Original Paper

Roles of Exosome-Like Vesicles Released from Inflammatory C2C12 Myotubes: Regulation of Myocyte Differentiation and Myokine Expression

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Abstract
Background/Aims: The complicated differentiation processes of cells in skeletal muscle against inflammation that induce muscle atrophy are not fully elucidated. Given that skeletal muscle is a secretory organ, we evaluated the effects of inflammation on myogenic signals and myokine expression, and the roles of inflammatory exosomes released by myotubes in myogenic differentiation. *Methods:* Inflammation was induced by treatment of fully differentiated C2C12 myotubes with a cytokine mixture of TNF-α and INF-γ. Exosome-like vesicles (ELVs) were isolated from conditioned media of control or inflamed myotubes and incubated with myoblasts. The expression of molecular switches that contribute to myogenic differentiation, including several kinases, their downstream targets, and myokines, were evaluated using immunoblot analysis in inflamed myotubes and in myoblasts treated with ELVs. *Results:* Inflammation activated molecular mechanisms contributing to muscle atrophy, including AMPK, p-38 MAPK and JNK, while inhibiting Akt-mediated myogenic signals. In addition, inflammation induced myostatin expression with suppression of a myostatin-counteracting myokine, decorin. Well-characterized ELVs released from inflamed myotubes induced myoblast inflammation and inhibited myogenic mechanisms while stimulating atrophic signals. *Conclusion:* Inflammation of skeletal muscle induces muscle atrophy via

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multiple mechanisms, including the regulation of myokines and kinases. Inflammatory ELVs are likely to contribute to inflammation-induced muscle atrophy.

Introduction

Inflammation in insulin-sensitive organs, including white adipose tissue (WAT), skeletal muscle, and liver, plays key roles in the development of chronic metabolic diseases. WAT is a source of obesity-induced chronic inflammation [1]; however, it is not the only organ that develops macrophage accumulation in obesity. In fact, recent studies have challenged the causal role of WAT inflammation in obesity-linked metabolic dysregulation of the whole body [2, 3]. In addition, it was suggested that local inflammation and ectopic lipid accumulation in skeletal muscle leads to impaired insulin signaling [2]. Furthermore, inflammation in skeletal muscle and the accumulation of macrophages are observed not only in metabolic diseases but also in certain conditions, such as aged muscle [4], acute muscle damage by high-intensity eccentric exercise [5, 6], or chronic diseases (including cancer cachexia) [7], which leads to physical inactivity and aggravates the loss of skeletal muscle oxidative capacity, and *vice versa* [8]. In contrast, regular exercise with optimal intensity decreases the levels of inflammation in skeletal muscle and mitigates aging-induced muscle atrophy [2, 9]. Skeletal muscle is the largest organ of the body in nonobese individuals and is important in the regulation of whole-body metabolism. Skeletal muscle cells dynamically respond to physiological and/or pathological stimuli, including inflammation, leading to either skeletal muscle atrophy or hypertrophy. Myogenic differentiation consists of complicated events that determine the cell cycle, the characteristic pattern of skeletal muscle gene expression, and myotube formation. The commitment of myogenic satellite cells to myotube formation is regulated by multiple kinases, including mitogen-activated protein kinases (MAPKs), 5'-AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), and PI3K/Akt [10-14], and by muscle regulatory transcription factors, including MyoD and myogenin [15, 16].

During the past decade, several lines of evidence have shown that skeletal muscle is also a secretory organ: the secretion of muscle-cell-derived effector molecules, "myokines", may influence metabolism in tissues through intercellular communication with an endocrine effect [17]. Moreover, probably in an autocrine or paracrine fashion, myokines may regulate skeletal muscle metabolism and differentiation. Using proteomics approaches, several myokines have been identified in human myotubes [18-20]. Importantly, various myokines were found in secretory intercellular communicating vesicles, particularly in exosomes [21, 22]. In addition, myokines are closely associated with muscle atrophy and with the regulation of inflammation in skeletal muscle [23, 24]. However, the role of inflammation in the expression of myokines in skeletal muscle cells has not been fully elucidated. Furthermore, the effects of inflammatory exosomes on the myogenic differentiation of myoblasts have not been evaluated. Therefore, we hypothesized that inflammation alters myokine expression in skeletal muscle cells and that exosomes released from inflammatory muscle cells are responsible for suppressing muscle differentiation. Among myokines, in particular, it has been reported that decorin downregulates the activity of the anti-myogenic myostatin/Smad [25-27] and is associated with muscle regeneration [10], hypertrophy [28], and myogenic differentiation [29]. Therefore, we evaluated the effects of inflammation on the expression of decorin and myostatin and on the molecular switches of muscle differentiation using C2C12 myotubes.

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Materials and Methods

Cell culture and treatment

Murine myoblasts (C2C12) were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in humid air containing 5% CO_2 at 37°C. Monolayers of undifferentiated C2C12 myoblasts were kept in a proliferative state in DMEM with 10% FBS, and myoblasts at ~100% confluency (day 0) were differentiated into myotubes by switching the proliferation medium to differentiation medium (DMEM supplemented with 2% horse serum), as described previously [30]. The differentiation medium was changed daily until day 5 when myotubes were clearly observed. We used serum-free DMEM containing a cytokine mixture of interferon-γ (IFN-γ; R&D Systems, Minneapolis, MN, USA) and tumor necrosis factor-α (TNF-α; R&D Systems) to induce the inflammatory responses of myotubes [31]). Inflammatory exosome-like vesicles (ELVs) were enriched from the conditioned medium (CM) of myotubes that were treated with the cytokine mixture or vehicle for 24 h. Undifferentiated myoblasts were treated with serum-free DMEM with ELVs enriched from inflammatory CM or control CM for the indicated time (see below). For ELV treatment, culture media of C2C12 myoblasts at ~100% confluency were replaced every 24 h by serum-free media containing ELVs with 10 μg of exosomal proteins.

Enrichment of exosome-like vesicles

To enrich for ELVs, C2C12 myoblasts were plated on 100 mm dishes at a density of 1 x 10^6 cells and differentiated for 6 days as described above. After washing the C2C12 myotubes with serum-free DMEM, to clear exogenous ELVs in FBS, cells were treated with the cytokine mixture or phosphate-buffered saline (PBS) for 24 h in serum-free medium. We enriched ELVs by differential centrifugation of CM combined with microfiltration, as described previously [32]. Briefly, CM either from control or inflamed myotubes were collected from six 100 mm dishes per group and were centrifuged at 300 x g for 10 min at room temperature (RT), to discard dead cells and bulky cellular debris. Subsequently, the supernatants were centrifuged at 2000 x g for 10 min at 4°C. To remove contaminating apoptotic bodies, microvesicles, and small cell debris, supernatants were filtered through a Millex syringe filter fitted with a 0.22 μm membrane (Merck Millipore, Temecula, CA, USA). The filtrate was then subjected to 100, 000 x g centrifugation in a Type70 Ti rotor for 90 min at 4°C, to pellet ELVs. The supernatant was carefully removed, and pellets were washed with PBS by 100, 000 x g centrifugation for 70 min, resuspended in 200 μL of PBS and stored at –80°C until use. The purity of ELVs was determined by detecting the presence of an exosomal marker (Alix) and the absence of a nonexosomal marker (golgin subfamily A member 2; GM130) by immunoblot analysis.

Western blot

To prepare whole-cell lysates, cells were washed twice with ice-cold PBS, scraped into ice-cold radioimmunoprecipitation (RIPA) buffer (Sigma-Aldrich) containing protease inhibitors and phosphatase inhibitors (Sigma-Aldrich), and sonicated to homogenize the cell suspension. The cell debris of the homogenates was removed by centrifugation at 13, 000 x g for 10 min and total protein content were quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA). Aliquots of protein extracts (10–30 μg) were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane in transfer buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% methanol. The membrane was blocked with 5% bovine serum albumin or non-fat milk in Tris-buffered saline with 0.2% Tween 20 and incubated with specific primary antibodies (Table 1). After washing, membranes were incubated for 1 h at RT with horseradish-peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Pierce) and analyzed using image analysis software Quantity One® 4.6. The protein content in isolated ELVs was quantified by a BCA protein quantitation kit after 1:1 dilution with RIPA buffer, and myoblasts were treated with exosomes containing 10 μg of protein per 60-mm dish (3.3 microg/mL), as indicated. Ten micrograms of exosomal proteins were mixed with reducing sample buffer (50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 100 mM dithiothreitol,

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and 0.01% bromophenol blue), and proteins were resolved by SDS–PAGE, transferred to a nitrocellulose membrane, blocked with 5% non-fat milk in TBS-T (0.1% Tween 20), and probed with an anti-Alix antibody or an anti-GM130 antibody.

Characterization of ELVs by electron microscopy and dynamic light scattering analysis

ELVs were visualized using transmission electron microscopy (TEM) according to Lobb, et al. [32], with slight modifications. Briefly, two microliters of the fresh ELV solution without fixation was transferred onto Formvar-carbon-coated EM grids and allowed to dry at RT. Grids were then stained with 3% uranyl acetate for 10 min. Imaging was performed at an acceleration voltage of 75 kV using a CCD camera (5 x 5 k, Hamamatsu Photonics, Shizuoka, Japan) coupled to an H-7100 electron microscope (Hitachi High Technology, Tokyo, Japan).

The size distribution of ELVs was determined by dynamic light scattering (DLS) analysis. Aliquots of ELV suspensions containing 100 μg of protein were diluted in PBS to a total volume of 1 mL and 3 x 10 measurement runs were performed, with standard settings (Refractive Index = 1.331, viscosity = 0.89, temperature = 25°C). The shape of the particle size distribution and the intensity peak were measured to determine the agreement of ELVs with the known size distribution of exosomes (30–150 nm).

Statistics

All data are presented as means ± S.D. The effects of inflammation or inflammatory ELVs on the expression of proteins were compared via the Mann–Whitney U test or one-sample t-test,

Table 1. Antibodies used in this study

| Antibodies	Source	Dilution	Manufacturer	Catalog#
anti-AMPK	Rabbit	1:1000	Cell Signaling	#2532
anti-p-AMPK$_{Thr172}$	Rabbit	1:1000	Cell Signaling	#2535
anti- ACC	Rabbit	1:1000	Cell Signaling	#3662
anti-p-ACC$_{Ser79}$	Rabbit	1:1000	Cell Signaling	#3661
anti-iNOS	Rabbit	1:1000	Santa Cruz Biotechnology	Sc-651
anti-COX-2	Rabbit	1:1000	Abcam	ab15191
anti-raptor	Rabbit	1:1000	Cell Signaling	#2280
anti-p-raptor$_{Ser792}$	Rabbit	1:1000	Cell Signaling	#2083
anti-mTOR	Rabbit	1:1000	Cell Signaling	#2972
anti-p-mTOR$_{Ser2448}$	Rabbit	1:1000	Cell Signaling	#2971
anti- Akt	Rabbit	1:1000	Cell Signaling	#9272
anti-p-Akt$_{Ser473}$	Rabbit	1:1000	Cell Signaling	#9271
anti-P38MAPK	Mouse	1:1000	Cell Signaling	#9217
anti-p-P38MAPK$_{Thr180/tTyr182}$	Rabbit	1:1000	Cell Signaling	#9215
anti- SAPK/JNK	Rabbit	1:1000	Cell Signaling	#9252
anti-p- SAPK/JNK$_{Thr183/tTyr185}$	Rabbit	1:1000	Cell Signaling	#9251
anti-FOXO3a	Rabbit	1:1000	Cell Signaling	#2497
anti-p-FoxOI$_{Thr24}$/FoxO3a$_{Thr32}$	Rabbit	1:1000	Cell Signaling	#9464
anti-P70S6K	Rabbit	1:1000	Cell Signaling	#9202
anti-p-P70S6K$_{Thr389}$	Mouse	1:1000	Cell Signaling	#9206
anti-atrogin1	Mouse	1:1000	Santa Cruz Biotechnology	sc-166806
anti-myo-D	Rabbit	1:500	Santa Cruz Biotechnology	sc-304
anti-myogenin	Mouse	1:500	Santa Cruz Biotechnology	sc-12732
anti-decorin	Rabbit	1:500	Santa Cruz Biotechnology	sc-22753
anti-myostatin	Rabbit	1:500	Abcam	ab-71808
anti-Alix	Mouse	1:1000	Cell Signaling	2171
anti-GM130	Mouse	1:1000	BD Transduction	610822
anti-α-actinin	Rabbit	1:1000	Santa Cruz Biotechnology	sc-166524
anti-β-actin	Rabbit	1:10000	Prosci	PM-7547
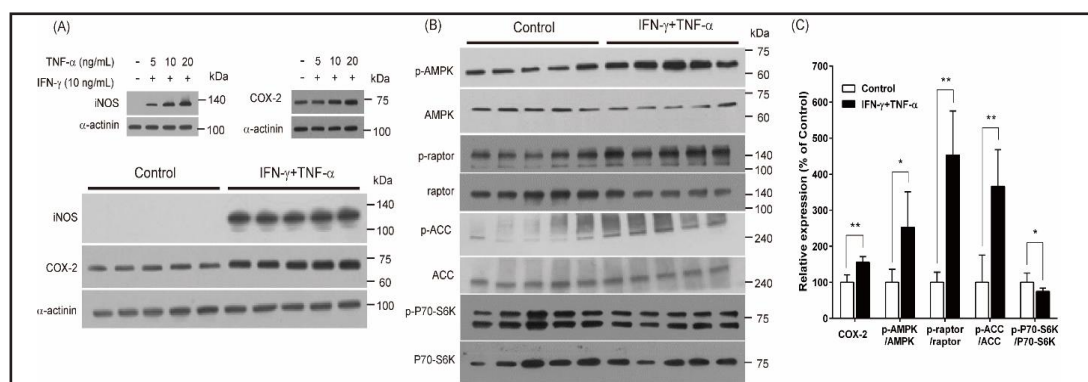
Fig. 1. Induction of cytokine-mediated inflammation with concurrent activation of AMPK signaling pathways in C2C12 myotubes. (A) Inflammation of C2C12 myotubes by treatment with a TNF-α and INF-γ mixture was evaluated by the induction of iNOS and COX-2 expression, which was dependent on the dose of TNF-α. (B, C) Treatment of myotubes with a cytokine mixture of IFN-γ (10 ng/mL) and TNF-α (20 ng/mL) significantly increased the level of p-AMPK and of its downstream effectors, p-ACC and p-raptor, while it significantly decreased the level of phosphorylated P70-S6K. Quantitation of the band densities showed statistical significance based on the Mann–Whitney U test. *p<0.05; **p<0.01 vs the control. Vertical bars indicate mean levels of protein expression relative to control group that were normalized to themselves to be 100% of mean value with S.D. regarding the variability in replicates.

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respectively, using the GraphPad Prism program version 6.0 (GraphPad Software, San Diego, CA, USA). We considered $P < 0.05$ as significant.

Results

Induction of inflammation activates AMPK signaling pathways in myotubes

As expected, when we treated C2C12 myotubes with a cytokine mixture of IFN-γ (10 ng/mL) and TNF-α (5–20 ng/mL) for 24 h, the expression of both inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) was dramatically increased compared with cells without treatment, indicating that the cytokine mixture successfully induced myotube inflammation (Fig. 1A). A slight induction of inflammatory proteins (iNOS and COX-2) at 4 h after treatment of cells with a cytokine mixture (TNF-α 20 ng/mL + IFN-γ 10 ng/mL) was also observed, but the induction was not apparent (data not shown); therefore, we selected 24 h as the time point of inflammation for further experiments. Given that AMPK is an important regulator of pathways associated with intracellular metabolism and inflammatory responses [33], we

Fig. 2. Effects of serum starvation on the levels of autophagy-related proteins. Differentiated C2C12 myotubes were treated with serum-free media for 4, or 24 hrs, and then proteins contributing to phagophore elongation in the autophagy pathway (Atg5 and Atg7), autophagy initiation which is activated by AMPK (ULK1), and marker of autophagosome (LC3) were evaluated, and found no significant activation of autophagy by serum starvation.

evaluated the effects of inflammation on AMPK activity and on its downstream molecules of mTORC1 [34]. Consistent with a previous study [35], the induction of inflammation in differentiated C2C12 myotubes significantly increased p-AMPK at Thr172 and its downstream molecules p-ACC at Ser79 and p-raptor at Ser792, which was accompanied by a decreased phosphorylation of P70-S6K at Thr421/Ser424 (Fig. 1B, C). The AMPK-mTOR pathway is also associated with autophagy, hence we tested whether autophgy signals are activated in myotubes treated with serum-free complete DMEM as compared with myotubes without serum starvation. However, we couldn't detect a significant activation of autophagy by serum starvation (Fig. 2), indicating that the effects of cytokines in serum-free media on AMPK pathway is likely to be induced by inflammation, not by activation of autophagy.

Effects of inflammation on myoblast differentiation and myokine expression

To evaluate the effect of inflammation on the molecules that are involved in muscle atrophy, we measured the expression of MAFbx (also known as atrogin-1 or F-box only protein 32), a key molecule in skeletal muscle atrophy [36] in inflamed myotubes. As shown in Fig. 3A, treatment of differentiated C2C12 with a cytokine mixture for 24 h increased the expression of MAFbx in a TNF-α dose-dependent manner (5–20 ng/mL), concurrent with iNOS induction. Conversely, the level of MyoD protein, a marker of myogenic differentiation, in myotubes treated with a cytokine mixture was significantly lower than that observed in vehicle-treated myotubes. Another marker of myogenic differentiation, myogenin, was slightly downregulated by inflammatory stimuli. In addition, the expression of decorin, a myokine with myogenic activity [10, 29], was suppressed in a dose-dependent manner (Fig. 3A).
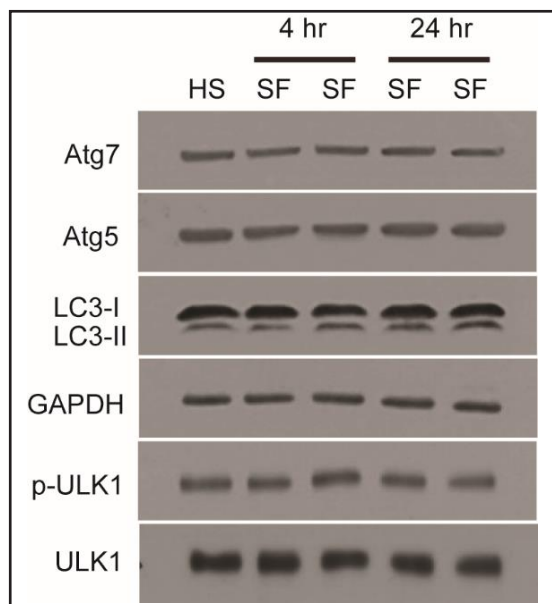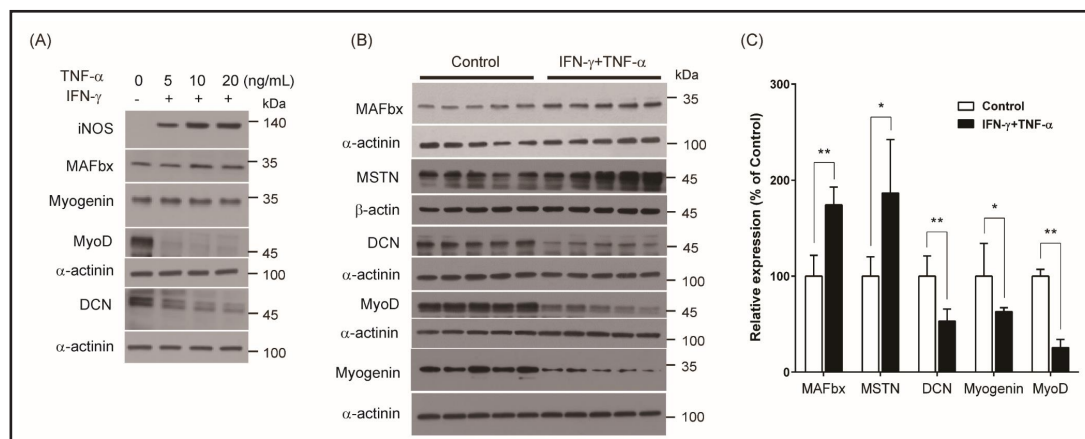
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Fig. 3. Inflammation of myotubes suppresses myogenic signals but increases the levels of proteins responsible for muscle atrophy. (A) Treatment of myotubes with a cytokine mixture remarkably suppressed the expression of Myo-D and decorin (DCN) but increased the level of MAFbx. A representative blot from a duplicate experiment is presented. (B, C) Induction of inflammation significantly increased the levels of myostatin (MSTN) and MAFbx, but significantly decreased the levels of decorin (DCN), MyoD, and myogenin. Quantitation of the band densities showed statistical significance based on the Mann–Whitney U test. *p<0.05; **p<0.01 vs the control. Vertical bars indicate mean levels of protein expression relative to control group that were normalized to themselves to be 100% of mean value with S.D. regarding the variability in replicates.

Given that myostatin, another molecule that is involved in muscle atrophy, is counteracted by decorin [25, 27, 37] and is closely associated with AMPK-mediated energy metabolism and atrophy [38-41], we evaluated the expression of myostatin in inflamed C2C12 myotubes. We found that the expression levels of myostatin and MAFbx in C2C12 myotubes was significantly increased, while the levels of decorin, MyoD, and myogenin were significantly decreased after treatment with a cytokine mixture for 24 h (Fig. 3B, C).

Effects of inflammation on p38-MAPK, JNK, and Akt phosphorylation in myotubes

Independently of the AMPK/mTOR signaling pathways, the activation of PI3K/Akt is crucial for muscle differentiation [42], while the c-jun NH2 terminus kinase (JNK) [43] and p38-MAPK [11] are anti-myogenic MAPKs. Therefore, we evaluated the effects of inflammation on these signaling pathways. As shown in Fig. 4, the levels of phosphorylated p38-MAPK and p-JNK were significantly higher in myotubes treated with a cytokine mixture than they were in the vehicle-treated control. In contrast, we observed a significant suppression of the expression of p-Akt at Ser473 and its downstream p-FOXO3a at Thr24/32 and p-mTOR at Ser2448 in inflamed myotubes compared with the controls (Fig. 4).

Exosome-like vesicles released from inflamed myotubes inhibit expression of MyoD and myogenin

We confirmed that the 100, 000 x *g* pellets were enriched with exosomes via the by detection of an exosomal marker and exclusion of a non-exosomal marker through immunoblotting, TEM imaging, and analysis of the size distribution of ELVs by DLS. The expression of Alix in the ELV fraction was clearly observed, and the level of Alix in ELVs from inflamed myotubes was higher than that detected in control ELVs; in addition, we did not detect GM130, a non-exosomal protein, in the two types of ELVs (but found it in cell lysate) (Fig. 5A). Furthermore, the size distribution of particles in the ELV suspension (z-ave = 145.9 ± 25.6 nm), with a peak intensity around 90 nm, was compatible with the known size distribution of exosomes (Fig. 5B). A morphological analysis using TEM also revealed a heterogeneous population of vesicles with a heterogeneous diameter size ranging from 30 to 120 nm and round or cup-shaped particles (Fig. 5C). To evaluate the effects of ELVs from
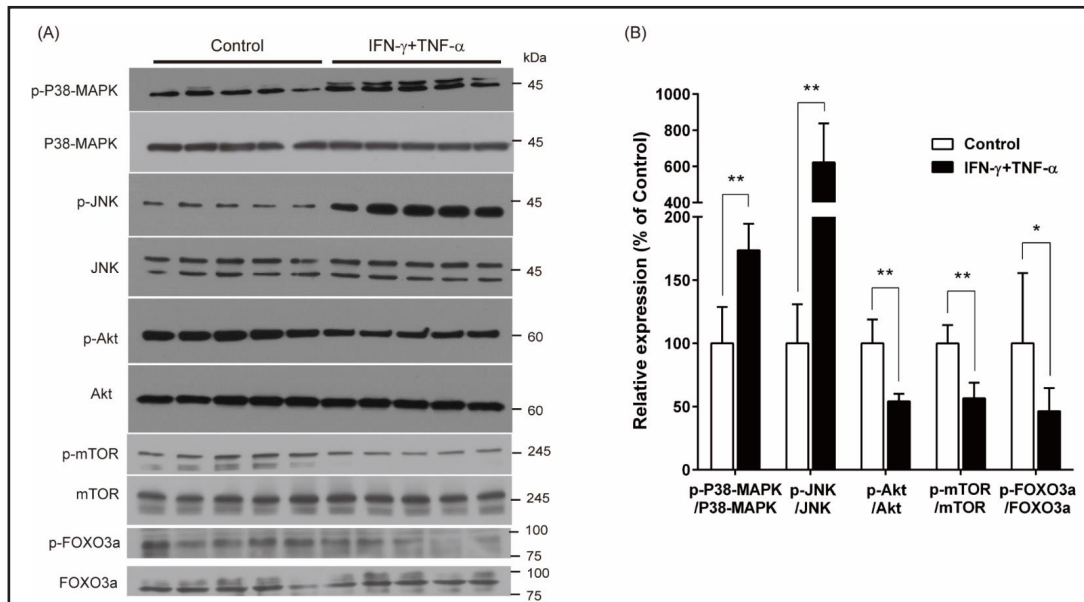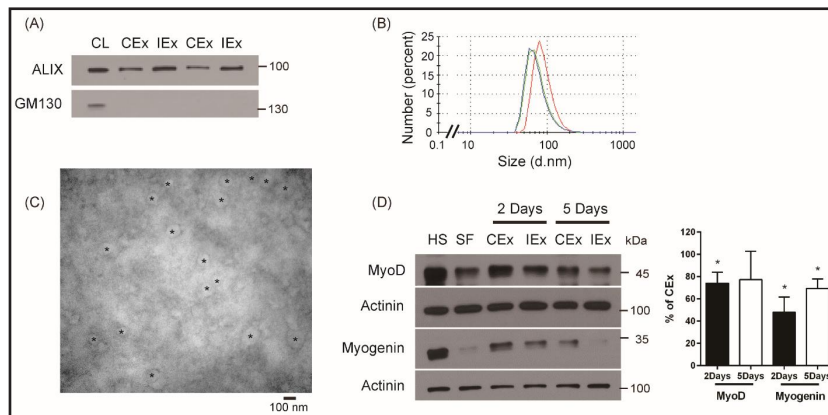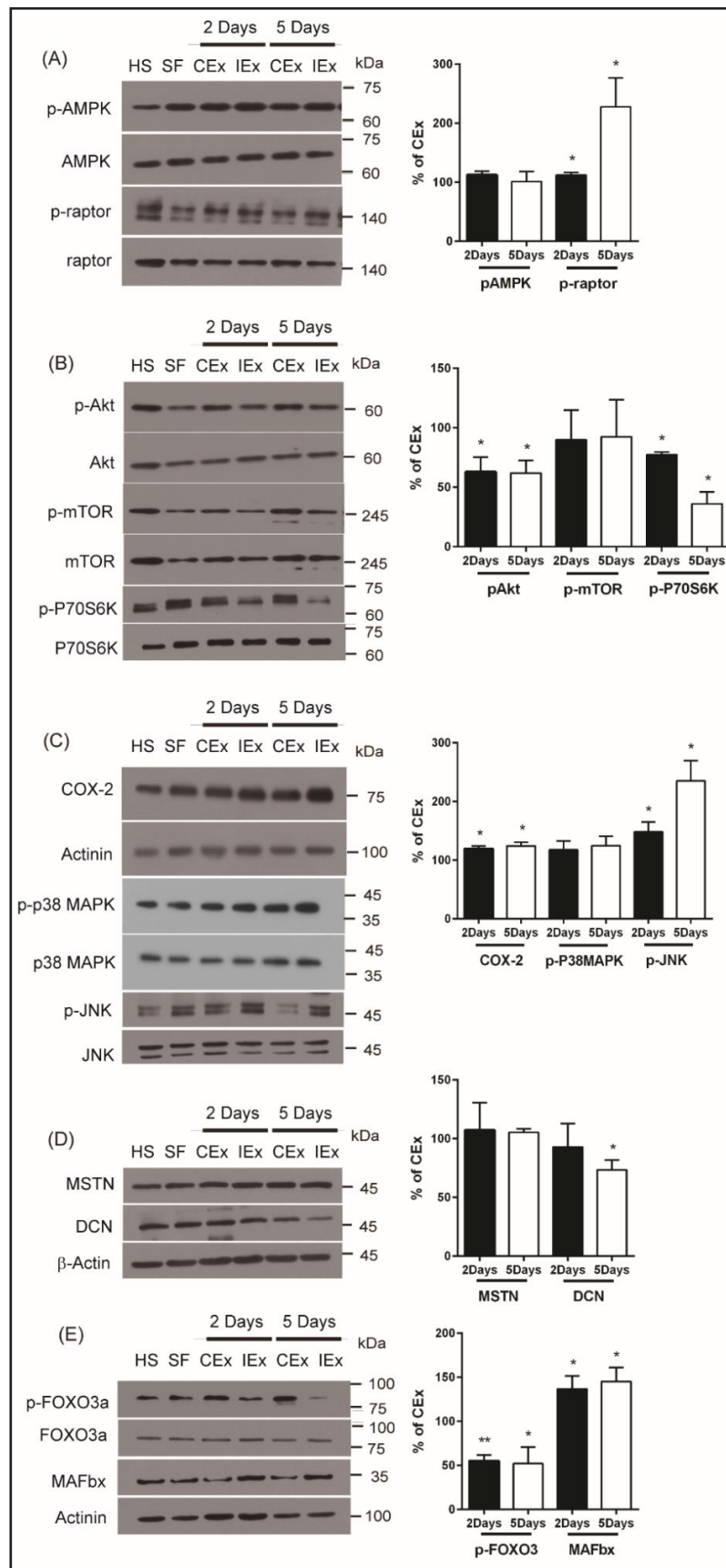
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Fig. 4. Effects of inflammation on the phosphorylation of anti-myogenic MAPKs, and p-Akt-mediated phosphorylation of mTOR and FOXO3a. (A) Immunoblots of anti-myogenic MAPK phosphorylation and Akt-mediated myogenic signaling. Treatment of myotubes with a cytokine mixture dramatically increased MAPK activity, while it significantly inhibited Akt-mediated pathways. Inhibition of the Akt-mediated phosphorylation of FOXO3 by cytokines may cause MAFbx upregulation (Fig. 2B and 2C). (C) Quantitation of the band densities showed statistical significance based on the Mann–Whitney U test. *p<0.05; **p<0.01 vs the control. Vertical bars indicate mean levels of protein expression relative to control group that were normalized to themselves to be 100% of mean value with S.D. regarding the variability in replicates.

Fig. 5. Characterization of exosome-like vesicles (ELVs) and the effects of ELVs released from myotubes on the levels of proteins associated with myoblast differentiation. (A) ELVs prepared from the conditioned medium of control myotubes (CEx) or inflamed myotubes (IEx) by differential centrifugation exhibited expression of an exosome marker (Alix) and absence of a non-exosomal marker (GM130). Ten micrograms of exosomal proteins were separated using SDS–PAGE followed by immunoblot, and a representative blot from duplicate experiments is shown. (B) Size distribution of ELVs measured by dynamic light scattering analysis. The size distribution showed a diameter that was within the range of that of exosomes (30–150 nm). We measured the size distribution of ELVs prepared from two independent experiments and obtained a similar result. (C) TEM imaging of ELVs with negative staining showed vesicles with a heterogeneous size of 30–120 nm (asterisks). A representative image from triplicate experiments is shown. (D) Effects of treatment with CEx- or IEx-containing serum-free DMEM on the level of the myogenic proteins myoD and myogenin. α-Actinin was used as a loading control. A representative blot from duplicate experiments is shown. *p<0.05 versus the levels in CEx-treated myoblasts (n=3, one-sample t-test). HS, myotubes fully differentiated by treatment with 2% horse serum for 5 days; SF, myoblasts treated with serum-free DMEM for 5 days.

Fig. 6. Effects of exosome-like vesicles (ELVs) released from myotubes on the molecular pathways related to myogenesis. Effects of control or inflammatory exosome-like vesicles on (A, B) the activation of the AMPK and Akt pathways, which are associated with myoblast differentiation; (C) the level of cyclooxygenase 2 (COX-2) and the phosphorylation of p38 MAPK and JNK; (D) the expression of myostatin (MSTN) and decorin (DCN); and (E) the FOXO3a-mediated MAFbx expression. α-Actinin and β-actin were used as a loading control. Representative blots from triplicate experiments are presented. HS, myotubes differentiated by treatment with 2% horse serum for 5 days; SF, myoblasts treated with serum-free DMEM for 5 days; CEx, myoblasts treated with control ELVs; IEx, myoblasts treated with inflammatory ELVs. *p<0.05; **p<0.01 versus the levels in CEx-treated myoblasts (n=3, one-sample t-test).

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inflamed myotubes, we treated myoblasts grown in 3 mL of serum-free media with exosomes containing 10 μg of proteins that were quantified by BCA assay. Interestingly, the levels of MyoD and myogenin in myoblasts treated with ELVs released from control myotubes (CEx) for 2 or 5 days were higher than those observed in cells treated with serum-free media for 5 days, indicating that CEx might induce myoblast differentiation [44]. The levels of MyoD and myogenin in myoblasts treated with CEx for 5 days were lower than those in 2-day-treated myoblasts, indicating that the relative effect size of serum starvation for different duration is larger than that of CEx treatment. Treatment of C2C12 myoblasts with ELVs released from inflamed myotubes (IEx) led to a decrease in the levels of both MyoD and myogenin compared with myoblasts treated with CEx or fully differentiated myotubes (Fig. 5D).

Effects of exosome-like vesicles on signaling pathways associated with differentiation

To test the anti-myogenic effects of inflammatory ELVs, we evaluated myogenic signals in ELV-treated myoblasts. When we treated myoblasts with CEx or IEx for 5 days, although p-AMPK levels were comparable, but p-raptor levels in IEx-treated myoblasts were 2.2-fold higher than those in CEx-treated cells (Fig. 6A). In contrast, p-Akt and p-P70S6K levels were significantly downregulated by IEx treatment compared with myoblasts treated with CEx, and p-mTOR levels in IEx-treated cells were slightly lower than CEx-treated cells (Fig. 6B). Compared with fully differentiated myotubes that were induced by 2% horse serum for 5 days, cells treated with CEx for 5 days showed slightly higher levels of p-AMPK, while the levels of phosphorylation of Akt and mTOR were comparable. The level of p-raptor in myoblasts treated with CEx for 2 days was comparable, while it was lower after 5 days than the level of fully differentiated myotubes. However, cells treated with IEx for 5 days showed lower levels of p-Akt and p-mTOR, but higher levels of p-AMPK compared with those observed in fully differentiated myotubes. We found that the levels of COX-2 in myoblasts treated with IEx for both 2 and 5 days were significantly higher than those detected in matched CEx-treated myoblasts or fully differentiated myoblasts. Considering the anti-myogenic MAPK pathways, myoblasts treated with IEx for 5 days showed the significantly higher level of p-JNK than that of CEx-treated myoblasts or fully differentiated myotubes (Fig. 6C). Furthermore, the expression level of decorin in myoblasts treated with IEx for 5 days was slightly but significantly lower than that detected in the matched CEx-treated myoblasts, but not in those treated for 2 days, while the levels of myostatin in IEx-treated myoblasts were not significantly different from those of CEx-treated cells (Fig. 6D). In addition, myoblasts treated with IEx for 5 days exhibited a reduced level of p-FOXO3a at Thr24/32, which is a target of Akt and causes upregulation of MAFbx [45], compared with CEx-treated myoblasts. Concurrently, the levels of MAFbx in IEx-treated myoblasts were significantly higher than those in CEx-treated cells (Fig. 6E).

Discussion

Inflammation of skeletal muscle has been implicated as a cause of the metabolic dysfunction and muscle atrophy that occur as a result of aging [46] and chronic diseases [7, 47], which are mediated by the elevation of pro-inflammatory cytokines. In contrast, exercise-induced, acute, localized, and transient inflammation promotes muscle repair and growth through, at least in part, the action of myokines [48]. In this study, we demonstrated that inflammation induced by treatment with a cytokine mixture turned on the molecular switches of muscle atrophy, but suppressed myogenic signaling. More importantly, ELVs from myotubes with inflammation is likely to contribute to the suppression of myogenic signaling pathways. In the face of stress or damages, an appropriate positive balance between the proliferation and differentiation of myogenic satellite cells in muscle against the loss of myocytes maintains the structure and function of skeletal muscles [49]. Our results are in agreement with previous studies [50, 51] in that inflammation can accelerate muscle atrophy in certain pathological conditions via mechanisms that inhibit both myogenic signals

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and energy-consuming protein synthesis. Recent studies provided evidence that myokines regulate myogenic differentiation and metabolism in skeletal muscle [27, 28, 52]. Consistently, our results clearly supported the contention that inflammation can lead to muscle atrophy, at least in part through the alteration of myokine expression; i.e., upregulation of myostatin associated with AMPK activation [41] and downregulation of counteracting decorin expression [27, 28]. Therefore, although this cause–effect relationship should be clarified in further studies, the myogenic vs muscle atrophic effect of decorin and myostatin, respectively, may contribute to the inhibition of myogenesis by inflammation. Although pharmacological activation of AMPK is a strategy that has been used to reinforce anti-inflammatory mechanisms [53], AMPK activation can suppress muscle differentiation and contribute to muscle atrophy [40, 54]. Therefore, the consequences of activation of AMPK by muscular contraction or by cytokine-induced inflammation for muscle differentiation might be different. In addition to the AMPK- and myokine-associated inhibition of myogenesis, the cytokine-induced alteration of multiple myogenic signaling pathways observed in our model, including the activation of p38-MAPK [11] and JNK [43] and the inhibition of Akt [45], may contribute to the suppression of myogenesis by inflammation. The activation of AMPK and inhibition of Akt leads to the inhibition of mTOR-mediated myogenesis [13, 14], which may also negatively regulate myogenesis in inflamed muscle.

To the best of our knowledge, this is the first report that ELVs from inflamed myotubes significantly inhibited myogenic signals. Although we cannot exclude the possibility that the preparation of ELVs by differential ultracentrifugation of CM was contaminated with non-exosomal extracellular vesicles, the characterization of ELVs by DLS analysis, western blot analysis, and TEM imaging showed a high purity of these exosomes (Fig. 5). Exosomes contain numerous molecules that are responsible for intercellular communication, including proteins, microRNAs, and lipids [55]. Decorin, but not myostatin, has been detected in human and mouse extracellular vesicles
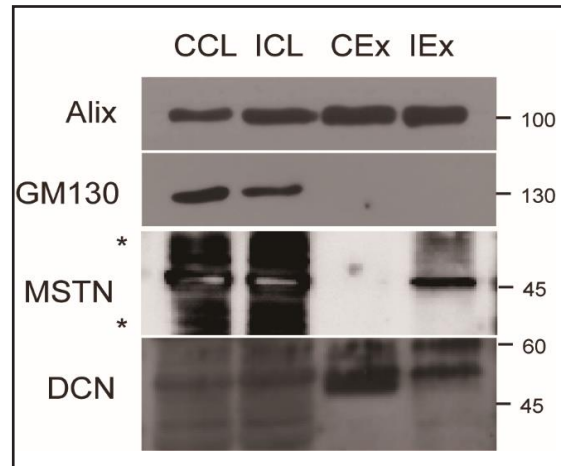

Fig. 7. Immunoblots of myostatin and decorin in exosome-like vesicles released from control or inflamed myotubes. The exosomal marker Alix was observed in cell lysates (CL) and exosome-like vesicles released from control myotubes (CEx) or inflamed myotubes (IEx), but non-exosomal protein GM130, a golgi matrix protein was not observed in exosomal fraction. In CEx, we observed the presence of decorin (DCN), but not myostatin (MSTN), however, both myokines were observed in IEx. Asterisks indicate nonspecific bands. CCL; control cell lysate, ICL; inflammatory cell lysate
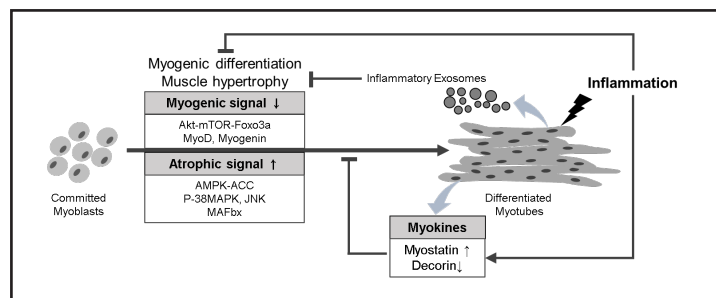

Fig. 8. Schematic summary of the mechanisms of inflammation-induced inhibition of myogenesis in C2C12 cells. Inflammation induced by cytokine treatment inhibits various myogenic signals while it activates atrophic signals and alters the expression of myokines related to myogenesis. In addition, exosome-like vesicles released from inflamed myotubes contribute to anti-myogenesis. Through multiple mechanisms, inflammation inhibits myoblast differentiation to myotubes, which leads to muscle atrophy.

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(www.exocarta.org and www.microvesicles.org). However, we detected myostatin in ELVs from inflammatory C2C12 myotubes, but not in control ELVs. The levels of decorin in both CEx and IEx were very low, but inflammation is likely to decrease the level of decorin in ELVs slightly (Fig. 7). Therefore, the possibility that the alteration of decorin and myostatin in ELVs contributes to the inhibition of myogenesis by inflammation in C2C12 could not be excluded. However, the biological roles of myostatin and decorin in inflammatory ELVs in myogenesis should be evaluated further, as we observed only a slight alteration in the levels of these myokines in myoblasts treated with inflammatory ELVs (Fig. 6). In addition, the possibility that unidentified proteins, miRNAs, or lipids in ELVs from inflammatory myotubes regulating myogenesis and myokine expression could not be ruled out [56-58]. Interestingly, our results suggested that ELVs released from myotubes can contribute to the activation or maintenance of muscle differentiation [44]; however, ELVs from inflammatory myotubes might hinder the process of differentiation (Figs 5D and 6). Although the molecular components that regulate myogenesis in ELVs released from myotubes should be further identified, our results provided, at least in part, evidence that inflammatory ELVs contribute to the inhibition of myoblast differentiation via multiple mechanisms, including disinhibition of the Akt-mediated suppression of FOXO3-MAFbx transcription, activation of the AMPK, p38-MAPK, and JNK signaling pathways, and induction of inflammation (Fig. 8).

In conclusion, our results clearly demonstrated that cytokine-induced inflammation significantly inhibited myogenic signaling and induced proteins responsible for muscle atrophy in a cellular model. The inflammation-induced inhibition of myogenic signals may be associated with an alteration in the levels of myokines and in the AMPK-, Akt-, and MAPK-mediated pathways. Given the results that ELVs released from inflammatory myotubes inhibited myogenic signals, further characterization of the molecular components of ELVs is warranted for the elucidation of the mechanisms underlying inflammation-induced muscle atrophy.

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Disclosure Statement

No conflict of interests exists.

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