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An update on oxysterol biochemistry: New discoveries in lipidomics

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ABSTRACT

Oxysterols are oxidised derivatives of cholesterol or its precursors post lanosterol. They are intermediates in the biosynthesis of bile acids, steroid hormones and 1,25-dihydroxyvitamin D_3. Although often considered as metabolic intermediates there is a growing body of evidence that many oxysterols are bioactive and their absence or excess may be part of the cause of a disease phenotype. Using global lipidomics approaches oxysterols are underrepresented encouraging the development of targeted approaches. In this article, we discuss recent discoveries important in oxysterol biochemistry and some of the targeted lipidomic approaches used to make these discoveries.

1. Introduction

Oxysterols came to prominence in the late 1970's with the *oxysterol hypothesis* which proposed that the suppressive effect of cholesterol on its own synthesis is mediated through oxysterols not by cholesterol itself [1]. This has proved to be only partly true, as cholesterol homeostasis in cells is modulated through oxysterols, side-chain oxysterols inhibiting the SREBP2 (sterol regulatory element-binding protein 2) pathway and also activating liver X receptors (LXRs) [2,3], although cholesterol itself is in fact the major regulator of its own synthesis through binding to SCAP (SREBP cleavage activating protein) and preventing transport of SERBP2 from the endoplasmic reticulum for processing to its active form as a transcription factor for genes of the cholesterol biosynthesis pathway [4]. In recent years oxysterols have been shown to have important functions in immunology [5–13], development [14,15] and cancer [16–20]. This has stimulated wide-spread interest in their analysis using lipidomics technology [21,22]. Although mostly thought of as oxidised forms of cholesterols, oxysterols can also be formed from precursors of cholesterol greatly widening the range of molecules required to be analysed in a lipidomic study.

2. Mass spectrometry-based technologies

Oxysterols tend not to be observed in global lipidomic analysis, whether shot-gun based electrospray ionisation – mass spectrometry (ESI-MS) or liquid chromatography (LC)-MS based. This is because of their comparatively low-abundance and poor ionisation characteristics. However, methods have been developed for shot-gun ESI – tandem MS (MS/MS) [23] and LC-MS/MS analysis [21,22]. Gas chromatography (GC)-MS also provides an excellent method for oxysterol and sterol analysis [24], but is less favoured in lipidomics laboratories.

2.1. Targeted LC-MS/MS analysis

Russell and McDonald and co-workers [21,22] have developed LC-MS/MS lipidomics protocols for oxysterol analysis based on the classical sample preparation method of Dzeletovic et al. [24] and multiple reaction monitoring (MRM) using a triple quadrupole mass spectrometer exploiting $[M + NH_4]^+$ adducts for fragmentation. They have made the largest study to date in terms of sample numbers, analysing 3230 serum samples for 60 sterols [22]. The McDonald and Russell method is the template on which many other LC-MS/MS studies have been made [25]. An inherent

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drawback, however, of targeted approaches is that only targeted oxysterol are detected and unexpected ones are missed.

2.2. LC-MS/MS with derivatisation

The concept of targeted oxysterol analysis has been extended with the use of derivatisation methods to enhance sensitivity. Derivatisation of hydroxy groups to dimethylglycine or *N,N*-dimethylaminobutyrate esters has recently gained popularity for the diagnosis of Niemann Pick (NP) type B and C disease where 7-oxocholesterol (7-OC) and cholestane-3β,5α,6β-triol (3β,5α,6β-triol, see Supplementary Table for a list of common and systematic names and abbreviations and Supplementary Figures 1 & 2 for structures) are elevated in plasma of NP patients [26,27], while derivatisation to picolinate esters has proved successful for analysis of both sterols and oxysterols [28]. “Primary” oxystrols have a 3β-hydroxy group, however, once 7α-hydroxylated, the 3β-hydroxy-5-ene function can be oxidised to a 3-oxo-4-ene by the enzyme hydroxysteroid dehydrogenase (HSD) 3B7 (Supplementary Figure 1). The 3-oxo group is readily derivatised by hydrazine reagents, e.g. quaternary nitrogen containing Girard hydrazines, (hydrazinocarbonylmethyl)trimethylammonium chloride, GT or 1-(hydrazinocarbonylmethyl)pyridinium chloride, GP [29], or hydroxylamine reagents, e.g. *O*-(3-trimethylammoniumpropyl) hydroxylamine bromide [30], to enhance sensitivity during plasma analysis, valuable for the identification of patients with inborn errors of metabolism. The 3β-hydroxy-5-ene function found in “primary” oxysterols can be oxidised *ex vivo* by bacterial cholesterol oxidase to the 3-oxo-4-ene function [31], this has been exploited with subsequent Girard derivatisation, using GP or GT, in numerous studies to enhance the sensitivity of oxysterol analysis [8,10,19,32–34]. Despite advantages, derivatisation technology is often criticised for being laborious and difficult to automate.

3. Lipidomics and oxysterol biochemistry

3.1. Cholesterol 25-hydroxylase, 25-hydroxycholesterol and the immune system

Cholesterol 25-hydroxylase (CH25H in man and Ch25h in mouse) is the enzyme which oxidises cholesterol to 25-hydroxycholesterol (25-HC) (Supplementary Figure 1). CH25H is not a cytochrome P450 (CYP), but a member of a family of enzymes that utilize diiron cofactors to catalyse hydroxylation. Some CYP enzymes also have some 25-hydroxylase activity, but often secondary to their main catalytic functions [35]. In a major LC-MS lipidomic study investigating the effect of Kdo2-lipid A, the active component of an inflammatory lipopolysaccharide (LPS), on the mouse macrophage cell line RAW264.7, Dennis et al. found 25-HC to be elevated in abundance, correlating with a 4-fold increase in *Ch25h* mRNA [36]. The mRNA increase was 15-fold in bone marrow derived macrophages. In a parallel study, Bauman et al. showed that stimulation of macrophage Toll-like receptor 4 (TLR4) induced *Ch25h* expression and 25-HC synthesis and that 25-HC suppressed interleukin-2 (IL-2) mediated stimulation of B-cell proliferation, repressed activation induced cytidine deaminase (AID) expression, and blocked class switch recombination, leading to markedly decreased IgA production [5] (Fig. 1). Suppression of IgA class switching in B cells in response to TLR activation provides a mechanism for local and systemic negative regulation of the adaptive immune response by the innate immune system [5]. *Ch25h* is an interferon (IFN) regulated gene and upon viral infection, or IFN-stimulation, 25-HC is synthesised by macrophages and acts as a potent paracrine inhibitor of viral infection demonstrating a role in the innate immune pathway [8].

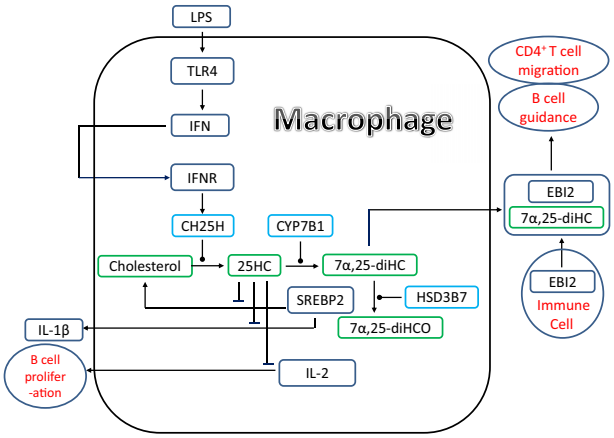

Fig. 1. Schematic representation of the regulation of immune responses by 25-HC and 7α,25-diHC.

Reboldi et al. have shown that activated macrophages from the *Ch25h*$^{-/-}$ mouse overproduce inflammatory IL-1 family cytokines [9]. They proposed that 25-HC represses *Il1b* transcription and repressed IL-1 activating inflammasomes by repressing the SREBP2 pathway [9]. Reboldi et al. also found that *Ch25h*$^{-/-}$ mice exhibited increased sensitivity to septic shock, exacerbated experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis, and a stronger ability to repress bacterial growth. They concluded that 25-HC is a mediator in the negative feedback pathway of IFN-signalling on IL1-family cytokine production and inflammasome activity. In agreement with the multiple sclerosis mouse model, Crick et al. in a LC-MS based sterolomic study exploiting GP-derivatisation found reduced plasma levels of 25-HC in plasma of multiple sclerosis patients [34].

Further understanding of the role of 25-HC in the inflammatory system comes from the work of Dang et al. [12], who confirmed that type 1 IFN restrains 1L-1β driven inflammation in macrophages by up-regulating *Ch25h* and 25-HC and repressing SREBP2 driven cholesterol synthesis. In the absence of *Ch25h*, cholesterol overload triggers mitochondrial DNA release and activation of AIM2 (absent in melanoma 2) inflammasomes in activated macrophages [12]. The inflammasome promotes the maturation of the inflammatory cytokines e.g. IL-1β by recruitment and activation of caspase-1 that processes IL-1β into its active form. Consequently, in *Ch25h*$^{-/-}$ macrophages there is an exaggerated response to pathogen derived activators.

There is just one report of CH25H deficiency in man. Goenka et al. reported on five unrelated infants born to consanguineous parents showing homozygous deletions of *CH25H* and the adjacent gene *LIPA* (lipase A, lysosomal acid type) in all five children [37]. *LIPA* encodes the enzyme lysosomal acid lipase, deficiency in which leads to Wolman disease in infants, a lysosomal storage disease characterised by accumulation of cholesterol esters. Neonatal BCG (Bacillus Calmette–Guérin) vaccination against *Mycobacterium tuberculosis* infection was performed on four of the children and in three local BCG abscesses developed. Otherwise, none of the children had any other history of prolonged, unusual or recurrent infections [37]. It is tempting to speculate that the absence of macrophage derived 25-HC leads to an exaggerated response to the BCG vaccination and abscess.

In contrast to the findings of Reboldi et al. [9], Chalmin et al. found that deletion of *Ch25h* (*Ch25h*$^{-/-}$) attenuated EAE [11], limiting the trafficking of CD4^{+} T cells to the central nervous

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