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Research Paper

Probes for protein adduction in cholesterol biosynthesis disorders: Alkynyl lanosterol as a viable sterol precursor

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ABSTRACT

The formation of lipid electrophile-protein adducts is associated with many disorders that involve perturbations of cellular redox status. The identities of adducted proteins and the effects of adduction on protein function are mostly unknown and an increased understanding of these factors may help to define the pathogenesis of various human disorders involving oxidative stress. 7-Dehydrocholesterol (7-DHC), the immediate biosynthetic precursor to cholesterol, is highly oxidizable and gives electrophilic oxysterols that adduct proteins readily, a sequence of events proposed to occur in Smith-Lemli-Opitz syndrome (SLOS), a human disorder resulting from an error in cholesterol biosynthesis. Alkynyl lanosterol (*a*-Lan) was synthesized and studied in Neuro2a cells, *Dhcr7*-deficient Neuro2a cells and human fibroblasts. When incubated in control Neuro2a cells and control human fibroblasts, *a*-Lan completed the sequence of steps involved in cholesterol biosynthesis and alkynyl-cholesterol (*a*-Chol) was the major product formed. In *Dhcr7*-deficient Neuro2a cells or fibroblasts from SLOS patients, the biosynthetic transformation was interrupted at the penultimate step and alkynyl-7-DHC (*a*-7-DHC) was the major product formed. When *a*-Lan was incubated in *Dhcr7*-deficient Neuro2a cells and the alkynyl tag was used to ligate a biotin group to alkyne-containing products, protein-sterol adducts were isolated and identified. In parallel experiments with *a*-Lan and *a*-7-DHC in *Dhcr7*-deficient Neuro2a cells, *a*-7-DHC was found to adduct to a larger set of proteins (799) than *a*-Lan (457) with most of the *a*-Lan protein adducts (423) being common to the larger *a*-7-DHC set. Of the 423 proteins found common to both experiments, those formed from *a*-7-DHC were more highly enriched compared to a DMSO control than were those derived from *a*-Lan. The 423 common proteins were ranked according to the enrichment determined for each protein in the *a*-Lan and *a*-7-DHC experiments and there was a very strong correlation of protein ranks for the adducts formed in the parallel experiments.

1. Introduction

The post-lanosterol pathway for the biosynthesis of cholesterol involves a complex series of chemical transformations [1]. Over 30 discrete compounds are intermediates between lanosterol and cholesterol, with the Bloch and Kandutsch-Russell parallel pathways differing only by unsaturation in the tail of the molecule. An abbreviated Scheme showing a simplified pathway is described in Fig. 1. A number of human disorders have been linked to deficiencies in the enzymes that catalyze the various demethylations, oxidations and reductions in the biosynthetic pathway [2–4]. Desmosterolosis, for example, derives from mutations in the enzyme involved in the conversion of desmosterol to cholesterol [5] while a more common disorder, Smith-Lemli-Opitz syndrome (SLOS), results from errors in the gene that encodes 7-dehydrocholesterol reductase, the enzyme that promotes the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol. SLOS is an autosomal recessive disorder with a *DHCR7* mutation carrier frequency of about 1% [6–10]. The phenotype is broad, with severe cases suffering pre-term demise and mild cases having minor physical findings with associated learning and behavioral problems [11]. A

Abbreviations: 7-DHC, 7-dehydrocholesterol; Lath, lathosterol; Lan, lanosterol; Chol, cholesterol; SLOS, Smith-Lemli-Opitz syndrome; DHCR7, 7-dehydrocholesterol reductase; MS, mass spectrometry; APCI, atmospheric pressure chemical ionization; HPLC-, high pressure liquid chromatography; MeOH, methanol; NMR, nuclear magnetic resonance; FBS, fetal bovine serum; DMEM, Dulbecco's Modified Eagle Medium; PBS, phosphate buffered saline; DMSO, dimethylsulfoxide

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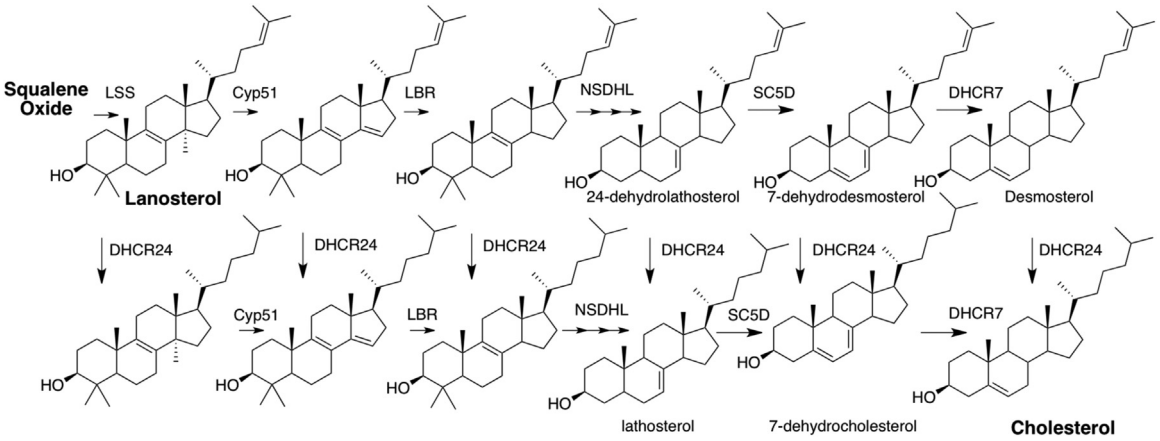

Fig. 1. Partial Scheme for Cholesterol Biosynthesis. Bloch pathway, Top Row of Sterols; Kandutsch-Russell Pathway, Bottom Row.

plasma sterol analysis showing an elevated dehydrocholesterol level and reduced cholesterol level forms the primary biochemical basis of a positive SLOS diagnosis.

Plasma and tissue levels of 7-DHC found in SLOS patients can be over 10,000-fold higher than those found in control individuals and recent evidence suggests that the pathology associated with the syndrome may be due in large part to the elevated levels of this sterol [12,13]. 7-DHC has the highest rate constant for the propagation of free radical peroxidation of any lipid studied to date [14,15] and the oxysterols formed in the process are toxic to primary cortical neuronal and glial cells in vitro and accelerate differentiation and arborization of cortical neurons [16,17]. The result of a mutation in *DHCR7* then, is a sequence of events that includes: 1. An increase of 7-DHC levels in tissues and fluids. 2. Oxidation of this vulnerable sterol to give a set of oxysterols, some of which are highly electrophilic. 3. Adduction of the reactive 7-DHC-derived electrophiles to target proteins.

To enquire if 7-DHC-derived electrophiles interact with the cellular proteome, cholesterol precursors bearing attached alkynyl groups were prepared and these compounds were evaluated as sterol surrogates in cell culture models and then used to identify lipid-adducted proteins. Protein adduction by reactive electrophiles likely contributes to chemical toxicities and oxidative stress, but the functional impact of adduction across proteomes is poorly understood [18]. Indeed, the isolation and identification of protein adducts of lipid electrophiles is a not a trivial exercise and various strategies have been developed to aid in this effort [19]. In this regard, we and others have utilized various lipid analogs that are modified with an alkyne functional group which serves as a "tag" that can be used to visualize the sterols in cells or to pull-down lipid-protein adducts by "click" methods [20–23]. In our hands, this approach has permitted the identification of adduct proteomes of various reactive species derived from fatty acid peroxidation [18,19], and it has provided evidence that proteins are modified by reactive electrophiles derived from 7-DHC. We now report that an alkyne-modified lanosterol can serve as a viable surrogate in cholesterol biosynthesis, completing the sequence of eighteen enzymatically promoted steps required for its conversion to alkynyl cholesterol in Neuro2a cells and in human fibroblasts. This permits an *in situ* analysis of the proteome adducted by sterol electrophiles formed in a Neuro2a cellular model of SLOS.

2. Results

2.1. Alkynyl sterols

The four alkynyl sterols shown in Fig. 2 were available for use in our studies. Alkynyl-cholesterol (*a*-Chol) and alkynyl-7-dehydrocholesterol (*a*-7-DHC) had been previously reported [24], the starting material for the synthesis of both being 3β-hydroxy-5-cholenic acid. The starting material for preparation of alkynyl-lanosterol (*a*-Lan) was lanosterol itself, while alkynyl-lathosterol (*a*-Lath) was prepared starting from ergosterol. In both of these syntheses, the double bond in the tail of the starting material was oxidatively cleaved and the aldehyde so generated was subjected to coupling with an appropriate Grignard reagent, which was then reduced with lithium aluminum hydride, see Fig. 2B and C. Details of the syntheses of *a*-Lan (four steps) and *a*-Lath (eight steps) are provided in Supporting Information (Scheme S1 and S2).

2.2. a-Lathosterol and a-Lanosterol in Neuro2a and Dhcr7-deficient Neuro2a

Cells were grown in the presence or absence of alkynyl sterols for 24–48 h and lipids isolated as described in Materials and Methods. Alkynyl sterols and endogenous sterols were analyzed by reverse-phase HPLC-MS, the alkynyl analogs eluting earlier than the natural compounds. Like their natural counterparts, *a*-Lath and *a*-Chol did not separate under any of the HPLC chromatography conditions attempted. These two sterols could be separated by GC however, and a combination of the HPLC-MS and GC was required for a complete analysis of *a*-sterol and natural sterol product mixtures. The HPLC retention times for the *a*-sterols under the conditions described in Materials and Methods were *a*-7-DHC, 3.8 min; (*a*-Lath and *a*-Chol), 4.1 min; and *a*-Lan, 4.8 min. For comparison, natural cholesterol elutes at 9.1 min under the same chromatography conditions.

In Neuro2a cells, both *a*-Lath and *a*-Lan undergo bio-conversion to *a*-Chol as the major product when incubated with the cells for 24 h at concentrations < 10 μM. Our initial experiments were carried out with *a*-Lath at concentrations of 5 μM and 10 μM in Neuro2a cells, the *a*-sterol profiles found were as follows: For 5 μM *a*-Lath incubation for 24 h, mole fractions of *a*-Lath remaining=0.16; *a*-7-DHC=0.10; and *a*-Chol=0.74. For 10 μM *a*-Lath incubation for 24 h, mole fractions of *a*-Lath remaining=0.32; *a*-7-DHC=0.21; and *a*-Chol=0.47. The natural sterol levels found after 24 h incubations with *a*-Lath were somewhat suppressed compared to levels found in controls, the total sterol levels (*a*-sterols+natural sterols) totaled 70–80% of the natural sterols found in control cells. Higher concentrations of *a*-Lath had a larger effect on reducing the total sterol levels. The alkynyl sterols made up 25% and 40% of the total sterol levels at *a*-Lath concentrations of 5 and 10 μM, respectively.

After the exploratory experiments with *a*-Lath in Neuro2a cells, subsequent studies were carried out with *a*-Lan at 0.1, 1.0, 5.0 and 10 μM in both Neuro2a and *Dhcr7*-deficient Neuro2a cells. Fig. 3 presents a typical HPLC-MS chromatogram for a product mixture derived from an experiment of 10 μM of *a*-Lan incubated in Neuro2a for 24 h. Under these conditions *a*-Chol is the major alkynyl sterol found in the product mixture, panel E in Fig. 3. The starting material in the experiment, *a*-Lan (panel A, Fig. 3), is always detected in the mix of