Research article

Itaconate and its derivatives repress C2C12 myogenesis

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Abstract

A Krebs cycle intermediate metabolite, itaconate, has gained attention as a potential antimicrobial and autoimmune disease treatment due to its anti-inflammatory effects. While itaconate and its derivatives pose an attractive therapeutic option for the treatment of inflammatory diseases, the effects outside the immune system still remain limited, particularly in the muscle. Therefore, we endeavored to determine if itaconate signaling impacts muscle differentiation. Utilizing the well-established C2C12 model of *in vitro* myogenesis, we evaluated the effects of itaconate and its derivatives on transcriptional and protein markers of muscle differentiation as well as mitochondrial function. We found itaconate and the derivatives dimethyl itaconate and 4-octyl itaconate disrupt differentiation media-induced myogenesis. A primary biological effect of itaconate is a succinate dehydrogenase (SDH) inhibitor. We find the SDH inhibitors dimethyl malonate and harzianopyridone phenocopie the anti-myogenic effects of itaconate. Furthermore, we find treatment with exogenous succinate results in blunted myogenesis. Together our data indicate itaconate and its derivatives interfere with *in vitro* myogenesis, potentially through inhibition of SDH and subsequent succinate accumulation. More importantly, our findings suggest the therapeutic potential of itaconate and its derivatives could be limited due to deleterious effects on myogenesis.

Keywords: C2C12, myogenesis, itaconate, succinate, succinate dehydrogenase

Introduction

It is well appreciated that dramatic metabolic reprogramming occurs in response to inflammatory challenges. One of the most robust metabolic adaptations made in myeloid cells in response to inflammatory cues is the diversion of cis-aconitate away from α-ketoglutarate towards the synthesis of itaconate (1,2). One primary function itaconate serves is to reduce inflammation via the inhibition of succinate dehydrogenase (SDH) thereby reducing accumulation of reactive oxygen species (ROS) (1). Due to its potent anti-inflammatory properties, itaconate and its derivatives such as dimethyl itaconate (DMI) and 4-octyl itaconate (4-OI) have gained attention as potential treatments for autoimmune conditions such as psoriasis (3), multiple sclerosis (4), rheumatoid arthritis (5), and systemic lupus erythematosus (6).

While the signaling attributes of itaconate with regards to immune function are well delineated, not much is known on the actions of itaconate in non-immune organs. What is known is that itaconate possesses anti-inflammatory, anti-fibrotic and pro-survival attributes in organs such as the liver (7) and kidney (8). However, the effects of itaconate regulation of muscle function is yet unknown.

Given that chronic inflammation is hypothesized to incur sustained itaconate production, coupled to its potential therapeutic use for the treatment of a variety of diseases, it is of the upmost importance to understand potential off target tissues and effects in the face of elevated itaconate levels. To address this critical gap in knowledge we utilized the well-established C2C12 murine myoblast model system for *in vitro* myogenesis and studied the effects of itaconate and its derivatives on altering myoblast differentiation.

Results

Itaconate derivatives inhibits myogenesis in C2C12 cells

Past research has shown that Krebs cycle metabolites serve as signal transducers in the immune response within macrophages (9). We endeavored to evaluate how the presence of itaconate affects myogenic differentiation within C2C12 cells. Given its recent focus as a potential treatment for autoimmune conditions, the function of dimethyl itaconate (DMI) was first investigated. Gene expression markers for myogenesis (i.e. *Myh1*, *Myh2*, *Myh7*, *Tnnt1*, and *Tnnt3*) were measured in the presence of DMI versus vehicle over a 4-day differentiation protocol. As expected, vehicle group cells had a time-dependent increase in the transcription of myogenic genes (Fig. 1a). However, treatment with DMI significantly inhibited the induction of these myogenic markers during our differentiation protocol (Fig. 1a). Another analog of itaconate was also utilized since DMI cannot be endogenously converted back to intracellular itaconate (10). 4-octyl itaconate (4-OI) was subsequently used because it has been suggested as a possible treatment for autoimmune conditions, but unlike DMI, it can be endogenously converted to itaconate (10,11). We analyzed C2C12 cells treated with 4-OI over 4 days in the presence of differentiation media (DM). 4-OI significantly inhibited the induction of myogenic gene transcription as well (Fig. 1b).

To further determine how DMI and 4-OI impairs myogenesis, we next assessed the expression of the essential myogenic transcription factors *Myog* and *Myod.* The expression of *Myog* mRNA was significantly impaired by DMI throughout day 1 to day 4 of differentiation compared to the control (Fig. 1c). Transcription of *Myog* was also blunted in response to 4-OI exposure (Fig. 1d). Protein levels of MYOD were not different in the

DMI groups compared to the control in response to DM. However, MYOG expression was almost completely abolished by DMI (Fig. 1e). Consequently, DMI-treated C2C12 myoblasts failed to differentiate into myotubes. DMI-treated groups showed no trace of MYHC in response to DM cues, while vehicle-treated cells displayed a time-dependent increase in MYHC protein (Fig. 1e). 4-OI significantly blocked MYOG and MYHC expression levels although it did not change MYOD levels (Fig. 1f). MYHC was visualized by immunofluorescence at day 4 of differentiation (Fig. 1g) and myogenic index was significantly impaired by DMI (Fig. 1h) and 4-OI (Fig. 1i).

Fig. 1.

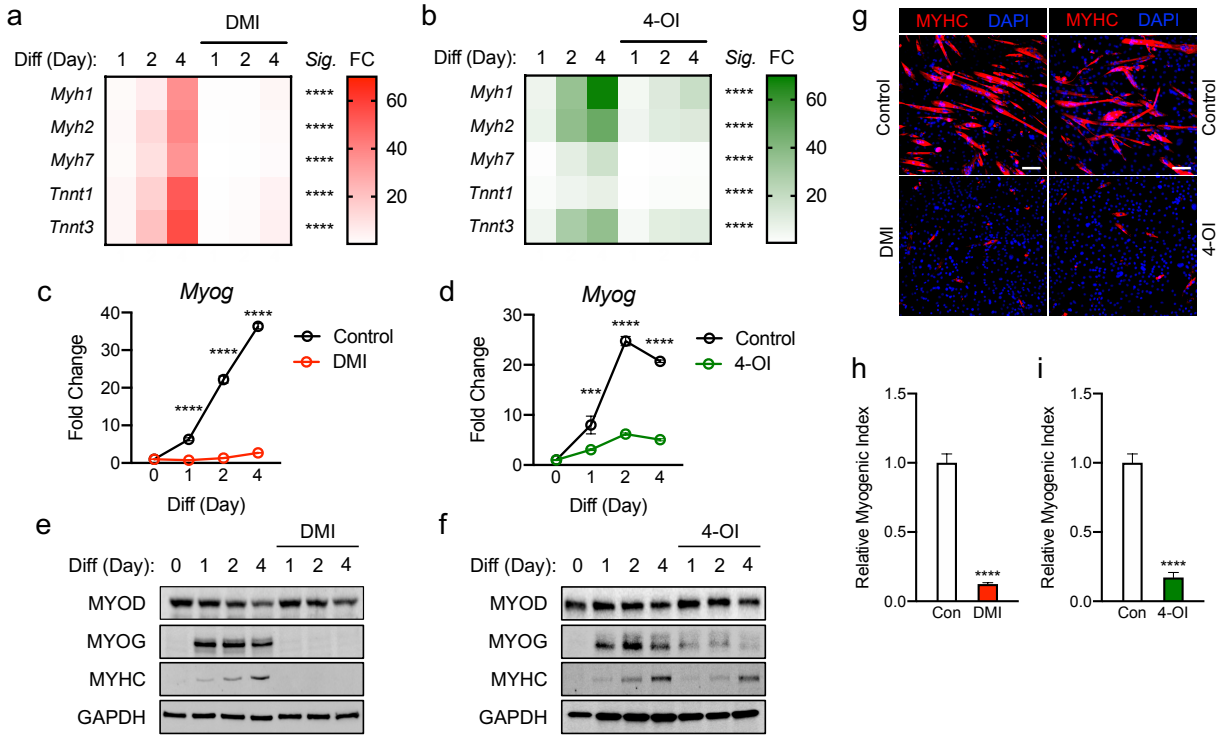

Fig. 1. Itaconate derivatives represse C2C12 myogenesis by modulating myogenic transcription mechanisms. Heatmap depiction of average fold change (*Sig.* denotes statistical differences at day 4, n = 3) in myogenic gene transcription levels detected via RT-qPCR when exposed to differential media with or without (a) dimethyl itaconate (DMI: 125 μM) and (b) 4-octyl itaconate (4-OI: 125 μM) relative to the untreated group over a 4-day time course. Fold change of *Myog* mRNA expression at various time points comparing differentiation media (DM) control groups to (c) DMI and (d) 4-OI groups relative to expression without either treatment (n = 3). Western blotting data showing differences in myogenic transcription factor expression between DM treated groups and groups treated with DM plus (e) DMI and (f) 4-OI over a 4 day time course as well as groups which were not exposed to either treatment for any period. (g) MYHC staining at day 4 of differentiation. Bar: 100 μm. Relative myogenic index (percentage of total nuclei associated with myotubes) of (h) DMI and (i) 4-OI. *** denotes $p < 0.001$, **** $p < 0.0001$ based on two-way ANOVA/t-test.

Physiological itaconate inhibits myogenesis

To investigate a role of physiological itaconate, C2C12 myoblast cells were then treated with DM only or DM plus itaconate and then examined over 4 days. It was reported that exogenous itaconate readily enters cells (9). Itaconate was used to portray physiological mechanisms more accurately. Transcription levels of *Myh2* and *Myh7* were not significantly impacted by itaconate exposure. However, the blunted induction of *Myh1*, *Tnnt1*, and *Tnnt3* was significant on the 4th day of exposure (Fig. 2a). Significant inhibition of *Myog* transcription on days 2 and 4 was also observed in itaconate groups (Fig. 2b). MYOG and MYHC expression were also inhibited in itaconate groups (Fig 2c). Itaconate treatment reduced MYHC staining (Fig. 2d) and myogenic index (Fig. 2e).

Overall DMI shows the strongest myogenic inhibitory response and itaconate has the weakest amount of myogenic suppression among itaconate and derivatives tested. Taken together, these results suggest that itaconate and its derivatives are sufficient to inhibit myogenesis in C2C12 cells, modulating myogenic regulating factors at the transcription and protein levels.

Malonate and pharmacological succinate dehydrogenase inhibitor obstruct myogenesis

Given that both itaconate and malonate are Krebs cycle metabolites that are shown to inhibit SDH (1,12), our next step was to determine whether malonate exposure could also inhibit C2C12 myogenesis. Dimethyl malonate (DMM) was administrated instead of itaconate under similar parameters to those discussed in Figures 1 through 2.

Fig. 2.

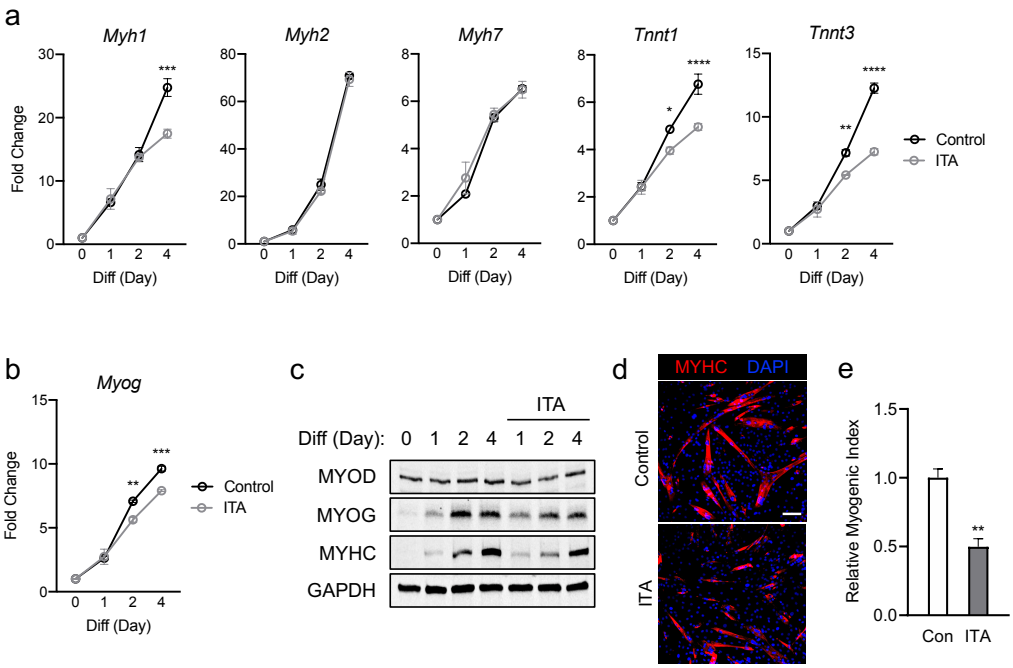

Fig. 2. Itaconate represses C2C12 myogenesis by modulating myogenic transcription mechanisms. (a) 4-day time course showcasing fold change differences of myogenic gene transcription levels detected via RT-qPCR when exposed to differential media with or without itaconate (7.5 mM) relative to the untreated group (n = 3). (b) Fold change of *Myog* mRNA expression at various time points comparing DM control groups to DM and itaconate groups relative to expression without either treatment (n = 3). (c) Western blotting data showing differences in myogenic transcription factor expression between DM treated groups and groups treated with both DM and itaconate over a 4 day time course as well as groups which were not exposed to either treatment for any period. (d) MYHC staining at day 4 of differentiation. Bar: 100 μm. (e) Relative myogenic index (percentage of total nuclei associated with myotubes). * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ based on two-way ANOVA/t-test.

Malonate inhibited the induction of myogenic markers during myogenesis (Fig. 3a). This inhibition was more severe than itaconate but not as extreme as the inhibition caused by itaconate derivatives. In parallel, a potent and specific inhibitor of SDH, which is Harzianopyridone (Harz) possessing antibiotic and antifungal effects (13), was tested to determine whether this specific shared inhibition was the primary inducer of myogenesis repression. We examined C2C12 cells exposed to Harz using the same methods to

elucidate whether SDH inhibition independent of the Krebs cycle might also elicit a dramatic obstruction of myogenesis. Harz treatment resulted in significant suppression of myogenic markers (Fig. 3b). Malonate lowered *Myog* levels compared to the control at all the time points (Fig. 3c). Consistent suppression of *Myog* expression was also observed following Harz treatment throughout the time course (Fig. 3d). MYOD levels were similar in malonate-treated cells during differentiation but malonate led to a reduction in MYOG levels, followed by a significant decrease in MYHC levels (Fig. 3e). Harz showed significantly reduced MYOG and MYHC levels as well (Fig. 3f). Malonate showed similar extent of reduction in MYHC staining (Fig. 3g) and myogenic index (Fig. 3h) as itaconate. Harz displayed the most robust inhibition of myogenesis shown by MYHC staining (Fig. 3g) and myogenic index (Fig. 3i). The degree of suppression caused by Harz was most comparable to the amount of inhibition by DMI. In tandem, these findings support that inhibition of SDH affects myogenesis in C2C12 cells.

Myogenesis is inhibited by exogenous succinate

Inhibition of SDH activity can lead to an increase in intracellular succinate levels (14). Given that SDH inhibition alone is able to significantly inhibit myogenesis, we next asked if an abundance of succinate could be the connecting factor leading to myogenic inhibition. Thus, we tested if exogenous succinate can cause myogenic failure in C2C12 cells treated with DM. All the data with diethyl succinate (DES) represented a very similar trend to previous findings. Myogenic gene expression (Fig. 6a), *Myog* (Fig. 6b), and

Fig. 3.

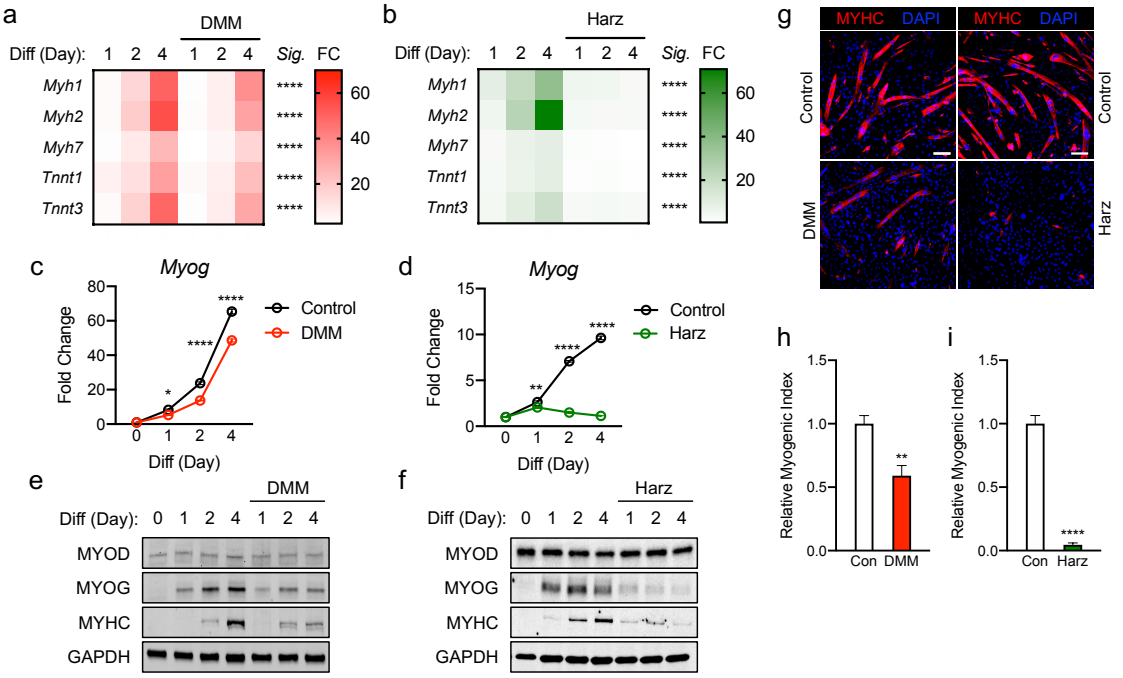

Fig. 3. Malonate and pharmacological inhibitor of succinate dehydrogenase repress C2C12 myogenesis via similar mechanism of action to that of itaconate and its derivatives. Heatmap depiction of average fold change (*Sig.* denotes statistical differences at day 4, n = 3) in myogenic gene transcription levels detected via RT-qPCR when exposed to differential media with or without (a) dimethyl malonate (DMM: 5 mM) and (b) harzianopyridone (Harz: 4 μM) relative to the untreated group over a 4-day time course. Fold change of *Myog* mRNA expression at various time points comparing differentiation media (DM) control groups to (c) DMI and (d) 4-OI groups relative to expression without either treatment (n = 3). Western blotting data showing differences in myogenic transcription factor expression between DM treated groups and groups treated with DM plus (e) DMM and (f) Harz over a 4 day time course as well as groups which were not exposed to either treatment for any period. (g) MYHC staining at day 4 of differentiation. Bar: 100 μm. Relative myogenic index (percentage of total nuclei associated with myotubes) of (h) DMM and (i) Harz. * denotes $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ based on two-way ANOVA/t-test.

myogenic proteins (Fig. 6c) were all inhibited by the exogenous succinate treatment. Reduced MYHC expression (Fig. 6d) and myogenic index (Fig. 6e) by succinate indicate its inhibitory potency of myogenesis. The degree of suppression was most similar to those results observed in groups treated with itaconate or malonate. These results support a possibility that inhibition of myogenesis by itaconate or malonate is at least partly due to accumulated succinate caused by SDH inhibition.

Fig. 4.

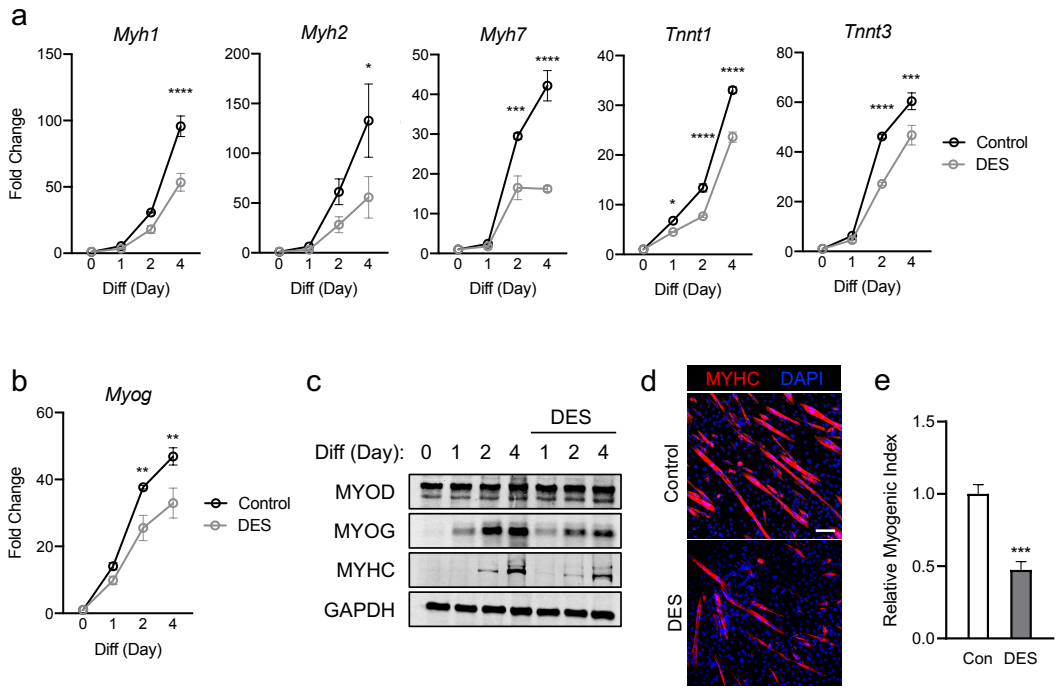

Fig. 4. Elevated exposure to succinate elicits inhibition of C2C12 myogenesis via mechanism similar to those observed when exposing cells to itaconate and malonate. (a) 4-day time course showcasing fold change differences of myogenic gene transcription levels detected via RT-qPCR when exposed to differential media with or without diethyl succinate (DES: 5 mM) relative to the untreated group (n = 3). (b) Fold change of *Myog* expression at various time points comparing DM control groups to DM and DES groups relative to expression without either treatment (n = 3). (c) Western blotting data showing differences in myogenic transcription factor expression between DM treated groups and groups treated with both DM and DES over a 4 day time course as well as groups which were not exposed to either treatment for any period. (d) MYHC staining at day 4 of differentiation. Bar: 100 μm. (e) Relative myogenic index (percentage of total nuclei associated with myotubes). * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ based on two-way ANOVA/t-test.

Discussion

Itaconate has become the epitome of metabolites that also have immunomodulation properties. It is a decarboxylated cis-aconitate which is primarily known for its SDH inhibiting activity in the TCA cycle. While initially used as a polymer for industrial purposes, it has been researched to have both antimicrobial and anti-inflammatory properties. Some bacteria (e.g. *Yersinia pestis*) have itaconate degrading enzymes and itaconate itself has been observed to restrict the growth of bacteria by inhibiting isocitrate lyase activity (15). It also regulates host mechanisms. Its anti-inflammatory role has gained the most attention recently. Itaconate is observed to significantly increase in response to macrophage activation (12). It acts to reduce inflammation by inhibiting the release of proinflammatory cytokines through a plethora of mechanisms, inhibiting ROS production through suppression of SDH activity (1), activating the regulator of antioxidant expression NRF2 (16), and activating the anti-inflammatory signaling transcription factor ATF3 (3). These effects in tandem have attracted scientists to get approval for itaconate derivatives as potential treatments to limit microbial pathogenicity and to attenuate autoimmune disease symptoms.

DMI and 4-OI have been researched to have promising effects on psoriasis, rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus due to their ability to suppress IL-17 signaling and reduce proinflammatory cytokine production (3,6). While seemingly propitious as a potential treatment, the fact is that knowledge is very limited on the effects of itaconate and its derivates outside of those relating to the immunomodulatory axis. A consequence of itaconate derivative use may be one or more harmful side effects which could lessen enthusiasm for their utilization as treatments.

Blocking itaconate signaling, which has yet to be proposed, might also be used to attenuate symptoms of other conditions. The focus of our research is to showcase the additional effects of itaconate on muscle regulation. Chronic inflammation has been linked to muscle wasting. Prolonged itaconate exposure resulting from chronic inflammation may cause or exacerbate muscle wasting.

Our hypothesis is that itaconate obstructs myogenesis via inhibition of SDH. This hypothesis arose from research done on the effects of succinate abundance on muscle wasting. Succinate is a substrate for SDH, so its intracellular concentration is directly linked to SDH activity. Succinate abundance has been proposed as a causative agent of muscle wasting. Treatments to elevate intracellular succinate concentration has been observed to decrease total muscle protein expression by ~25%, inhibit in vitro C2C12 myoblast cell myogenesis, decrease myofiber diameter in murine models, inhibit myogenesis in vivo following barium chloride injury, reduce respiration capacity by ~35% in myoblasts, and also disrupt a number of other metabolic processes within muscle cells (17). SDH has also been shown to have significantly reduced activity in sarcopenic muscle (18). Given that anti-inflammatory activity of itaconate is incurred via SDH inhibition, we endeavored to determine if itaconate treatment led to similar effects seen in succinate abundant cells.

We first tested the proposed treatment forms of itaconate, DMI, and 4-OI. We then examined itaconate alone followed by malonate, a pharmacological inhibitor of SDH, and then lastly diethyl succinate. Treatment incurred transcriptional modulation of *Myh1, Myh2, Myh7, Tnnt1, and Tnnt3* along with alteration of myogenic transcription factor (mainly MYOG) and myogenesis marker MYHC protein expression were examined. Our

204 results showcased substantial inhibition of myogenesis in C2C12 cells by DMI. 4-OI
205 treatment also exhibited significant inhibition, but not as aggressive as DMI. Itaconate
206 treatment also resulted in significant myogenesis inhibition, but not to the degree seen in
207 either DMI or 4-OI treated groups. It is possible that inability of DMI conversion to
208 itaconate leads to a strong inhibition of myogenesis exhibiting sustained activity because
209 4-OI can be converted to itaconate, but DMI cannot (10). It is established that itaconate
210 can be converted to itaconyl-CoA by succinate-CoA ligase (19). A possible mechanism
211 to explain this tiered order of inhibition severity may involve itaconate conversion to
212 itaconyl-CoA. Regardless of potency, all itaconate treatments inhibited *in vitro*
213 myogenesis.

214 We show that SDH suppression and subsequent succinate accumulation
215 significantly inhibit myogenesis via myogenic transcription suppression. Given that
216 MYOG expression is dependent upon MYOD activity, it is hypothesized that MYOD
217 activity was also suppressed in the treatment groups, but via post translational
218 modification that leads to changes in MYOD activity such as phosphorylation and
219 acetylation (20,21) rather than transcriptional inhibition. Inactive MYOD in response to
220 SDH inhibition and succinate causes blunted induction of *Myog* thereby suppressing the
221 transcription of myogenic genes. Together, our results support that itaconate may
222 contribute to impairment of myogenesis via its effects on SDH which modulates myogenic
223 transcription mechanisms.

224 Sarcopenia patients have been observed to have inhibited mitochondrial
225 functioning via transcriptional downregulation of key genes involved in oxidative
226 phosphorylation and mitochondria proteostasis (18). 4-OI has been shown to increase

aerobic respiration by reducing glycolytic function through inhibition of GAPDH (11). This data may implicate that in addition to subsequent muscle wasting induced by increased succinate concentration, prolonged itaconate activity may exacerbate issues relating to mitochondrial function. What demands further study is the determination of whether itaconate and its derivatives induce mitochondrial activity suppression which may cause subsequent inhibition of the oxidative phosphorylation pathway and thus reduce energy demand accommodation capability.

In this study, we find that itaconate and derivatives contribute to suppression of myogenesis. What remains to be researched is how itaconate affects muscle regulation outside of succinate accumulation. Our results showcase functions of itaconate outside of immunomodulation and implicate that derivative use to treat autoimmune diseases or microbial infections should be cautioned due to a potential for deleterious effects on myogenesis. Further elucidation of these functions may lead to the development of treatments to attenuate muscle wasting via possible itaconate signaling inhibition.

241 ## Materials and methods

242 ### Antibodies and chemical reagents

243 Primary antibodies used in this study are as follows: GAPDH (Santa Cruz
244 Biotechnology, sc-32233), MYHC (Sigma, 05-716), MYOD (Santa Cruz Biotechnology,
245 sc-377460), and MYOG (Santa Cruz Biotechnology, sc-12732). Reagents added to the
246 media are as follows: Diethyl succinate (Sigma, 112402), Dimethyl itaconate (Sigma,
247 592498), Dimethyl malonate (Sigma 136441), Harzianopyridone (Santa Cruz
248 Biotechnology, sc-280769), Itaconic acid (Sigma, I29204), and 4-octyl itaconate (Tocris
249 Bioscience, 6662).

250

251 ### Cell culture and differentiation

252 The mouse myogenic C2C12 myoblasts were maintained on plastic cell culture
253 plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal
254 bovine serum and 1% penicillin-streptomycin in a humidified incubator kept at 37 °C and
255 5% CO_2. Cells were used up until passage 9. For differentiation, cells at 90% confluency
256 were serum restricted with differentiation medium (DMEM, 2% horse serum, 1% penicillin-
257 streptomycin) for up to 4 days. Growth or differentiation medium was replenished every
258 day.

259

260 ### RNA isolation and RT-qPCR

RNA isolation was performed using the commercially available Aurum RNA miniprep kit (Bio-rad, 732-6820). Gene expression analysis was conducted with 50 ng RNA using iTaq Universal One-Step RT-qPCR Kit (Bio-rad, 1725140). The reaction was carried out according to the manufacturer's instructions using CFX Connect Real-Time PCR Detection System (Bio-rad, 1855200). Probes used for TaqMan® Gene Expression Assays (ThermoFisher) were as follows: *Myog* (Mm00446194_m1), *Myh1* (Mm01332489_m1), *Myh2* (Mm01332564_m1), *Myh7* (Mm00600555_m1), *Ppib* (Mm00478295_m1), *Tnnt1* (Mm00449089_m1), and *Tnnt3* (Mm01137842_g1).

Western blot

Cells were lysed in RIPA buffer (ThermoFisher, 89901) in the presence of Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoFisher, 78440), and protein concentration was measured using Pierce™ BCA Protein Assay Kit (ThermoFisher, 23227). Lysates were boiled for 5 min with 4x Laemmli Sample Buffer (Bio-Rad, 1610747) and 2-mercaptoethanol (Bio-Rad, 1610710). Lysates were resolved on SDS polyacrylamide gels and blotted onto PVDF membranes using Trans-Blot Turbo RTA Midi 0.45 µm LF PVDF Transfer Kit (Bio-Rad, 1704275). Transfer was run in Trans-Blot Turbo Transfer System (Bio-Rad, 1704150) using Mixed MW (1.3A-25V-7M) protocol. The membranes were blocked with Intercept™ (TBS) Blocking Buffer (LI-COR, 927-60001) for 1 h at room temperature and incubated with appropriate primary antibodies diluted 1:1000 in blocking buffer at 4 °C overnight. After 3 washes with 0.05% tween20 (Bio-Rad) in TBS buffer (Bio-Rad), the membrane was incubated with IRDye® 800CW Goat anti-Mouse IgG (LI-COR, 926-32210) and IRDye® 680RD Goat anti-Rabbit IgG (LI-COR, 926-

68071) secondary antibodies diluted 1:10000 in blocking buffer for 1 h at room temperature. Band images were visualized using ChemiDoc™ MP Imaging System (Bio-Rad, 12003154).

Immunofluorescence and calculation of myogenic index

Cells were seeded in a 24-well cell culture plate and followed by above-mentioned differentiation protocol. At day 4 of differentiation, cells were rinsed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Then, cells were permeablized with 0.5% Triton™ X-100 (Fisher, BP151-100) in PBS for 10 min at room temperature. Next, cells were submerged with Intercept® (PBS) Blocking Buffer (LI-COR, 927-70001) for 1 h at room temperature. MYHC primary antibody was diluted to 1:100 in blocking buffer and incubated overnight at 4 °C with the fixed cells. Next day, after 3 washes with 0.05% tween20 in PBS, Alexa Fluor 594 secondary antibody (Fisher, A11005) was diluted to 1:500 in blocking buffer and incubated with the cells for 1 h at room temperature. Followed by further washes, cells were mounted with VECTASHIELD® Antifade Mounting Medium with DAPI (Vectalabs, H-1200) and covered with circular cover glass. Images were taken using ZOE™ Fluorescent Cell Imager (Bio-rad, 1450031). Myogenic index was calculated as the percentage of nuclei in fused myotubes (MYHC positive) out of the total nuclei in given images. Distribution of nuclei in myoblasts and myotubes was measured by counting over 100 nuclei at 4 distinct locations. The number of nuclei was counted using imageJ.

Statistical analysis

Unpaired t-test or two-way ANOVA with multiple comparisons was performed to determine significant relationships between groups in the experiments. Data are represented as mean ± SEM.

References

1. Lampropoulou, V., Sergushichev, A., Bambouskova, M., Nair, S., Vincent, E. E., Loginicheva, E., Cervantes-Barragan, L., Ma, X., Huang, S. C., Griss, T., Weinheimer, C. J., Khader, S., Randolph, G. J., Pearce, E. J., Jones, R. G., Diwan, A., Diamond, M. S., and Artyomov, M. N. (2016) Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation. *Cell Metab* **24**, 158-166
2. Mills, E. L., Ryan, D. G., Prag, H. A., Dikovskaya, D., Menon, D., Zaslona, Z., Jedrychowski, M. P., Costa, A. S. H., Higgins, M., Hams, E., Szpyt, J., Runtsch, M. C., King, M. S., McGouran, J. F., Fischer, R., Kessler, B. M., McGettrick, A. F., Hughes, M. M., Carroll, R. G., Booty, L. M., Knatko, E. V., Meakin, P. J., Ashford, M. L. J., Modis, L. K., Brunori, G., Sevin, D. C., Fallon, P. G., Caldwell, S. T., Kunji, E. R. S., Chouchani, E. T., Frezza, C., Dinkova-Kostova, A. T., Hartley, R. C., Murphy, M. P., and O'Neill, L. A. (2018) Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. *Nature* **556**, 113-117
3. Bambouskova, M., Gorvel, L., Lampropoulou, V., Sergushichev, A., Loginicheva, E., Johnson, K., Korenfeld, D., Mathyer, M. E., Kim, H., Huang, L. H., Duncan, D., Bregman, H., Keskin, A., Santeford, A., Apte, R. S., Sehgal, R., Johnson, B., Amarasinghe, G. K., Soares, M. P., Satoh, T., Akira, S., Hai, T., de Guzman Strong, C., Auclair, K., Roddy, T. P., Biller, S. A., Jovanovic, M., Klechevsky, E., Stewart, K. M., Randolph, G. J., and Artyomov, M. N. (2018) Electrophilic properties of itaconate and derivatives regulate the IkappaBzeta-ATF3 inflammatory axis. *Nature* **556**, 501-504
4. Kuo, P. C., Weng, W. T., Scofield, B. A., Paraiso, H. C., Brown, D. A., Wang, P. Y., Yu, I. C., and Yen, J. H. (2020) Dimethyl itaconate, an itaconate derivative, exhibits immunomodulatory effects on neuroinflammation in experimental autoimmune encephalomyelitis. *J Neuroinflammation* **17**, 138
5. Daly, R., Blackburn, G., Best, C., Goodyear, C. S., Mudaliar, M., Burgess, K., Stirling, A., Porter, D., McInnes, I. B., Barrett, M. P., and Dale, J. (2020) Changes in Plasma Itaconate Elevation in Early Rheumatoid Arthritis Patients Elucidates Disease Activity Associated Macrophage Activation. *Metabolites* **10**
6. Tang, C., Wang, X., Xie, Y., Cai, X., Yu, N., Hu, Y., and Zheng, Z. (2018) 4-Octyl Itaconate Activates Nrf2 Signaling to Inhibit Pro-Inflammatory Cytokine Production in Peripheral Blood Mononuclear Cells of Systemic Lupus Erythematosus Patients. *Cell Physiol Biochem* **51**, 979-990
7. Yi, Z., Deng, M., Scott, M. J., Fu, G., Loughran, P. A., Lei, Z., Li, S., Sun, P., Yang, C., Li, W., Xu, H., Huang, F., and Billiar, T. R. (2020) IRG1/Itaconate Activates Nrf2 in Hepatocytes to Protect Against Liver Ischemia-Reperfusion Injury. *Hepatology*
8. Tian, F., Wang, Z., He, J., Zhang, Z., and Tan, N. (2020) 4-Octyl itaconate protects against renal fibrosis via inhibiting TGF-beta/Smad pathway, autophagy and reducing generation of reactive oxygen species. *Eur J Pharmacol* **873**, 172989
9. Swain, A., Bambouskova, M., Kim, H., Andhey, P. S., Duncan, D., Auclair, K., Chubukov, V., Simons, D. M., Roddy, T. P., Stewart, K. M., and Artyomov, M. N. (2020) Comparative evaluation of itaconate and its derivatives reveals divergent inflammasome and type I interferon regulation in macrophages. *Nat Metab* **2**, 594-602
10. ElAzzouny, M., Tom, C. T., Evans, C. R., Olson, L. L., Tanga, M. J., Gallagher, K. A., Martin, B. R., and Burant, C. F. (2017) Dimethyl Itaconate Is Not Metabolized into Itaconate Intracellularly. *J Biol Chem* **292**, 4766-4769
11. Liao, S. T., Han, C., Xu, D. Q., Fu, X. W., Wang, J. S., and Kong, L. Y. (2019) 4-Octyl itaconate inhibits aerobic glycolysis by targeting GAPDH to exert anti-inflammatory effects. *Nat Commun* **10**, 5091
12. O'Neill, L. A. J., and Artyomov, M. N. (2019) Itaconate: the poster child of metabolic reprogramming in macrophage function. *Nat Rev Immunol* **19**, 273-281
13. Dickinson, J. M., Hanson, J. R., Hitchcock, P. B., and Claydon, N. (1989) Structure and biosynthesis of harzianopyridone, an antifungal metabolite of Trichoderma harzianum. *Journal of the Chemical Society, Perkin Transactions 1*, 1885-1887
14. Cordes, T., Wallace, M., Michelucci, A., Divakaruni, A. S., Sapcariu, S. C., Sousa, C., Koseki, H., Cabrales, P., Murphy, A. N., Hiller, K., and Metallo, C. M. (2016) Immunoresponsive Gene 1 and

Itaconate Inhibit Succinate Dehydrogenase to Modulate Intracellular Succinate Levels. *J Biol Chem* **291**, 14274-14284

15. Sasikaran, J., Ziemski, M., Zadora, P. K., Fleig, A., and Berg, I. A. (2014) Bacterial itaconate degradation promotes pathogenicity. *Nat Chem Biol* **10**, 371-377
16. Mills, E. L., Kelly, B., Logan, A., Costa, A. S. H., Varma, M., Bryant, C. E., Tourlomousis, P., Dabritz, J. H. M., Gottlieb, E., Latorre, I., Corr, S. C., McManus, G., Ryan, D., Jacobs, H. T., Szibor, M., Xavier, R. J., Braun, T., Frezza, C., Murphy, M. P., and O'Neill, L. A. (2016) Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell* **167**, 457-470 e413
17. Arneson, P. C., Hogan, K. A., Shin, A. M., Samani, A., Jatoi, A., and Doles, J. D. (2020) The wasting-associated metabolite succinate disrupts myogenesis and impairs skeletal muscle regeneration. *JCSM Rapid Commun* **3**, 56-69
18. Migliavacca, E., Tay, S. K. H., Patel, H. P., Sonntag, T., Civiletto, G., McFarlane, C., Forrester, T., Barton, S. J., Leow, M. K., Antoun, E., Charpagne, A., Seng Chong, Y., Descombes, P., Feng, L., Francis-Emmanuel, P., Garratt, E. S., Giner, M. P., Green, C. O., Karaz, S., Kothandaraman, N., Marquis, J., Metairon, S., Moco, S., Nelson, G., Ngo, S., Pleasants, T., Raymond, F., Sayer, A. A., Ming Sim, C., Slater-Jefferies, J., Syddall, H. E., Fang Tan, P., Titcombe, P., Vaz, C., Westbury, L. D., Wong, G., Yonghui, W., Cooper, C., Sheppard, A., Godfrey, K. M., Lillycrop, K. A., Karnani, N., and Feige, J. N. (2019) Mitochondrial oxidative capacity and NAD(+) biosynthesis are reduced in human sarcopenia across ethnicities. *Nat Commun* **10**, 5808
19. Nemeth, B., Doczi, J., Csete, D., Kacso, G., Ravasz, D., Adams, D., Kiss, G., Nagy, A. M., Horvath, G., Tretter, L., Mocsai, A., Csepanyi-Komi, R., Iordanov, I., Adam-Vizi, V., and Chinopoulos, C. (2016) Abolition of mitochondrial substrate-level phosphorylation by itaconic acid produced by LPS-induced Irg1 expression in cells of murine macrophage lineage. *FASEB J* **30**, 286-300
20. Di Padova, M., Caretti, G., Zhao, P., Hoffman, E. P., and Sartorelli, V. (2007) MyoD acetylation influences temporal patterns of skeletal muscle gene expression. *J Biol Chem* **282**, 37650-37659
21. Song, A., Wang, Q., Goebl, M. G., and Harrington, M. A. (1998) Phosphorylation of nuclear MyoD is required for its rapid degradation. *Mol Cell Biol* **18**, 4994-4999

393 **Figure legend**

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