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

43 **Introduction**

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

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

59 Given that chronic inflammation is hypothesized to incur sustained itaconate
60 production, coupled to its potential therapeutic use for the treatment of a variety of
61 diseases, it is of the upmost importance to understand potential off target tissues and
62 effects in the face of elevated itaconate levels. To address this critical gap in knowledge
63 we utilized the well-established C2C12 murine myoblast model system for *in vitro*
64 myogenesis and studied the effects of itaconate and its derivatives on altering myoblast
65 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.

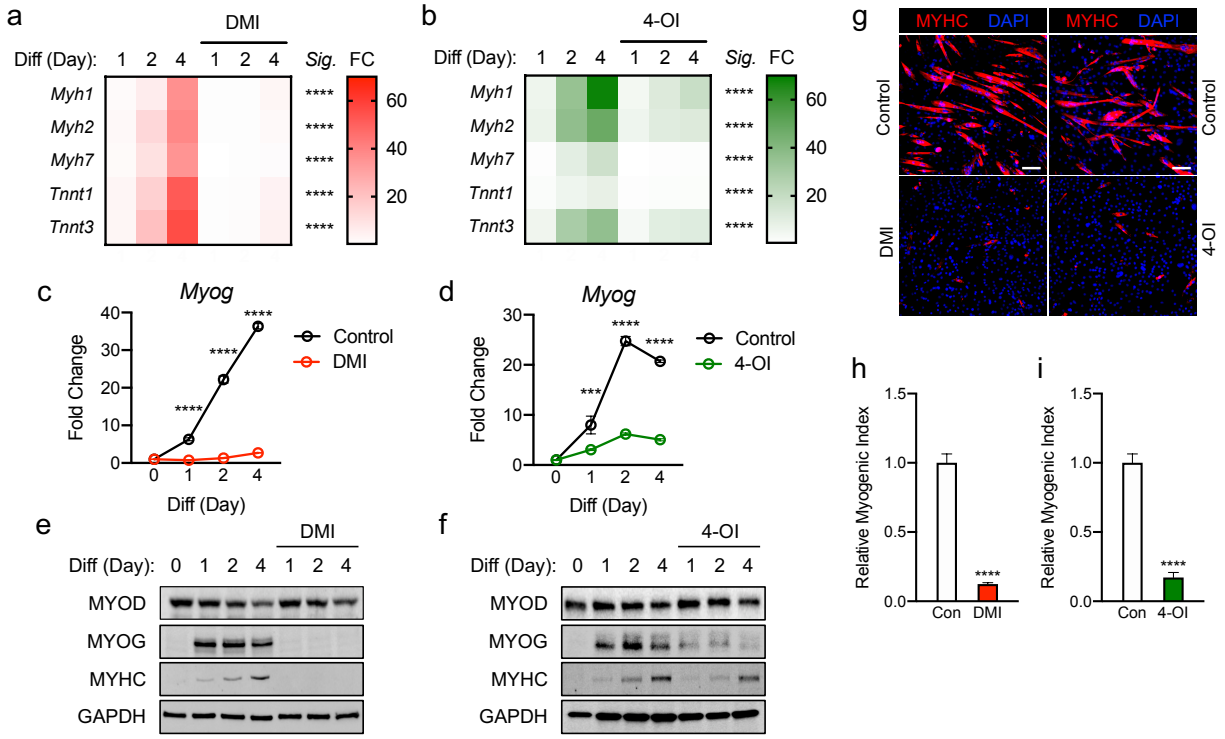

Fig. 1. Itaconate derivatives represse C2C12 myogenesis by modulating myogenic transcription mechanisms. Heatmap depiction of average fold change (*Sig.* denotes statistical differences at day 4, n = 3) in myogenic gene transcription levels detected via RT-qPCR when exposed to differential media with 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.

97 **Physiological itaconate inhibits myogenesis**

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

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

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113 **Malonate and pharmacological succinate dehydrogenase inhibitor obstruct**
114 **myogenesis**

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

Fig. 2.

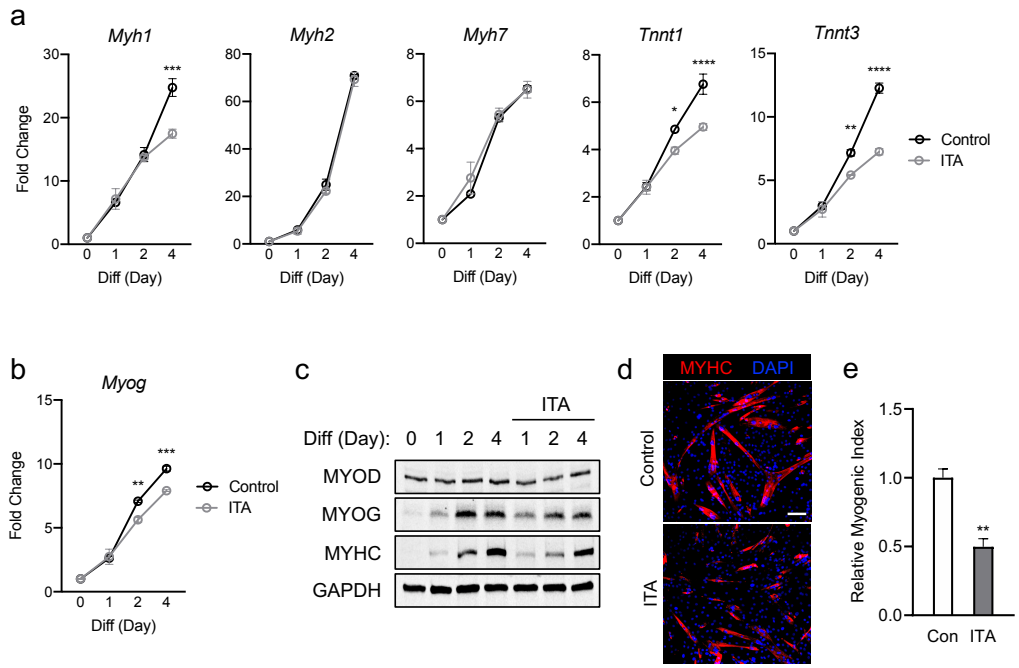

Fig. 2. Itaconate represses C2C12 myogenesis by modulating myogenic transcription mechanisms. (a) 4-day time course showcasing fold change differences of myogenic gene transcription levels detected via RT-qPCR when exposed to differential media with or without itaconate (7.5 mM) relative to the untreated 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.

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

125 elucidate whether SDH inhibition independent of the Krebs cycle might also elicit a
126 dramatic obstruction of myogenesis. Harz treatment resulted in significant suppression of
127 myogenic markers (Fig. 3b). Malonate lowered *Myog* levels compared to the control at all
128 the time points (Fig. 3c). Consistent suppression of *Myog* expression was also observed
129 following Harz treatment throughout the time course (Fig. 3d). MYOD levels were similar
130 in malonate-treated cells during differentiation but malonate led to a reduction in MYOG
131 levels, followed by a significant decrease in MYHC levels (Fig. 3e). Harz showed
132 significantly reduced MYOG and MYHC levels as well (Fig. 3f). Malonate showed similar
133 extent of reduction in MYHC staining (Fig. 3g) and myogenic index (Fig. 3h) as itaconate.
134 Harz displayed the most robust inhibition of myogenesis shown by MYHC staining (Fig.
135 3g) and myogenic index (Fig. 3i). The degree of suppression caused by Harz was most
136 comparable to the amount of inhibition by DMI. In tandem, these findings support that
137 inhibition of SDH affects myogenesis in C2C12 cells.

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140 **Myogenesis is inhibited by exogenous succinate**

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

Fig. 3.

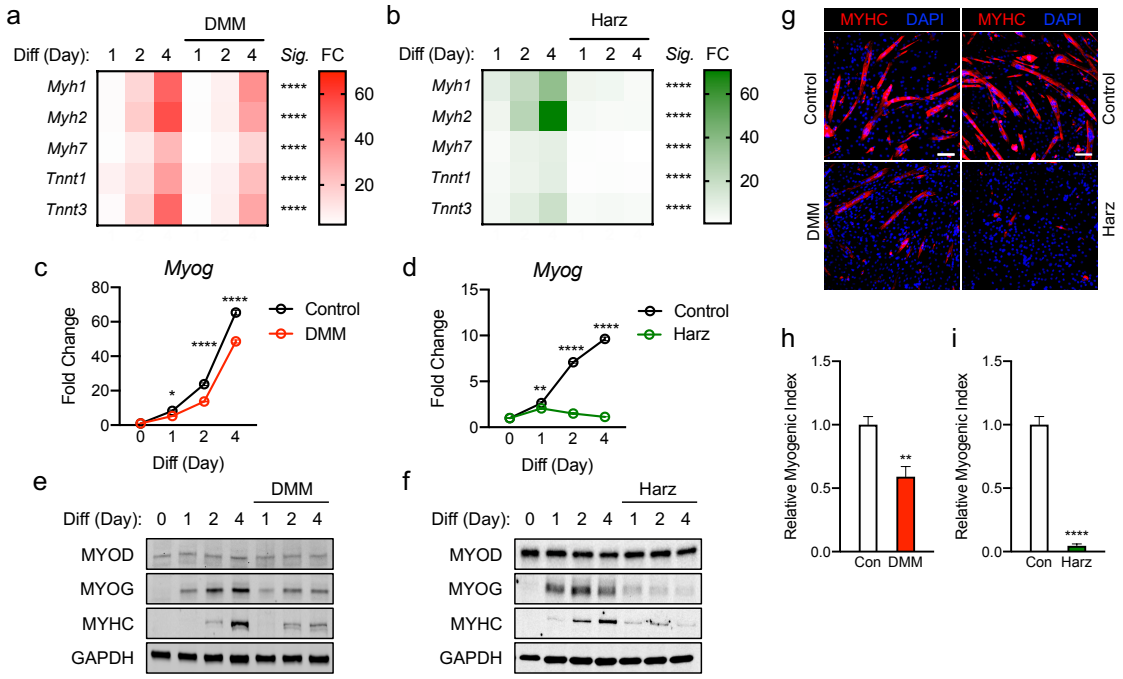

Fig. 3. Malonate and pharmacological inhibitor of succinate dehydrogenase repress C2C12 myogenesis via similar mechanism of action to that of itaconate and its derivatives. Heatmap depiction of average fold change (*Sig.* denotes statistical differences at day 4, n = 3) in myogenic gene transcription 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.

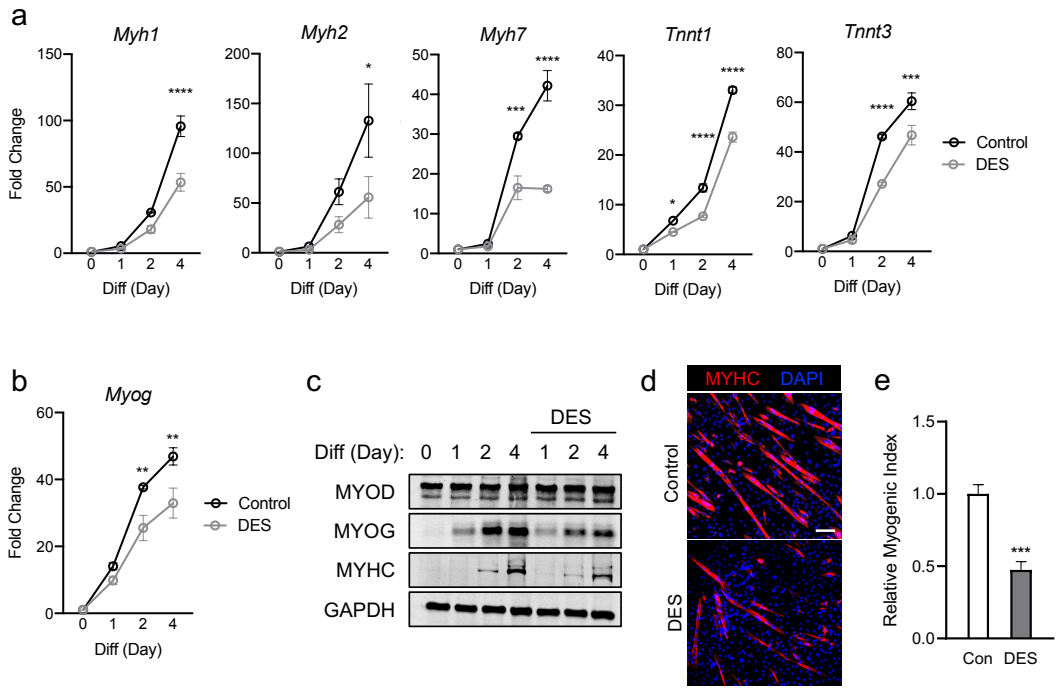

Fig. 4. Elevated exposure to succinate elicits inhibition of C2C12 myogenesis via mechanism similar to those observed when exposing cells to itaconate and malonate. (a) 4-day time course showcasing fold change differences of myogenic gene transcription levels detected via RT-qPCR when exposed to differential 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.

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158 **Discussion**

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

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

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

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

199 We first tested the proposed treatment forms of itaconate, DMI, and 4-OI. We then
200 examined itaconate alone followed by malonate, a pharmacological inhibitor of SDH, and
201 then lastly diethyl succinate. Treatment incurred transcriptional modulation of *Myh1,*
202 *Myh2, Myh7, Tnnt1, and Tnnt3* along with alteration of myogenic transcription factor
203 (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

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

234 In this study, we find that itaconate and derivatives contribute to suppression of
235 myogenesis. What remains to be researched is how itaconate affects muscle regulation
236 outside of succinate accumulation. Our results showcase functions of itaconate outside
237 of immunomodulation and implicate that derivative use to treat autoimmune diseases or
238 microbial infections should be cautioned due to a potential for deleterious effects on
239 myogenesis. Further elucidation of these functions may lead to the development of
240 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**

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

269

270 **Western blot**

271 Cells were lysed in RIPA buffer (ThermoFisher, 89901) in the presence of Halt™
272 Protease and Phosphatase Inhibitor Cocktail (ThermoFisher, 78440), and protein
273 concentration was measured using Pierce™ BCA Protein Assay Kit (ThermoFisher,
274 23227). Lysates were boiled for 5 min with 4x Laemmli Sample Buffer (Bio-Rad, 1610747)
275 and 2-mercaptoethanol (Bio-Rad, 1610710). Lysates were resolved on SDS
276 polyacrylamide gels and blotted onto PVDF membranes using Trans-Blot Turbo RTA Midi
277 0.45 µm LF PVDF Transfer Kit (Bio-Rad, 1704275). Transfer was run in Trans-Blot Turbo
278 Transfer System (Bio-Rad, 1704150) using Mixed MW (1.3A-25V-7M) protocol. The
279 membranes were blocked with Intercept™ (TBS) Blocking Buffer (LI-COR, 927-60001)
280 for 1 h at room temperature and incubated with appropriate primary antibodies diluted
281 1:1000 in blocking buffer at 4 °C overnight. After 3 washes with 0.05% tween20 (Bio-Rad)
282 in TBS buffer (Bio-Rad), the membrane was incubated with IRDye® 800CW Goat anti-
283 Mouse IgG (LI-COR, 926-32210) and IRDye® 680RD Goat anti-Rabbit IgG (LI-COR, 926-

284 68071) secondary antibodies diluted 1:10000 in blocking buffer for 1 h at room
285 temperature. Band images were visualized using ChemiDoc™ MP Imaging System (Bio-
286 Rad, 12003154).

287

288 **Immunofluorescence and calculation of myogenic index**

289 Cells were seeded in a 24-well cell culture plate and followed by above-mentioned
290 differentiation protocol. At day 4 of differentiation, cells were rinsed with PBS and fixed
291 with 4% paraformaldehyde for 10 min at room temperature. Then, cells were
292 permeablized with 0.5% Triton™ X-100 (Fisher, BP151-100) in PBS for 10 min at room
293 temperature. Next, cells were submerged with Intercept® (PBS) Blocking Buffer (LI-COR,
294 927-70001) for 1 h at room temperature. MYHC primary antibody was diluted to 1:100 in
295 blocking buffer and incubated overnight at 4 °C with the fixed cells. Next day, after 3
296 washes with 0.05% tween20 in PBS, Alexa Fluor 594 secondary antibody (Fisher,
297 A11005) was diluted to 1:500 in blocking buffer and incubated with the cells for 1 h at
298 room temperature. Followed by further washes, cells were mounted with VECTASHIELD®
299 Antifade Mounting Medium with DAPI (Vectalabs, H-1200) and covered with circular
300 cover glass. Images were taken using ZOE™ Fluorescent Cell Imager (Bio-rad,
301 1450031). Myogenic index was calculated as the percentage of nuclei in fused myotubes
302 (MYHC positive) out of the total nuclei in given images. Distribution of nuclei in myoblasts
303 and myotubes was measured by counting over 100 nuclei at 4 distinct locations. The
304 number of nuclei was counted using imageJ.

305

306 **Statistical analysis**

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

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Figure legend