Dimethyl Itaconate Is Not Metabolized into Itaconate Intracellularly*

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Itaconic acid is an important metabolite produced by macrophages after stimulation with LPS. The role of itaconate in the inflammatory cascade is unclear. Here we used [13C]itaconate and dimethyl [13C]itaconate (DMI) to probe itaconate metabolism, and find that [13C]DMI is not metabolized to itaconate. [13C]Itaconate in the cell culture medium leads to elevated intracellular levels of unlabeled succinate, with no evidence of intracellular uptake. The goal of this study is to encourage the development of effective pro-drug strategies to increase the intracellular levels of itaconate, which will enable more conclusive analysis of its action on macrophages and other cell and tissue types.

Itaconic acid is a dicarboxylic acid polar metabolite originally characterized in *Aspergillus terreus*, but is also produced in mammalian cells (1). After LPS stimulation, itaconic acid is secreted by macrophages, where it can inhibit bacterial cell growth (1). In macrophages, itaconate synthesis is catalyzed by the immune-responsive gene 1 (IRG1) protein, which mediates the decarboxylation of *cis*-aconitate to itaconate (2). Metabolomic and fluxomic analysis of LPS-stimulated macrophages demonstrated reduced Isocitrate dehydrogenase-1 (IDH1) expression and increased IRG1 expression, resulting in a diversion of citrate from the TCA cycle[2] toward itaconate production (3). This metabolic remodeling results in glutamate serving as the anaplerotic substrate to maintain or elevate succinate levels (3). Moreover, elevated succinate acts as an inflammatory signal that induces secretion of IL-1β and stabilization of HIF-1α (4). Based on the similarity between itaconate (methyl-

ene succinic acid) and succinate, recent investigations have focused on the link between itaconate synthesis and succinate accumulation. Two complementary studies reported that itaconate inhibits succinate dehydrogenase and drives succinate accumulation (5, 6). Studying the role of itaconate requires either lowering its intracellular concentration by using IRG1 knock-out mouse models (5, 6) or increasing its intracellular concentration by using either itaconate (5) or a "cell-permeable" analog, dimethyl itaconate (DMI) (6). However, there is no direct evidence that itaconate or DMI can cross cell membranes and increase intracellular itaconate. Without direct evidence of intracellular delivery, it remains unclear whether itaconate-mediated metabolic and inflammatory effects are induced by increasing intracellular itaconate or by an extracellular mechanism.

Here we synthesized isotopically labeled [13C]itaconate and dimethyl [13C]itaconate ([13C]DMI) to directly profile itaconate metabolism and uptake (Fig. 1). This analysis suggests that exogenous itaconate is not taken up into cells and [13C]DMI is not metabolized into [13C]itaconate in bone marrow-derived macrophages. We also report that [13C]itaconate in the cell culture medium leads to elevated intracellular levels of unlabeled succinate, yet there is no evidence of intracellular uptake. Overall, this study highlights current limitations in intracellular itaconate delivery, and emphasizes the development of effective pro-drug strategies to conclusively define its action on macrophages and other cell and tissue types.

Results and Discussion

Itaconate is a highly polar metabolite that is unlikely to cross into cells without the presence of an active transporter. Because no transporter has been identified, it remains unclear whether the addition of exogenous itaconate can affect intracellular itaconate levels. To test whether exogenous itaconate can enter cells, we treated RAW-264.7 cells with 10 mM itaconate neutralized with sodium hydroxide (pH 7). Cells were incubated for 2–3 h, and then rinsed and quenched. To reduce background signal from residual extracellular and cell surface-associated metabolites, cells were washed either once or twice with 150 mM ammonium acetate before freezing in liquid nitrogen as described previously (7). The extracted metabolites were then analyzed by HILIC-LC/MS. The levels of citrate and succinate did not change significantly following the second wash. Conversely, itaconate levels fell more than 50% after the second wash, suggesting that much of the cell-associated itaconate signal is due to nonspecific association (Fig. 2A). Thus, analysis of cellular uptake is prone to misleading results, because any itaconate adsorbed to the cell surface would contribute to the measured cellular uptake.

As reported previously (5), we also found that the addition of exogenous itaconate elevates succinate levels in RAW cells and bone marrow-derived macrophage (BMDM) cells (data not shown). To evaluate whether this effect is caused by itaconate conversion to succinate, we treated mouse BMDM with increasing concentrations of [13C]itaconate (0–1000 μM). Simi-

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[2] The abbreviations used are: TCA cycle, tricarboxylic acid cycle; DMI, dimethyl itaconate; HILIC, hydrophilic interaction liquid chromatography; BMDM, bone marrow-derived macrophage.
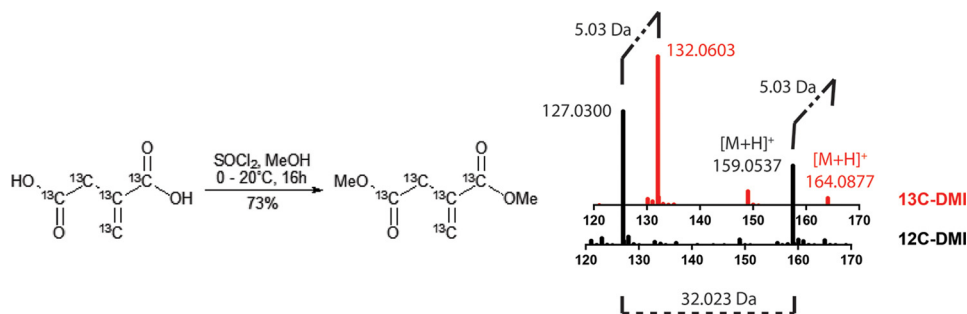
FIGURE 1. **Synthesis of [¹³C]DMI from [¹³C₅]itaconic acid.** Shown is an overlay of the mass spectra of unlabeled (*black*) and labeled (*red*) DMI showing the +5 mass shift in the parent $[M+H]^+$ and its fragment.
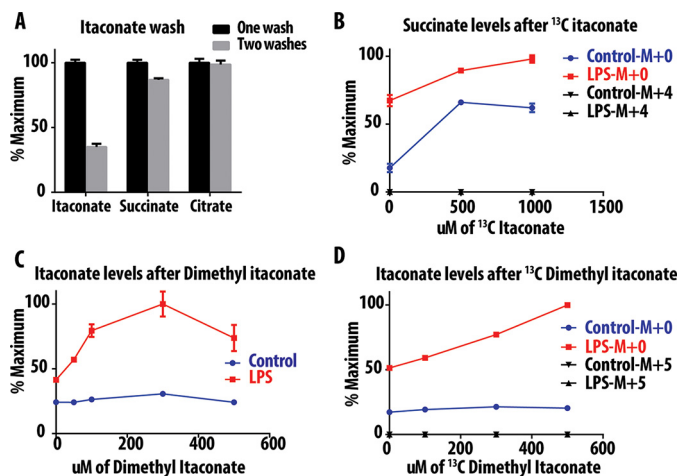

FIGURE 2. **Itaconate and succinate levels after treatment with dimethyl itaconate.** *A*, levels of different metabolites in RAW cells after incubation with itaconic acid and subjecting plates to one or two wash steps. *B*, levels of succinate in mice BMDM after treatment with serial concentrations of [¹³C]itaconic acid. *C*, levels of itaconate in mice BMDM after treatment with serial concentration of DMI. *D*, levels of itaconate in mice BMDM after treatment with serial concentration of [¹³C]DMI. Data indicate S.E.

larly, the addition of [¹³C]itaconate increased levels of succinate in both LPS-stimulated and unstimulated cells. However, the accumulated succinate lacked the M+4 label, and thus cannot be the product of [¹³C]itaconate metabolism (Fig. 2*B*). Based on these results, itaconate is not metabolized by cells to form succinate.

Intracellular delivery of polar carboxylate metabolites is typically achieved by synthesis of methyl or ethyl ester analogues, as has been done for succinate (8, 9) and glutamate (10). This pro-drug strategy relies on methyl or ethyl ester hydrolysis by intracellular esterases, leaving the unmasked polar metabolite trapped inside cells. Methyl ester derivatives of DMI have been used to presumably deliver intracellular itaconate; however, the conversion of DMI to itaconate has not been demonstrated (6). To address this possibility, BMDM cells were incubated with vehicle or LPS along with a range of DMI concentrations, followed by LC-MS quantitation of intracellular itaconate (Fig. 2*C*). DMI treatment had no effect on itaconate levels in vehicle-treated cells (Fig. 2*C*). In contrast, LPS-stimulated cells revealed a dose-dependent increase in itaconate levels when incubated with increasing concentrations of DMI (Fig. 2*C*). From these data, we concluded either that DMI potentiates LPS-mediated *de novo* itaconate syn-

thesis or that LPS stimulates cellular esterases to accelerate DMI methyl ester hydrolysis.

To trace the intracellular fate of DMI, we synthesized isotopically labeled dimethyl [¹³C]itaconate, which, after esterase hydrolysis, would release the M+5 isotopomer of [¹³C]itaconate (see "Materials and Methods"). Vehicle-treated and LPS-stimulated BMDM macrophages were incubated with increasing concentrations of [¹³C]DMI. Again, only LPS-treated cells demonstrated a dose-dependent increase in unlabeled (M+0) itaconate (Fig. 2*D*). However, there was no measurable ¹³C-labeled (M+5) itaconate in either vehicle-treated or LPS-treated cells, suggesting either that DMI is not converted to itaconate or that the resulting itaconate is quickly excreted or further metabolized, without intracellular accumulation (Fig. 2*D*).

Based on these findings, we conclude that exogenous DMI is not directly metabolized to itaconate, but instead somehow potentiates the effects of LPS activation to increase itaconate biosynthesis. How cells achieve alteration in metabolism, including amplification of itaconate and succinate production, following itaconate or DMI treatment will require further investigation. The findings here appear to reveal an alternative mode of dimethyl itaconate action. Importantly, although itaconate is not very electrophilic, methyl esterification is predicted to activate the electrophilic acrylate. Once introduced to cells, DMI could potentially alkylate active site of cysteine residues or alter redox homeostasis. Thus, we agree with previous studies (5, 6) that treatment of cells with either itaconate or DMI has a broad effect on cellular metabolism. However, these metabolic effects do not appear to be due to accumulation of intracellular itaconate, and could be the result of electrophilic stress and covalent inactivation of select metabolic enzymes. Alternatively, itaconate or DMI could bind to surface receptors, such as succinate receptor 1 (SUCNR1/GPR91), which is expressed on macrophages (11), or an as yet unknown receptor to modulate macrophage activation, and thus act entirely extracellularly. It is tempting to speculate that accumulation of extracellular itaconate, which may occur following macrophage death, could provide an amplification signal to enhance the inflammatory activity of local macrophages. Indeed, a recent study has found that succinate release into the extracellular fluid in a model of antigen-induced arthritis can increase macrophage production of IL-β, in a GPR91-dependent manner (12).

Finally, suitable pro-drugs are needed to manipulate intracellular itaconate levels to probe its exact role in the mechanism

of macrophage activation and stimulation. Future pro-drug strategies to introduce itaconate into cells should avoid enhancing the electrophilicity of the conjugated acrylate, and focus on selective esterification or amidation of the distal carboxylate.

Materials and Methods

Dimethyl Itaconate and Itaconic Acid—Dimethyl itaconate and itaconic acid were purchased from Sigma (part numbers 109533 and I29204). [$^{13}C_5$]Itaconic acid was synthesized by the Metabolite Standards Synthesis Core at SRI International, arranged through the National Institutes of Health Common Fund's Metabolomics Initiative (13).

[$^{13}C_5$]Itaconic acid dimethyl ester ([^{13}C]DMI) was synthesized by the following procedure. A magnetic stir bar was added to a 10-ml dry round-bottom flask. Next, 5 ml of methanol was added, the flask was cooled to 0 °C on ice, and 1 ml of thionyl chloride was slowly added while stirring. [$^{13}C_5$]Itaconic acid (22 mg, 0.17 mmol) was dissolved in 1 ml of methanol, and then added dropwise to the mixture under a positive pressure of nitrogen for 1 h. The reaction was then warmed to room temperature and stirred overnight. TLC analysis indicated the consumption of starting materials. The solvent was gently removed by rotary evaporation, and the residue was directly purified by flash chromatography eluted with MeOH in dichloromethane (0–5%). Pure fractions of [$^{13}C_5$]itaconic acid dimethyl ester were collected (20 mg, 73%). The product was confirmed by NMR. Using ^{1}H NMR (401 MHz, CDCl$_3$): δ 6.75 − 6.00 (m, 1H), 5.98 − 5.36 (m, 1H), 3.75 (dd, J = 3.9, 1.0 Hz, 3H), 3.68 (dd, J = 3.9, 1.0 Hz, 3H), 3.32 (ddt, J = 130.2, 8.4, 4.7 Hz, 2H), while for ^{13}C NMR (101 MHz, CDCl$_3$) δ 171.28 (dt, J = 58.7, 3.3 Hz), 166.76 (dd, J = 71.8, 3.2 Hz), 133.74 (tdd, J = 72.1, 46.6, 3.0 Hz), 131.05 − 126.04 (m), 37.62 (dddd, J = 58.7, 46.7, 3.3, 1.8 Hz). Product was also confirmed by high resolution mass spectrometry (Fig. 1).

[$^{13}C_5$]Itaconic acid was synthesized by pyrolyzing [$^{13}C_6$] citric acid to [$^{13}C_5$]itaconic anhydride followed by hydrolysis to give [$^{13}C_5$]itaconic acid with an isotopic purity of 99%.[3]

Bone Marrow-derived Macrophages—Bone marrow-derived macrophages were derived from bone marrow of C57/BL mice and cultured with 30% L-Cell-enriched medium for 6 days prior to the experiment. Macrophage stimulation was performed in RPMI containing 10 mM glucose, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 10% FBS overnight using LPS (20 ng/ml) and IFN-γ (50 ng/ml). RAW 264.7 cells were obtained from the ATCC and cultured until a maximum passage number of 10. RAW cells were grown in DMEM supplied with 100 units/ml penicillin/streptomycin and 10% FBS.

Targeted Metabolite Profiling by LC-MS—Polar metabolites were separated by hydrophilic interaction chromatography using a Luna NH$_2$ column and detected using 6520 Agilent Q-TOF (Santa Clara, CA) as described previously (14–17). Metabolites were identified by accurate mass and by matching their retention time with standards.

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[3] L. Jong, L. Olson, and J. J. Tanga, manuscript in review.

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