The conversion of the two-carbon acetyl-CoA to the twenty-seven-carbon, tetracyclic cholesterol via the mevalonate pathway is a remarkable feat of anabolic engineering. Its earliest steps yield mevalonate, followed by isoprenoid precursors that condense to produce the squalene backbone of cholesterol (Fig. 1). Oxygenation and cyclization form the steroid nucleus, upon which the pathway bifurcates into two parallel branches, Bloch and Kandutsch-Russell, each involving successive rounds of reduction and demethylation. Ultimately, the synthesis of a single cholesterol molecule expends approximately one hundred ATP equivalents and eleven oxygen molecules.

It comes as no surprise, then, that the complexity of cholesterol synthesis is matched only by its exquisite regulation. During sterol excess, pathway flux is curtailed in the short-term by accelerated degradation of the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), which generates mevalonate, and in the long-term by inactivation of sterol regulatory element binding protein-2 (SREBP-2), a master transcriptional regulator that promotes cholesterol synthesis and uptake (1). In a classic example of feedback inhibition, cholesterol suppresses its own production by preventing the proteolytic cleavage and maturation of SREBP-2 (2). However, it does not induce HMGCR turnover (2). Indeed, since the pioneering work of Kandutsch and Chen over forty years ago, it has been understood that cholesterol is not the sole sterol regulating this pathway (3, 4). For instance, oxidized derivatives of cholesterol such as 25-hydroxycholesterol are potent inducers of both HMGCR degradation and SREBP-2 inactivation (5). Yet the contribution of mevalonate pathway intermediates to cholesterol homeostasis has remained elusive—and even controversial.

LANOSTEROL AND HMGCR

With respect to feedback regulation, lanosterol is perhaps the closest-studied pathway intermediate. Named for its 1930 isolation from lanolin, a wax found in sheep’s wool, it is the first sterol to be formed during cholesterol synthesis. Lanosterol was directly implicated in regulating this pathway when its addition to cell culture medium accelerated the degradation of HMGCR, an activity that was shared by its Kandutsch-Russell counterpart 24,25-dihydrolanosterol (6). The activity of these molecules was reinforced by their accumulation during hypoxia, when HMGCR undergoes rapid turnover (7). Curiously, neither lanosterol nor 24,25-dihydrolanosterol influenced SREBP-2 activation (6, 7), implying a feedback mechanism distinct from both cholesterol and oxysterols. However, later studies attributed the apparent activity of exogenous lanosterol to contamination with 24,25-dihydrolanosterol, proposing that lanosterol itself has no effect on HMGCR (8) and cholesterol synthesis (9).

In the subsequent decade, research into the regulatory functions of pathway intermediates such as lanosterol has been stymied by a reliance on exogenous sterols. Such an approach cannot guarantee the uptake and transport of hydrophobic intermediates to the endoplasmic reticulum, where sterol-sensing occurs, nor their delivery at concentrations required for activity. As highlighted by the lanosterol debate, impurities in commercial preparations can also be confounding. A preferable alternative is to study endogenous sterol intermediates; however, this carries unique challenges owing to their low abundance. While accumulation can be achieved by the knockdown or pharmacological inhibition of biosynthetic enzymes, the efficiency of these methods may vary between targets.

A NEW CLASS OF ENDOGENOUS REGULATORS

In this issue of the Journal of Lipid Research, Chen and colleagues (10) use an elegant genetic approach to address
the challenge of elevating endogenous mevalonate pathway intermediates. First, the overexpression of a mevalonate transporter enables pathway flux to be upregulated by mevalonate treatment, thereby increasing intermediate levels by over an order of magnitude. This is coupled with systematic CRISPR-Cas9 knockout of select pathway enzymes, which accumulates intermediates upstream of the blockage. Finally, preincubation in sterol-depleted medium increases SREBP-2 activity and upregulates cholesterol synthesis genes, thereby maximizing intermediate formation and steady-state levels of HMGCR. Following mevalonate treatment and lipidomic confirmation of intermediate accumulation, changes in the rate of HMGCR degradation and SREBP-2 inactivation are determined.

The authors first establish that intermediates of the committed cholesterol synthesis pathway are essential for feedback regulation by ablating squalene synthase (FDEFT1) (10). This blocks the mevalonate-induced inactivation of HMGCR and SREBP-2, corroborating previous reports that products of the isoprenoid branch of the mevalonate pathway augment, but do not directly induce downregulation of cholesterol synthesis (11). Chen et al. (10) next knock out DHCR24, an enzyme that catalyses the C24-reduction of sterol side chains to dictate their entry into the Kandutsch-Russell pathway, as well as the final conversion of desmosterol to cholesterol. Loss of DHCR24 expectedly depletes Kandutsch-Russell intermediates; however, HMGCR and SREBP-2 regulation persists, indicating that Bloch pathway sterols are sufficient for feedback control. To narrow down the responsible intermediates, the authors also target MSMO1, the initiating enzyme of the sterol C4-demethylation complex. The resultant accumulation of C4-dimethylated intermediates, including lanosterol and 24,25-dihydrolanosterol, dramatically accelerates HMGCR degradation while maintaining the rate of SREBP-2 inactivation. Simultaneous knockout of DHCR24 does not reduce this sensitivity to mevalonate, further reinforcing that Bloch pathway intermediates are capable of regulating HMGCR and SREBP-2. In this manner, Chen et al. establish C4-dimethylated sterols as endogenous regulators of cholesterol homeostasis.

The potent induction of HMGCR turnover by these intermediates is consistent with a previous study using a panel of exogenous sterols, where a 4,4-dimethyl moiety was the major determinant of HMGCR regulation (6). Accordingly, synthetic sterol analogs containing this functional group efficaciously reduce HMGCR levels in vivo (12). Chen et al. (10) also show that mevalonate-induced pathway flux preferentially accumulates C4-dimethylated intermediates, lending credence to their regulatory function. Intriguingly, these sterol intermediates are translocated to the plasma membrane during cholesterol synthesis, where they are preferentially exported from the cell (13). This may serve as an additional layer of regulation that restricts the access of these intermediates to homeostatic effectors in the endoplasmic reticulum, thereby averting rapid downregulation of HMGCR. The effect of these sterols on active SREBP-2 is comparatively less pronounced, however, suggesting that downstream intermediates or cholesterol itself are the major regulators of this aspect of homeostasis.

Furthermore, while Bloch pathway intermediates are certainly sufficient for feedback regulation, their precise contribution remains unclear. Relative to cells with an intact mevalonate pathway, mevalonate treatment of DHCR24-null cells induces a several-fold accumulation of Bloch sterols, but only minor downregulation of HMGCR and SREBP-2. Likewise, mevalonate sensitivity is similar between MSMO1 and MSMO1-DHCR24 knockouts, despite dramatic elevation of Bloch intermediates in the latter. This may represent saturation of the effectors that respond to these intermediates. Alternatively, Kandutsch-Russell sterols may exhibit greater potency than Bloch sterols. This possibility is intriguing, given that flux through the Kandutsch-Russell pathway is...
pathway is considered constitutive, whereas Bloch pathway activity is altered depending on sterol availability (14). The tissue specificity of these pathways—with Kandutsch-Russell favored in muscle, skin and brain, yet minimal in the testes and adrenal gland (14)—further suggests that shifts in pathway utilization may fine-tune cholesterol synthesis by generating intermediates with higher or lower regulatory activity. This possibility awaits further investigation.

**LANOSTEROL AND 24,25-DIHYDROLANOSTEROL: STRUCTURALLY RELATED YET FUNCTIONALLY DISTINCT**

Importantly, Chen et al. (10) also address the controversial contributions of lanosterol and 24,25-dihydrolanosterol to HMGCR degradation. Using dual-knockout of CYP51A1 and DHCR24, they show that the accumulation of lanosterol alone can potentially induce HMGCR turnover, thus validating initial reports of its regulatory capacity (6, 7). However, SREBP-2 regulation is abolished in these cells, suggesting that 24,25-dihydrolanosterol and other intermediates downstream of CYP51A1 are required for inactivation of SREBP-2. The ability of CYP51A1 knockout cells to maintain negative regulation of SREBP-2 is unexpected, given previous reports that 24,25-dihydrolanosterol has no effect on its activity (6). However, this may reflect differences between exogenous and endogenous sterol.

One outstanding question is why, in stark contrast to other C4-dimethylated intermediates, lanosterol is selective in regulating HMGCR but not SREBP-2. Oxysterols regulate both of these proteins by binding with Insigs, which facilitate the ubiquitination of HMGCR and bind Scap to retain SREBP-2 in the endoplasmic reticulum (5). A similar mechanism may account for the broad action of C4-dimethylated sterols, warranting investigation into whether these molecules associate with Insigs. On the other hand, the SREBP-2-specificity of cholesterol results from direct binding to Scap, which promotes interaction with Insig (2). It is possible that lanosterol similarly achieves HMGCR specificity by directly binding the enzyme. In this case, C24-saturation by DHCR24 to form 24,25-dihydrolanosterol would confer quite different activity, raising the question of whether other pairs of Bloch and Kandutsch-Russell intermediates display divergent mechanisms of regulation, despite sharing structural similarities.

Beyond SREBP-2 and cholesterol biosynthesis enzymes, a third element of cholesterol homeostasis is the activity of the liver X receptor (LXR), which controls genes that counter cholesterol acquisition and promote its efflux. The cholesterol synthesis intermediates 24,25-dihydrolanosterol, FF-MAS, and desmosterol have previously been recognized as endogenous LXR agonists (4). Therefore, the genetic approach devised by Chen et al. (10) would be useful to further characterize these molecules, or indeed any cellular process modulated by mevalonate pathway intermediates. For instance, C4-dimethylated sterols are implicated in the activation of the RORγ nuclear receptor (15), while FF-MAS and T-MAS are thought, albeit tentatively, to function in gamete formation (16). To further characterize intermediate activity, overexpression of preceding enzymes (17) may be able to complement the knockout of downstream enzymes. Altogether, Chen and colleagues have extended our understanding of the finely tuned feedback control of cholesterol homeostasis, as well as clarifying the long-debated regulatory activity of lanosterol. Moreover, they lay a methodological groundwork for studying the functions of lowly abundant, and often underappreciated, intermediates of cholesterol synthesis.

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