubated at 30 °C for 30 min. Mitochondria were recovered by centrifugation (16,000 x *g*) through 600 μl of 20% sucrose in 0.1 M potassium chloride, 2 mM magnesium chloride, and 20 mM HEPES (pH 7.4) at 4 °C for 15 min. For Tom40 import, mitochondrial pellets were resuspended in freshly prepared 0.1 M sodium carbonate (Na_2CO_3; pH 11.5) and incubated on ice for 30 min. For standard import reactions, mitochondrial pellets were then resuspended in 0.6 M sorbital, and 20 mM HEPES-KOH (pH 7.4), and equal volumes of sample buffer (10% (v/v) glycine, 80 mM SDS, 62.5 mM Tris–HCl, pH 6.8), 5% (v/v) 2-mercaptoethanol and 5% (v/v) dye added. Samples were denatured for 5 min and electrophoresed through an 8 or 12% SDS-polyacrylamide gel for OCT and Tom40, respectively. Following electrophoresis, gels were treated for 5 min in boiling 5% trichloroacetic acid, followed by a 30 s wash in distilled water, 5 min in 10 mM Tris base (pH 9.0), and 30 min in 1 M sodium salicylate. Gels were subsequently dried with a vacuum gel dryer (Model 583; Bio-Rad). Radiolabeled precursor proteins were detected using

phosphorimaging (Pharos FX; Bio-Rad) and quantified using Quantity 1 software.

2.5. Blue-native PAGE

For analysis of [^{35}S]hTom40 import and assembly into the TOM complex, samples were prepared as previously described (Johnston et al., 2002; Schagger and von, 1991). Briefly, following centrifugation of the sample through a sucrose gradient, the pellet was washed in 50 µl of import buffer (250 mM sucrose, 5 mM magnesium acetate, 80 mM potassium acetate, 10 mM sodium succinate, 1 mM dithiothrietol, 0.1 mM ADP, 20 mM Hepes-KOH, pH 7.4) and centrifuged at 10,000×*g* for 5 min. The supernate was removed and the pellet resuspended in 50 µl of digitonin buffer (1% (w/v), 50 mM NaCl, 10% (v/v) glycerol, 20 mM BisTris, pH 7.0) and incubated on ice for 10 min. The samples were then centrifuged at 16,000×*g* for 5 min and the supernate transferred to a new tube. Sample buffer (5% (w/v) Coomassie Brilliant Blue G-250, 500 mM -amino-*n*-caproic acid, 160 mM BisTris, pH 7.0) was added to the supernate and the samples were subjected to 5–13% BN-PAGE (Ryan et al., 2001; Schagger and von, 1991) and electrophoresed overnight at 23 V. The following day, the gels were destained (50% methanol, 10% acetic acid) and fixed (7% methanol, 7% acetic acid, 1% (v/v) glycerol). The gels were then dried with a vacuum gel dryer and imaged as described above.

2.6. Antibody shift assay

The procedure was performed as described previously (Johnston et al., 2002). Briefly, following centrifugation through the sucrose gradient the mitochondrial pellet was washed in 50 µl of Import Buffer and centrifuged for 5 min at 10,000×*g*. The mitochondrial pellet was resuspended in 50 µl 1% (w/v) digitonin and 5 µl of the specific antibody. The samples were incubated on ice for 20 min and centrifuged for 5 min at 16,000×*g*. The supernate was transferred to a new tube, and loading buffer added to the sample prior to BN-PAGE.

2.7. Contribution of cyosolic components in protein import

To establish the role of cytosolic components in Tom40 import and assembly, 10 µg of cytosol isolated from control or stimulated muscle was preincubated with lysate and allowed to equilibrate at 30 °C for 10 min before the addition to mitochondria and the initiation of the import reaction. Novobiocin (Sigma Aldrich), a specific Hsp90 inhibitor, was added to the cytosol-containing lysate at a concentration of 1 mM and allowed to equilibrate before the addition to mitochondria.

2.8. Immunoblotting

Whole muscle extracts, isolated mitochondria, as well as cytosolic fractions were used for Western blotting analyses as previously described (Takahashi and Hood, 1993). Briefly, protein samples were separated by gel electrophoresis on 8–15% polyacrylamide gels and transferred to nitrocellulose membranes. Blots were blocked for 1 h in 5% skim milk in 1X Tris-buffered saline-Tween 20-Tris·HCl (TBST, pH 7.4) and probed with the appropriate primary antibody (1:350 for Sam50; 1:500 for Tim17, Tim23, hTom40, Hsp90; 1:1000 for Tom22, porin; 1:5000 for GAPDH). Blots were then incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase at a dilution of 1:3000 (hTom40), anti-mouse secondary at a dilution of 1:1,000 (Tom22, Sam50, porin), 1:1500 (Tim23), and 1:25,000 (GAPDH), anti-goat secondary at 1:2000 (Tim17), and anti-rat secondary at 1:1000 (Hsp90). Blots were washed in TBST (3×5 min), and proteins subsequently visualized with an enhanced chemiluminescence kit (Santa Cruz) and quantified with SigmaScan software. Immunoblotting of porin and GAPDH, or Ponceau S staining was used to normalize for the amount of protein loaded. Staining of BN gels was used to correct for loading of assembly experiments.

2.8.1. Antibodies

For immunoblotting experiments, the Tom40 antibody was provided by Dr. M.T. Ryan (La Trobe University, Melbourne, Australia) and the Tom20 antibody for the BN-shift experiments was provided by Dr. M. Mori (Kumamoto University School of Medicine). Antibodies were obtained from Abnova (Sam50), Santa Cruz (Tim17), BD Transduction Laboratories, (Tim23), Assay Designs (Hsp90), Sigma (Tom22), and Mitosciences (porin).

2.9. Statistics

Data were presented as means ± SEM and analyzed with a paired Student's *t*-test or a one- or two-way ANOVA. Differences were considered statistically significant if $P<0.05$.

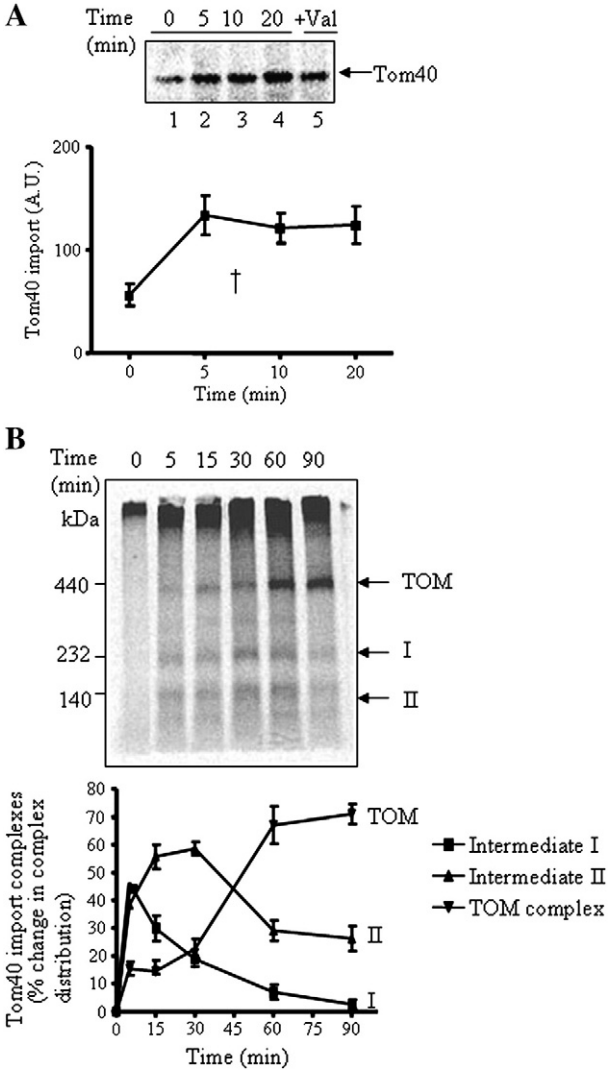

Fig. 1. Import of radiolabeled Tom40 precursor in skeletal muscle mitochondria. A. Radiolabeled Tom40 was imported into SS mitochondria and subjected to SDS-PAGE. In addition, mitochondria were incubated with valinomycin (Val) and imported for 5 min (Lane 5). Graphical representation of the data (n = 6) is shown below, with values expressed as arbitrary units (A.U.). †$P<0.05$, main effect of time. B. Assembly of Tom40 precursor imported into mitochondria for 0, 15, 30, 60 and 90 min. Solubilized mitochondria were reisolated with digitonin and loaded on a 5–13% Blue Native (BN) gel. A summary of multiple experiments (n = 5–7) is shown below where over time the progression of the Tom40 precursor protein into intermediate I (I), intermediate II (II) and the TOM complex (TOM) is observed. Values are expressed as a percentage change in the distribution of Tom40 into each intermediate complex.

3. Results

3.1. Tom40 import and assembly dynamics in mitochondria

To characterize the import of Tom40 in isolated mitochondria from rat skeletal muscle, we first examined the kinetics of Tom40 import into mitochondria using a standard import assay. A progressive increase ($P<0.05$) in the import of the Tom40 precursor into the outer membrane was evident over time (Fig. 1A). As expected, the dissipation of the mitochondrial membrane potential with valinomycin had no effect on the import of Tom40 into the outer membrane (lane 2 vs. lane 5). To directly monitor the assembly of the radiolabeled Tom40 precursor protein into its intermediate complexes, BN-PAGE was employed. The progression of Tom40 into a number of assembly intermediates was observed over the 90 min time course. At 5 min, 45% of the radiolabeled Tom40 was incorporated into a ~230 kDa intermediate I complex (labeled "I"). This was followed by its assembly into a smaller ~120 kDa intermediate II complex (labeled "II") before its final incorporation into a ~380 kDa mature complex (labeled "TOM"). At 90 min, ~70% of Tom40 was present in the TOM complex (Fig. 1B). This pattern of Tom40 assembly closely resembled that found in yeast, which exhibit a ~250 kDa complex composed of a Tom40 precursor and the SAM complex (4.5Meisinger et al., 2004; Model et al., 2002; Wiedemann et al., 2003).

To determine the localization of Tom40 within the TOM complex at the outer membrane, mitochondria were subjected to protease treatment. As shown in Fig. 2A, treatment with proteinase K resulted in Tom40 being resolved within a lower molecular weight TOM complex at 90 min. This suggests the association of the Tom40 precursor with protein import machinery components containing accessible cytosolic domains. Such proteins likely may include the receptor protein Tom22, which has been reported to be present within the TOM complex and which is essential for the assembly of Tom40 (Humphries et al., 2005; Johnston et al., 2002; van et al., 1999). Antibody shift experiments verified the presence of Tom40, as well as Tom22 within the TOM complex (Fig. 2B). The inability of the Tom20 antibody to shift the complex may be due to the nature of the gel which does not allow weakly associated proteins to be resolved within the complex (Johnston et al., 2002). Antibodies against porin (VDAC), an outer membrane protein that forms part of the mitochondrial permeability transition pore, also did not shift the Tom40-containing complex. To determine the energy requirements of the Tom40 import process, the lysate and mitochondria were depleted of ATP using the ATP diphosphohydrolase, apyrase. Similar to observations in different species (Humphries et al., 2005; Rapaport and Neupert, 1999), we found that the import and assembly of the Tom40 precursor protein into its intermediate complexes was drastically reduced, if not abolished, in the absence of ATP, suggesting the importance of energy-requiring processes in Tom40 import and assembly (Fig. 2C).

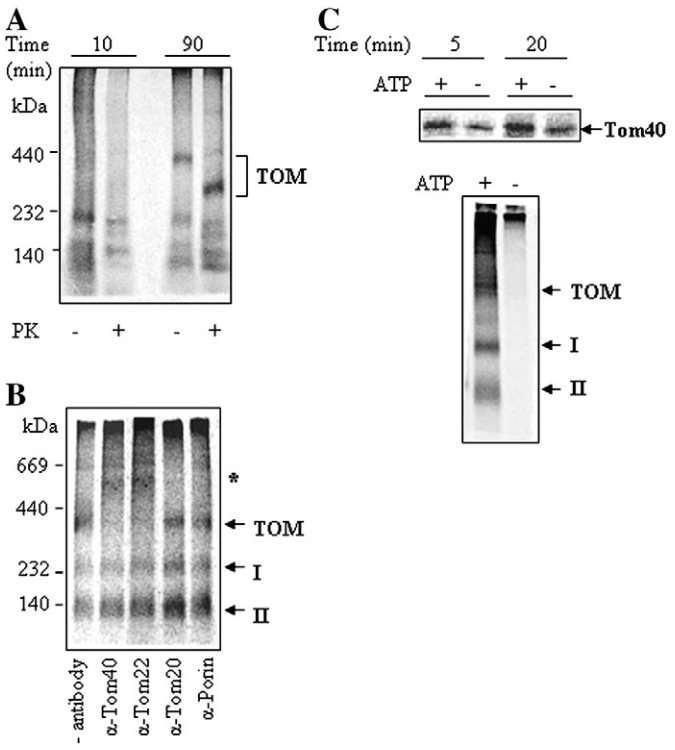

Fig. 2. Characterization of Tom40 assembly intermediates. A. Radiolabeled Tom40 was incubated with skeletal muscle mitochondria for 10 and 90 min. Samples were treated with proteinase K (n = 5), solubilized with digitonin and analyzed by BN-PAGE. B. Radiolabeled Tom40 was incubated with mitochondria for 90 min, solubilized in digitonin-containing buffer, and incubated with 5 μl of antibodies specific for Tom40, Tom22, Tom20, and porin, prior to BN-PAGE (n = 5). Supershift complexes are indicated by asterisks. C. Tom40 was imported into mitochondria for 5 and 20 min in the presence or absence of apyrase and subjected to SDS-PAGE (top). Import of Tom40 into mitochondria for 90 min, treated with apyrase, and reisolated with digitonin prior to BN-PAGE (bottom; n = 5).

3.2. Chronic contractile activity-induced adaptations in Tom40 import and assembly

To assess the adaptability of the TOM complex to chronic contractile activity, we measured Tom40 import and assembly into the outer

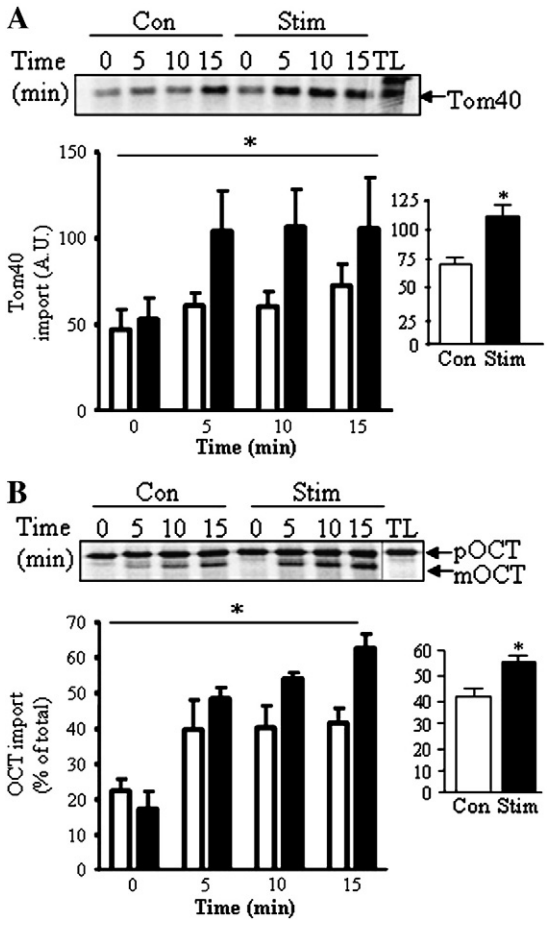

Fig. 3. Import of Tom40 and OCT precursors in isolated mitochondria following chronic contractile activity. Representative autoradiograms of Tom40 (*A*) and OCT import (*B*) in mitochondria from control (Con) and stimulated (Stim) skeletal muscle. Graphical representation of Tom40 and OCT import (n = 7–9) is shown below with the import of Tom40 expressed as arbitrary units (A.U.) and OCT as the amount of mature protein as a percentage of total available precursor. *$P<0.05$, main effect of time. Inset graphs on the right represent average values taken over the time course. *$P<0.05$, Stim vs. Con.

membrane, as well as the import of the liver matrix enzyme OCT which has previously been shown to be responsive to energy demanding perturbations (Craig et al., 1998). Tom40 and OCT import into mitochondria were increased progressively over time (Fig. 3A, B). Following 7 days of chronic contractile activity there was a further increase ($P<0.05$) in Tom40 and OCT precursor protein import over the 20 min time course (Fig. 3A, B). Radiolabeled Tom40 and OCT precursor protein import was 1.6-fold and 1.4-fold greater ($P<0.05$) in stimulated muscle, respectively, when compared to control (Fig. 3A, B).

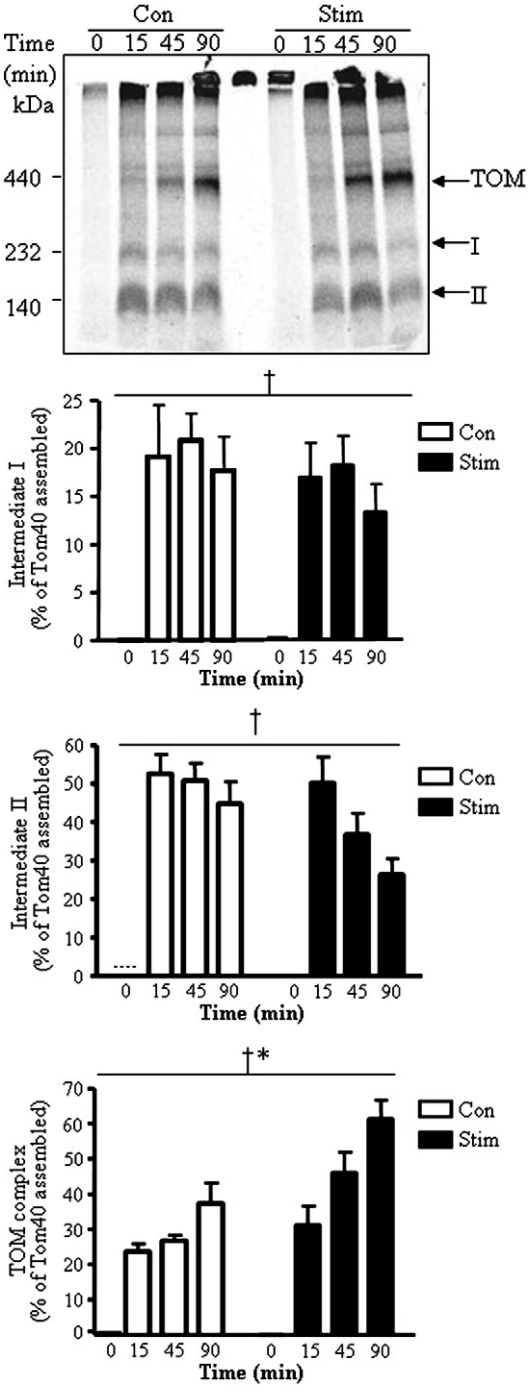

Fig. 4. Chronic contractile activity-induced changes in Tom40 assembly in isolated mitochondria. Radiolabeled Tom40 precursor import and assembly into its intermediate complexes was measured in skeletal muscle mitochondria isolated from control (Con) and stimulated (Stim) muscle at 0, 15, 45 and 90 min. Mitochondrial pellets were solubilized in digitonin buffer and subjected to BN-PAGE (n = 9). Graphs representing the percentage change of Tom40 incorporated into each of the intermediate complexes is shown. †$P<0.05$, main effect of time; *$P<0.05$, Stim vs. Con.

The adaptability of the Tom40 assembly pathway was then characterized in mitochondria isolated from control and stimulated skeletal muscle. We found that Tom40 assembly into intermediate I, II, and the TOM complex occurred in a time-dependent manner ($P<0.05$; Fig. 4). Incorporation of radiolabeled Tom40 into the final TOM complex was significantly greater (64%; $P<0.05$) in mitochondria from the stimulated muscle when compared to the control muscle over the 90 min import reaction (Fig. 4).

3.3. Enhanced levels of protein import machinery components with chronic contractile activity

The assembly of Tom40 into the outer membrane is dependent on preexisting components of the protein import pathway (Model et al., 2002). We therefore investigated potential regulatory factors that may be mediating the accelerated import and assembly of Tom40 observed in response to chronic muscle use. Interestingly, we found that Tom40, Tim17, and Tim23 protein levels were 1.6-fold, 3.3-fold and 1.6-fold higher ($P<0.05$) in mitochondria from stimulated muscle, respectively, when compared to control muscle (Fig. 5A). The involvement of the TIM complex in Tom40 import has recently been highlighted with the finding that small TIM proteins such as the Tim8/Tim13 complex and the Tim9/Tim12 complex target the Tom40 precursor to be assembled into the Intermediate I complex (Hoppins and Nargang, 2004; Wiedemann et al., 2004). Interestingly, Sam50 remained unaltered in response to chronic muscle use in isolated mitochondria (Fig. 5B). Furthermore, in the stimulated muscle, Tom22 levels were 2.1-fold greater ($P<0.05$) in the mitochondrial fraction when compared to mitochondria from control muscle (Fig. 5B). There was no effect of contractile activity on the levels of Tom34 protein (data not shown).

3.4. Importance of cytosolic components in Tom40 import and assembly

We next examined the cytosolic chaperone protein, Hsp90 which has previously been shown to be involved in Tom40 import (Humphries et al., 2005). Following chronic contractile activity, Hsp90 levels were 1.3-fold greater ($P<0.05$) at 7 days of stimulation, when compared to control (Fig. 5B). A 1.5-fold increase in the level of this chaperone was observed after only 5 days of stimulation (data not shown). These data suggest that the early inducibility of this protein may be an important event for Tom40 import and assembly in skeletal muscle. To determine the contribution of cytosolic components in the import of Tom40, the cytosolic fraction was isolated from control and stimulated skeletal muscle. The incubation of lysate with cytosol prior to the addition to mitochondria resulted in a significant acceleration ($P<0.05$) in the amount of radiolabeled Tom40 imported into to the outer membrane following 20 min of import (Fig. 6A). However, no difference was observed in Tom40 import between cytosol isolated from stimulated and control muscle, suggesting that increased levels of cytosolic chaperones found within stimulated muscle alone could not accelerate Tom40 import into mitochondria. The addition of novobiocin, a specific Hsp90 inhibitor, resulted in a 64% ($P<0.05$) reduction in the amount of Tom40 imported into the outer membrane, substantiating a role of this chaperone in mediating Tom40 import in skeletal muscle. To confirm the specificity of this import reaction, OCT import in the presence of cytosolic fractions was also investigated (Fig. 6B). In contrast to Tom40 import, the addition of cytosolic fraction exerted an inhibitory effect on OCT import, suggesting compartment-specific differences in the modulation of precursor protein import into mitochondria.

4. Discussion

Skeletal muscle mitochondria readily adapt to chronic conditions of muscle use and disuse. In particular, those mitochondria localized

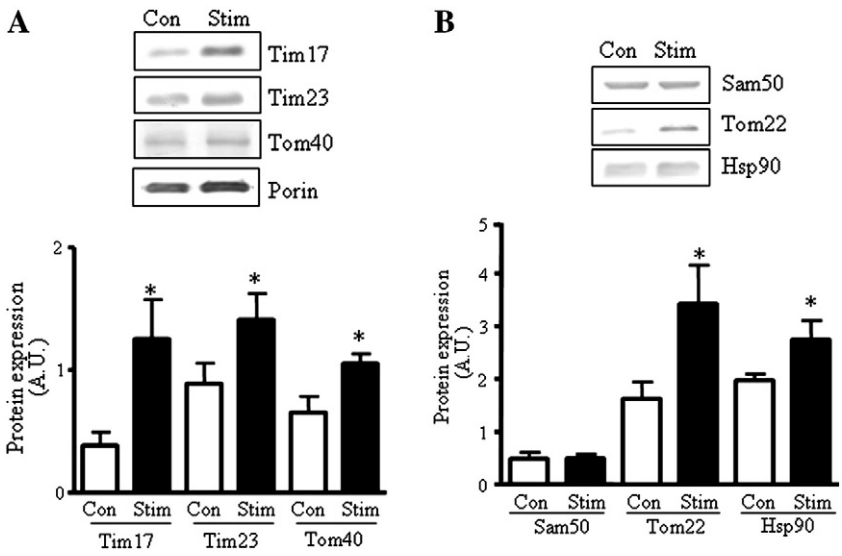

Fig. 5. Effect of chronic contractile activity on protein import machinery components. A. Western blot analyses of Tim17, Tim23, Tom40 and porin protein in isolated mitochondria from both control (Con) and 7 day stimulated (Stim) animals (n = 6). Graphical representation of the data is shown below. *P<0.05, Stim vs. Con. Porin was used as an appropriate control for loading of isolated mitochondria. B. Immunoblotting of Sam50 and Tom22 from isolated mitochondria and Hsp90 from cytosol of control (Con) and stimulated (Stim) muscle. Porin and ponceau staining was used as an appropriate control for loading. *P<0.05, Stim vs. Con.

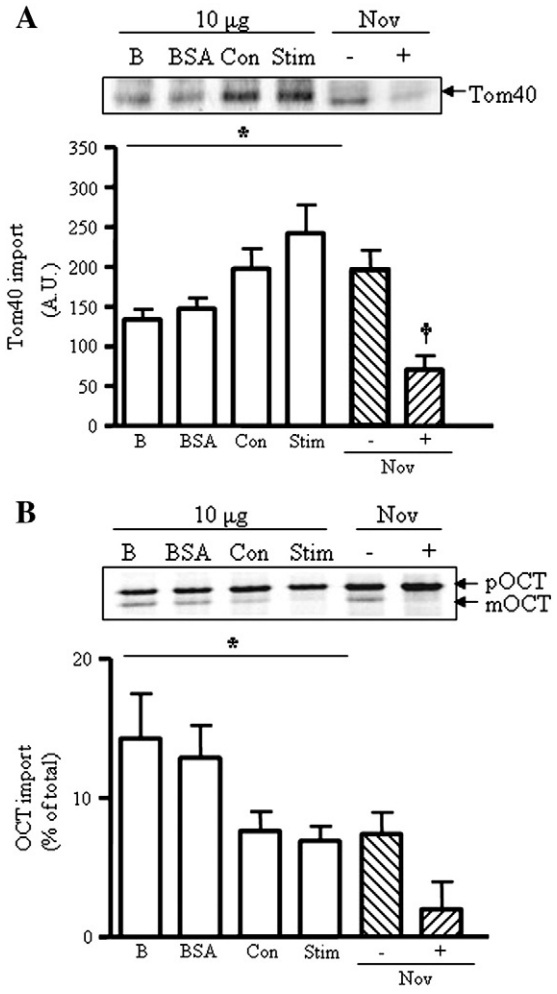

Fig. 6. Effect of cytosol on Tom40 import. Radiolabeled Tom40 (Fig. 6A) and OCT (Fig. 6B) in the presence of 10 µg of buffer (B), bovine serum albumin (BSA), cytosol from control animals (Con) and cytosol from stimulated animals (Stim) was imported into mitochondria for 20 min. In addition, mitochondria were incubated with lysate and control cytosol (10 µg) in the absence (−) or presence (+) of novobiocin (nov). Graphical representation of the data is shown below (n = 8), with values expressed as arbitrary units (A.U.; *P<0.05, main effect of treatment; †P<0.05, (+) Nov vs. (−) Nov).

near the plasma membrane, termed subsarcolemmal mitochondria, are particularly labile (Howald et al., 1985; Krieger et al., 1980; Martin, 1987; Riley et al., 1990). Thus, in order to investigate the potential plasticity of the protein import and assembly process, we chose to use this mitochondrial fraction. The protein import pathway is responsible for targeting nuclear-encoded proteins into the organelle, and is therefore essential for organelle assembly. The importance of the protein import pathway has been further exemplified with the finding of its adaptability to metabolic and genetic stress signals. Whether it is in response to chronic muscle use (Takahashi et al., 1998) or in patients cells with a mtDNA defect where mitochondrial function is compromised (Joseph et al., 2004; Rungi et al., 2002), precursor protein import into the organelle is enhanced to increase ATP production within the cell.

Mitochondrial protein import in skeletal muscle has primarily been examined in the context of proteins involved in cellular metabolism, and not in the actual import and assembly of proteins comprising organelle multisubunit complexes (e.g., TOM and TIM complexes, ETC complex). We recently showed that Tom40 import and assembly is altered in muscle with aging and specifically that Tom40 precursor protein assembly into the final TOM complex is higher in mitochondria from aged, compared to young animals (Joseph et al., 2010). These data suggest that the greater assembly rate of Tom40 in mitochondria from aged animals might be a compensatory mechanism to offset decrements in mitochondrial content observed in aging, and illustrate the potential importance of the Tom40 import and assembly process in skeletal muscle function. In this study, we wished to further investigate the biogenesis of the TOM complex in mammalian skeletal muscle mitochondria. Our purpose was to investigate the effects of chronic contractile activity on TOM complex assembly, as well as some of the mechanisms involved.

Similar to other precursor proteins, Tom40 is encoded in the nucleus and is then targeted and imported into the organelle via preexisting import machinery proteins (Dekker et al., 1998; Model et al., 2001). The biogenesis of Tom40 in skeletal muscle employs a pathway of import into the outer membrane, utilizing several assembly intermediate complexes before its final incorporation into the mature TOM complex. The ~230 kDa intermediate I complex most likely represents the Tom40 precursor bound to the SAM complex (Wiedemann et al., 2003). The lower molecular weight TOM complex that results with protease treatment may represent the cleavage of the cytosolic domain of Tom22,

which as shown in this study, forms part of the TOM complex in muscle. The import of precursor proteins is dependent on cytosolic chaperones such as Hsp70 and Hsp90 that maintain precursor proteins in an import competent state while delivering them to outer membrane receptor proteins (Hachiya et al., 1994; Mori and Terada, 1998). This was illustrated by the fact that cytosol isolated from muscle had a stimulatory effect on Tom40 import, implicating cytosolic factors in the targeting of Tom40 to the outer membrane. Inhibition of the ability of Hsp90 to bind to the precursor with novobiocin, markedly reduced Tom40 import into the outer membrane, confirming Hsp90 as one of the primary chaperone proteins responsible for Tom40 biogenesis in skeletal muscle. In addition, the depletion of ATP resulted in reduced import and assembly of Tom40 into the outer membrane. This is consistent with the energy-dependent nature of chaperone function in directing precursors to their organelle location. The divergent responses of matrix- (OCT), as compared to outer membrane (Tom40)-destined proteins to the effects of cytosol may be explained by several possibilities: 1) organelle compartment-specific differences in chaperone-mediated import, 2) variations in precursor protein targeting sequences affecting the affinity of cytosolic chaperones for the imported proteins (Craig et al., 1998; Terada et al. 1996), or 3) alternative degradation rates or stabilization of precursor proteins mediated by the cytosolic fraction, containing components of the ubiquitin-proteasome system or other proteases that can determine precursor protein availability (Wright et al., 2001). More research is required to investigate these possibilities.

A unique feature of skeletal muscle mitochondria is their ability to adapt to cellular demands that may arise from chronic muscle use or disuse. For example, using an in vivo animal model of chronic contractile activity, we have previously shown that following chronic muscle use, mitochondria have a greater capacity to import matrix-destined precursor proteins, and these adaptations are involved in the altered mitochondrial phenotype associated with chronic exercise (Takahashi et al., 1998). In this report, we show that Tom40 import and assembly dynamics can be enhanced by chronic contractile activity. Although the assembly of the Tom40 precursor is dependent on several factors within mitochondria, we believe that the increased assembly of Tom40 into the TOM complex is mediated, in part, by the chronic contractile activity-induced expression of endogenous levels of Tom22. Tom22 has been shown to be required for Tom40 assembly in mammalian cells. Overexpression of Tom22 has been shown to result in the accelerated import of Tom40 into its intermediate complexes (Humphries et al., 2005; Johnston et al., 2002; van et al., 1999). Interestingly, Sam50, a key component of the sorting and assembly machinery (SAM) located at the outer mitochondrial membrane (Wiedemann et al., 2003) has also been implicated in Tom40 assembly. Depletion experiments of Sam50 in HEK293T cells resulted in a lower amount of Tom40 assembled into the final TOM complex (Humphries et al., 2005). However, in our study we found no change in the level of Sam50 with chronic contractile activity, despite a clear acceleration of the assembly process with this treatment. Thus, either Sam50 exists in sufficient quantities within muscle mitochondria, or the essentiality of its role is cell type-specific.

Finally, Blesa et al. (2004, 2007, 2008) have shown that several import proteins including Tom20, Tom70, and Tom34 are transcriptionally activated by the well known mitochondrial biogenesis regulators nuclear respiratory factor (NRF)-1 and -2. The expression and DNA binding activity of these transcription factors is increased by exercise (Baar et al., 2002; Irrcher et al., 2003). This provides a link between exercise-induced signaling pathways and the adaptability of the protein import machinery. However, more work is required in this area to further elucidate upstream molecular factors regulating the protein import pathway during conditions of augmented mitochondrial biogenesis.

In conclusion, this study demonstrates that the adaptability of the TOM complex channel to chronic contractile activity suggests its importance as a potential regulator of the mitochondrial response that occurs during altered states of mitochondrial biogenesis. This finding has significant implications given the increasing reports of defects in protein import machinery components and their culmination in human disease (Agsteribbe et al., 1993; Devi et al., 2006; Koehler et al., 1999). Chronic contractile activity, therefore, through its ability to increase the expression of import machinery components and accelerate protein import and assembly may serve as a potential therapeutic modality for the treatment of mitochondrial-associated pathologies.

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