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Detection of nonsterol isoprenoids by HPLC–MS/MS

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ABSTRACT

Isoprenoids constitute an important class of biomolecules that participate in many different cellular processes. Most available detection methods allow the identification of only one or two specific nonsterol isoprenoid intermediates following radioactive or fluorescent labeling. We here report a rapid, nonradioactive, and sensitive procedure for the simultaneous detection and quantification of the eight main nonsterol intermediates of the isoprenoid biosynthesis pathway by means of tandem mass spectrometry. Intermediates were analyzed by HPLC–MS/MS in the multiple reaction monitoring mode using a silica-based C_{18} HPLC column. For quantification, their stable isotope-labeled analogs were used as internal standards. HepG2 cells were used to validate the method. Mevalonate, phosphomevalonate, and the six subsequent isoprenoid pyrophosphates were readily determined with detection limits ranging from 0.03 to 1.0 μmol/L. The intra- and interassay variations for HepG2 cell homogenates supplemented with isoprenoid intermediates were 3.6–10.9 and 4.4–11.9%, respectively. Under normal culturing conditions, isoprenoid intermediates in HepG2 cells were below detection limits. However, incubation of the cells with pamidronate, an inhibitor of farnesyl pyrophosphate synthase, resulted in increased levels of mevalonate, isopentenyl pyrophosphate/dimethylallyl pyrophosphate, and geranyl pyrophosphate. This method will be suitable for measuring profiles of isoprenoid intermediates in cells with compromised isoprenoid biosynthesis and for determining the specificity of potential inhibitors of the pathway.

The isoprenoid biosynthesis pathway (Fig. 1) plays an important role in cellular metabolism. It provides the cell with a variety of compounds serving a number of different functions. In addition to sterols involved in maintaining membrane fluidity and required for the synthesis of hormones, bile acids, and oxysterols, the pathway produces a variety of nonsterol isoprenoids. Examples of these are the side chains of ubiquinone-10 and heme A (which function in the mitochondrial respiratory chain), dolichol (required for protein glycosylation), isopentenyl tRNA (involved in protein translation), and the farnesyl and geranylgeranyl moieties of isoprenylated proteins such as the small GTPases. Although isoprenoids are rather diverse in structure and function, they all are derived from the basic C5 isoprene units isopentenyl pyrophosphate (IPP)[1] and dimethylallyl pyrophosphate (DMAPP). These C5 isoprene units are synthesized in the nonsterol, pre-squalene part of the isoprenoid biosynthesis pathway, also known as the mevalonate pathway [1,2]. The mevalonate pathway starts with three acetyl-CoAs, which are converted into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in two consecutive enzyme steps. HMG-CoA is then converted into mevalonate (MVA) by the rate-limiting enzyme of the pathway, HMG-CoA reductase. Subsequently, MVA is phosphorylated twice, which produces 5-pyrophosphomevalonate (MVAPP). Decarboxylation of the latter compound yields IPP. After isomerization of IPP to DMAPP, a head-to-tail conden-

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[1] *Abbreviations used:* MVA, mevalonate; MVAP, 5-phosphomevalonate; MVAPP, 5-pyrophosphomevalonate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HPLC-MS/MS, high-performance liquid chromatography–tandem mass spectrometry; FPPS, farnesyl pyrophosphate synthase; MVAL, mevalonolactone; MBP, maltose-binding protein; MK, mevalonate kinase; PMK, phosphomevalonate kinase; IS, internal standard; MPD, mevalonate pyrophosphate decarboxylase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MRM, multiple reaction monitoring; LOQ, limit of quantification; LOD, limit of detection.

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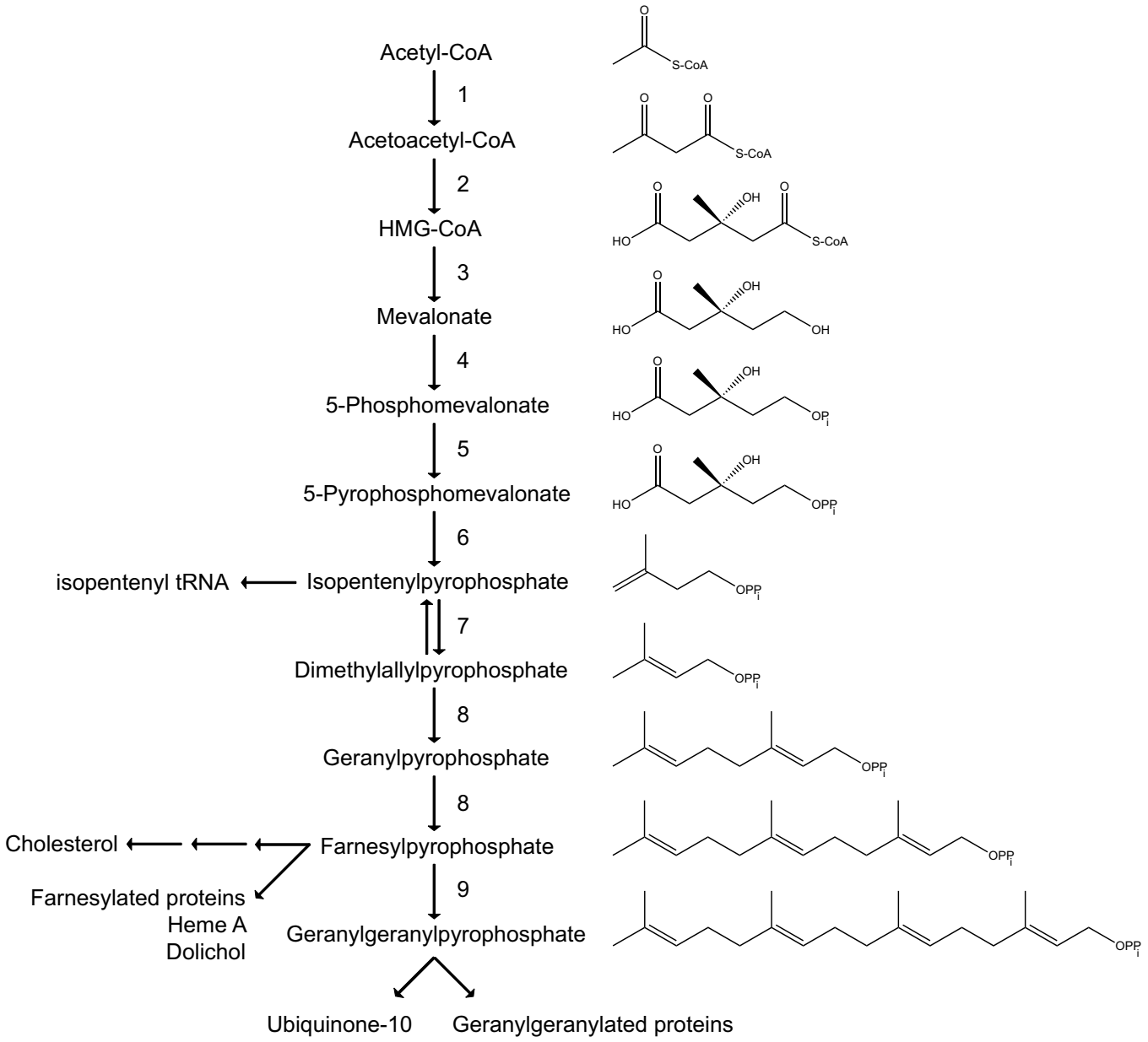

Fig. 1. Isoprenoid biosynthesis pathway. The different enzymes involved are numbered as follows: (1) acetoacetyl-CoA thiolase, (2) 3-hydroxy-3-methylglutaryl-CoA synthase, (3) 3-hydroxy-3-methylglutaryl-CoA reductase, (4) mevalonate kinase, (5) phosphomevalonate kinase, (6) mevalonate pyrophosphate decarboxylase, (7) isopentenyl pyrophosphate isomerase, (8) farnesyl pyrophosphate synthase, and (9) geranylgeranyl pyrophosphate synthase.

sation of IPP to DMAPP results in the formation of geranyl pyrophosphate (GPP). Addition of another IPP gives farnesyl pyrophosphate (FPP), the branch point metabolite of the pathway, which is the precursor of geranylgeranyl pyrophosphate (GGPP); GGPP is produced by the condensation of one FPP with one IPP molecule.

Different methods for the detection of intermediates of the mevalonate pathway have been described in the literature. Most of these methods allow the detection of only one specific compound, for example, the detection of MVA in human urine and plasma [3–7] and dog plasma [8]; DMAPP in plant leaves, yeast, and bacteria [9]; and FPP in human and dog plasma [10] and yeast [11]. In addition, methods have been described for the simultaneous determination of FPP and GGPP in rat liver [12] and cultured NIH3T3 cells [13] and the detection of IPP and FPP in mouse and rat liver [14]. Measuring all the intermediates of the mevalonate pathway in one procedure is a major challenge, because the metabolites differ markedly in structure and physical properties. Indeed, only McCaskill and Croteau [15] reported a procedure for the analysis of all 11 intermediates of the mevalonate pathway from acetyl-CoA through GGPP in plant cells, while Zhang and Poulter [16] described a method to analyze the phosphorylated isoprenoid intermediates. Both procedures require incubation of cells or purified enzymes with radiolabeled precursors, after which metabolites are detected by HPLC with radiodetection.

Here we report the development of a sensitive method using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) that allows the direct detection and quantification of all intermediates of the mevalonate pathway without the use of radioactive or fluorescent compounds. The applicability of our procedure was demonstrated by the analysis