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Itaconic acid is a mammalian metabolite induced during macrophage activation

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Abstract

Itaconic acid, or methylenesuccinic acid, is not generally classified as a mammalian metabolite. Using NMR based metabolomics and ¹³C-labeling, we have detected itaconic acid in both macrophage-like VM-M3 and RAW 264.7 tumor cell lines as well as stimulated and unstimulated primary murine macrophages. Macrophage activation by addition of lipopolysaccharide and IFN- γ markedly increased itaconic acid production and secretion. Crude cell extracts synthesize itaconic acid via decarboxylation of *cis*-aconitate, indicative of a novel mammalian *cis*-aconitic decarboxylase activity. Our results highlight a previously unidentified biosynthetic pathway related to TCA cycle metabolism in mammalian cells and a novel metabolite that likely plays a role in macrophage-based immune response.

Keywords

metabolomics; NMR; LC-MS; itaconic acid; tumor cells; macrophages

Small molecule effectors of cells are often overlooked. A case in point is itaconic acid (methylenesuccinic acid) – a metabolite made and secreted by the fungal organism *Aspergillus terreus*.^{1, 2} The biosynthesis of this dicarboxylic acid has been of interest because it can be used as a starting material for chemical synthesis of polymers.^{3, 4} More recently, itaconic acid (ITA) has been identified in a small number of cases of metabolomic analysis of mammalian tissue specimens^{5–7}. In those few cases, whether it was synthesized by mammalian cells or endogenous flora, or arose merely as an adventitious contaminant, was unknown. Despite the overall lack of study of ITA as a mammalian metabolite, there is evidence that ITA can be catabolized by both guinea pig and rat liver mitochondria.^{8,9}

The VM-M3 murine tumor cell line is derived from a spontaneously arising brain tumor in a VM mouse.¹⁰ This cell line, reported to be metastatic, is similar in morphology to the well-characterized RAW 264.7 murine macrophage cell line, and both share properties of macrophages including gene expression and phagocytic capability.^{11, 12} We detected itaconic acid (ITA) in 1D-gHSQC NMR experiments (obtained on a Varian 600 MHz

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Supporting Information. Details of VM-M3 and RAW 264.7 cell culture, murine peritoneal macrophage isolation, preparation of cell extracts, NMR and LC-MS methods are available as Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

VNMRS equipped with a triple resonance probe) with methanol/water (80:20) extracts of VM-M3 cells that had been incubated with [$^{13}\text{C}_5$]glutamine or [$^{13}\text{C}_6$]glucose. [$^{13}\text{C}_5$]glutamine resulted in detection of proton resonances at 3.15 ppm characteristic of the ITA signal for the $-\text{CH}_2-$ moiety; [$^{13}\text{C}_6$]glucose incubation gave rise to resonances consistent with labeled $-\text{CH}_2-$ and $=\text{CH}_2$ (at 5.37 and 5.85 ppm) groups¹³. The ^{13}C -labeled ITA was present at sufficiently high levels that it could easily be identified using 2D-gHSQC (Fig. 1A) and 2D-gHSQC-TOCSY experiments. The ^1H resonances arising from itaconate were also confirmed by doping cell extracts with an itaconate standard. Using the size of the VM-M3 cells and cell count used for the extracts, and quantifying the amount of ITA in the extracts using MS methods, we estimate the intracellular concentration of this compound to be 1.33 ± 0.16 mM.

^{13}C -labeled ITA was also detected by 1D-gHSQC in an extract of the culture medium (Fig. 2) in which cells were incubated with [$^{13}\text{C}_6$]glucose for 12 h. In order to detect the ITA, the large amount of [$^{13}\text{C}_6$]glucose was removed from the resolubilized extract with Strata-X-A solid phase extraction cartridges (see Supporting Information for detailed treatment). Thus, this molecule appears to be secreted by the cells.

ITA may be a metabolite associated with a wide variety of tumors, or it could occur in a unique subset. We therefore investigated ITA synthesis in the macrophage-like RAW 264.7 cell line and the unrelated MCF-7 breast cancer cell line. Although ^1H NMR has been used to profile the Raw 264.7 metabolome^{14, 15}, the relatively low concentration of ITA would make it difficult to detect in the absence of ^{13}C -labeling and selective analysis of protons coupled to ^{13}C . gHSQC spectra of extracts from unstimulated RAW 264.7 cells incubated with [$^{13}\text{C}_6$]glucose exhibited a detectable amount of ITA (Fig. 1B), while extracts from the MCF-7 cell line incubated with the labeled glucose did not contain NMR-detectable levels of ITA. Moreover, analysis of several unrelated cell types (*Escherichia coli* strain NCM3722, *Clostridium acetobutylicum* strain ATCC 824, *Saccharomyces cerevisiae* strain CEN-PK, cultured human foreskin fibroblasts, or pancreatic tumor tissue) by LC-MS failed to reveal detectable levels of ITA. It is noteworthy that stimulation of RAW 264.7 cells with lipopolysaccharide (LPS) resulted in an increased level of detectable ^{13}C -ITA (Fig. 1C); ITA was also detected in the tissue culture media of the RAW 264.7 cells as well as VM-M3 cells (Fig. 2). Thus, ITA synthesis is not a generic characteristic of all tumor cell lines, and might instead be indicative of those of macrophage lineage.

To test the possibility that ITA synthesis is a characteristic of macrophages, we analyzed unstimulated mouse peritoneal macrophages and activated macrophages following *ex vivo* stimulation with IFN- γ and LPS that were then incubated with [$^{13}\text{C}_6$]glucose. While very low levels of ITA could be detected in the unstimulated cell extracts (Fig. 1D), a dramatic increase was observed in the activated cells (Fig. 1E). This is consistent with the findings of Shin et al.⁵ who detected ITA in the lungs of mice infected with *Mycobacterium tuberculosis*, the primary site of inflammation and macrophage response to infection, but not other organs. As with the VM-M3 cells, ITA was also detected in the culture medium, but only in cells that had been stimulated with LPS. Therefore, production of ITA may represent a previously unrecognized facet of the macrophage-mediated immune response.

Multiple pathways have been proposed for the biosynthesis of ITA^{1, 16, 17} (Supplemental Information, Fig. S2). To distinguish among these, we used isotope tracers. The low concentration of ITA in the ^{13}C -labeled extracts made direct ^{13}C -detection by NMR impractical. Therefore, we used LC-MS measurements¹⁸ of ITA and its potential precursors including acetyl CoA, pyruvate, citrate and aconitate after incubating cells with [$^{13}\text{C}_6$]glucose or [$^{13}\text{C}_5$]glutamine (Fig. 3).

If the biosynthesis of ITA in mammalian cells occurs through the same pathway as in *Aspergillus* (decarboxylation of *cis*-aconitate followed by a 1,3-allylic rearrangement catalyzed by *cis*-aconitic decarboxylase, Fig. 4A)^{1, 19, 20}, then incubation of the cells with [¹³C₅]glutamine should yield [¹³C₄]ITA as the primary isotopologue from the first turn of the TCA cycle. This results from glutamine hydrolysis to glutamate followed by transamination and production of α -ketoglutarate, which then enters the TCA cycle. This would ultimately result in [¹³C₄]*cis*-aconitate that is site specifically decarboxylated to [¹³C₄]ITA (Fig. 4B). [¹³C₄]ITA is indeed the major isotopologue of itaconic acid present under these conditions (see Supplement Fig. S4). This pathway is further supported by the fact that we see vastly more ¹³C label at the –CH₂– position than the =CH₂ position by 1D-gHSQC NMR. Incubation of cells with [¹³C₆]glucose, with the same biosynthetic scheme, should generate [¹³C₁]ITA resulting from the [¹³C₂]acetyl CoA that enters the TCA cycle and is converted to *cis*-aconitate, with one of the labeled carbons site-specifically lost during the *cis*-aconitate decarboxylase reaction (Fig. 4C). This ¹³C flux through pyruvate dehydrogenase (PDH) does indeed result in the major isotopologues observed by MS, although there is also appreciable flux through pyruvate carboxylase (PC).^{21, 22} PC generates [¹³C₃]oxaloacetate that will ultimately result in the [¹³C₄]ITA isotopologues (Fig. 4D). By comparing the percentage of total isotopologues of ITA resulting from PDH and PDH+PC flux ([¹³C₁] and [¹³C₄], respectively) to the percentage of total isotopologues of anticipated precursors citrate and aconitate from both PDH and PDH+PC fluxes ([¹³C₂] and [¹³C₅], respectively), we see that the three metabolites show comparable percentages from each flux. The isotope labeling data we have obtained (Fig. 4E) are thus fully consistent with a biosynthetic pathway in mammalian cells whereby ITA is synthesized via the specific decarboxylation of *cis*-aconitate from the TCA cycle. The NMR data, which shows significantly higher ¹³C labeling at the =CH₂ resonance but still an appreciable amount of label at the –CH₂– resonance in the 1D-gHSQC spectrum, further supports this pathway. This route requires the presence of a mammalian homologue of fungal *cis*-aconitic decarboxylase (cADC).^{23, 24} Other likely routes can be ruled out based on the labeling data (see Supporting Information).

The decarboxylation reaction catalyzed by cADC is unique amongst carboxy-lyases. cADC is the only carboxy-lyase in the ExPASy ENZYME database that catalyzes a decarboxylation resulting in both the reduction of a β - γ unsaturation as well as the formation of a methyldene group α to the decarboxylation. There are no identifiable mammalian sequence homologues of the *Aspergillus* cADC protein in the NCBI gene database and no structures of the fungal protein to suggest key motifs that could aid in identifying any structural homologues. Hence, we used crude cell extracts to assess *in vitro* synthesis of ITA. Because the RAW 264.7 cells generated a large amount of ITA when stimulated with LPS, we used cell extracts of these to assess *in vitro* synthesis of ITA. Crude RAW 264.7 cell lysates, prepared by Dounce homogenization in a hypotonic buffer, were incubated with 4 mM *cis*-aconitate for 3 h at 37°C. Metabolites were extracted, lyophilized, and examined by ¹H NMR. There was a 40-fold increase in the intensity of the ITA ¹H resonances compared to control extracts (Fig. 5), made either without the addition of *cis*-aconitate or using a boiled cell extract to inactivate any enzyme(s). VM-M3 lysates also generated significant amounts of ITA from *cis*-aconitate (Supplement Fig. S2). Thus, macrophage-derived cells possess a highly unusual enzymatic activity previously observed only in a few fungi.

Although ITA can be used to form synthetic polymers, that is unlikely to be its biological function. What is the purpose of the synthesis and excretion of this novel metabolite? ITA has been shown to be a potent inhibitor of isocitrate lyase^{25, 26}, an enzyme of the glyoxylate cycle found in bacteria, so its secretion could be part of an antibacterial response. However, ITA has also been shown to inhibit phosphofructokinase²⁷, and thus, could potentially be

involved in regulation of metabolism. It is also possible that ITA acts as a signaling molecule involved in recruitment or regulation of other cells. That ITA levels in cells and culture supernatant significantly increase in response to IFN- γ and LPS points to a potential role in macrophage activation and/or macrophage effector responses. Macrophages are currently known to secrete over 100 substances of various molecular weights that are involved in inflammatory cell activation, proliferation, and effector cell migration.²⁸ Interestingly, Raw 264.7 conditioned medium has been shown to induce migration and metastasis in colon cancer cells, due at least partially to secretion of chemokines.²⁹ That the ITA metabolite was first noticed in a metastatic tumor cell line hints that it may also have a role in tumor biology. Observation of this novel metabolite opens up an array of future studies to determine its physiological function, including in the mammalian immune system and in cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) 2D-gHSQC spectrum of methanol extracts of VM-M3 cells (8×10^6 cells) incubated with 25 mM [$^{13}\text{C}_6$]glucose for 12 h. Inset: structure of itaconate. Slices from 2D-gHSQC spectra diagnostic for ITA are shown for extracts of RAW 264.7 cells (10^7 cells) that were (B) unstimulated or (C) stimulated with 10 ng/mL LPS for 24 h, and extracts from murine macrophages (8×10^6 cells) that were (D) unstimulated or (E) stimulated by priming with 150 U/mL IFN- γ for 6 h followed by 24 h incubation with 10 ng/mL LPS prior to incubation with 25 mM [$^{13}\text{C}_6$]glucose for 12 hours. For spectra (A)–(C), nt=128 and ni=128. For spectra (D) and (E), nt=256 and ni=128.

Figure 2. 1D gHSQC spectra of culture media from (A) RAW 264.7 cell cultures stimulated with 10 ng/ml LPS and (B) VM-M3 cell cultures that were incubated with [U- ^{13}C]glucose for 12 h, then treated to isolate weak acids including ITA (*). 6400 transients were collected for each spectrum.

Figure 3.

Representative chromatographic traces for the detection of itaconic acid. (A) Standard of citraconic acid (5 $\mu\text{g/mL}$), a structural isomer of itaconic acid, has a retention time of 13.2 min and detected mass is 129.0196 (error of 2.3 ppm) compared to the theoretical mass of 129.0193, in negative ion mode. (B) Standard of itaconic acid (5 $\mu\text{g/mL}$), has a retention time of 12.70 min and detected mass of 129.0195 (1.5 ppm error). (C) Itaconic acid detected in the cellular extract of VM-M3 cell line, with a detected mass of 129.0195 (1.5 ppm error).

Figure 4.

ITA labeling schemes and isotopologues distribution in itaconate, aconitate and citrate for the first turn of the TCA cycle in VM-M3 cells incubated for 12 h with $[\text{U-}^{13}\text{C}]\text{glutamine}$ or $[\text{U-}^{13}\text{C}]\text{glucose}$. ITA labeling scheme from $[\text{U-}^{13}\text{C}]\text{glutamine}$ ((A, $\blacklozenge$)) and $[\text{U-}^{13}\text{C}]\text{glucose}$ (B and C) if the *cis*-aconitic decarboxylase (cADC) pathway is active. $[\text{U-}^{13}\text{C}]\text{glucose}$ enters glycolysis and results in $^{13}\text{C}_3$ -pyruvate, which can enter the TCA cycle as $^{13}\text{C}_2$ -acetyl CoA through pyruvate dehydrogenase (PDH), or as $^{13}\text{C}_3$ -oxaloacetate through pyruvate carboxylase (PC). The isotopologues of ITA synthesized by this pathway should be predominantly $^{13}\text{C}_1$ (PDH flux only, (B, $\bullet$)) or $^{13}\text{C}_4$ (both PC and PDH flux, (C, $\bullet$)). (D) Comparison of isotopologues of itaconic acid, citric acid and aconitate in VM-M3 cells after incubation with $[\text{U-}^{13}\text{C}]\text{glucose}$: isotopologues resulting from PDH flux $\blacksquare$, or PC/PDH flux $\hatchedbox$.

Figure 5.

Cis-aconitate decarboxylase (cADC) activity in RAW 264.7 crude cell lysates. Comparison of ¹H spectra (600 MHz) of stimulated RAW 264.7 crude extracts incubated at 37°C in the presence (A) or absence (B) of 4 mM *cis*-aconitate for 3 h. The resonance at 5.67 ppm is residual *cis*-aconitate (c-A).