Cellular Signalling 27 (2015) 487–497

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Hedgehog signaling stimulates the conversion of cholesterol to steroids

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ARTICLE INFO

Article history:
Received 23 November 2014
Received in revised form 19 December 2014
Accepted 5 January 2015
Available online 10 January 2015

Keywords:
Cholesterol
Hedgehog signaling
Steroids

ABSTRACT

Cholesterol modification of Hedgehog (Hh) ligands is fundamental for the activity of Hh signaling, and cholesterol biosynthesis is also required for intracellular Hh signaling transduction. Here, we investigated the roles and underlying mechanism of Hh signaling in metabolism of cholesterol. The main components of the Hh pathway are abundantly expressed in both human cytotrophoblasts and trophoblast-like cells. Activation of Hh signaling induces the conversion of cholesterol to progesterone (P4) and estradiol (E2) through up-regulating the expression of steroidogenic enzymes including P450 cholesterol side chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase type 1 (3β-HSD1), and aromatase. Moreover, inhibition of Hh signaling attenuates not only Hh-induced expression of steroidogenic enzymes but also the conversion of cholesterol to P4 and E2. Whereas Gli3 is required for Hh-induced *P450scc* expression, Gli2 mediates the induction of *3β-HSD1* and *aromatase*. Finally, in ovariectomized nude mice, systemic inhibition of Hh signaling by cyclopamine suppresses circulating P4 and E2 levels derived from a trophoblast-like choricarcinoma xenograft, and attenuates uterine response to P4 and E2. Together these results uncover a hitherto uncharacterized role of Hh signaling in metabolism of cholesterol.

1. Introduction

Hedgehog (Hh) signaling has conserved roles in the development of various organs in metazoans ranging from Drosophila to humans [1,2]. The mammalian Hh family of secreted proteins consists of sonic hedgehog (Shh), indian hedgehog (Ihh) and desert hedgehog (Dhh). In vertebrates, in the absence of Hh, patched 1 (Ptc1) receptor represses smoothened (Smo) activity, and the Gli transcription factors (Gli2 and Gli3) are proteolytically cleaved into repressors within the primary cilium. The cleavage requires the activities of suppressor of fused (SuFu) and kinesin family member 7 (Kif7) and is mediated by phosphorylation of Gli2 or Gli3 by protein kinase A (PKA), casein kinase 1 (CK1), and glycogen synthase kinase-3β (GSK3β). In the presence of Hh, binding of Hh to Ptc1 relieves the inhibition of Smo, resulting in activation of the Gli transcriptional factors that induce transcription of target genes including cyclin D, cyclin E, myc as well as Ptc1 and Gli1 [3,4]. Overall, the conserved effect of Hh is to switch the Gli transcription factors from repressors into activators and allow for well-coordinated transcriptional events [2].

Hh ligands are precursor proteins that are translocated to endoplasmic reticulum, and undergo the post-translationally covalent-modification including palmityl residue attachment at the N-terminus mediated by the palmityl transferase and cholesteryl residue attachment at the C-terminus by autocatalytic modification [5,6]. These two lipid modifications occur independently and are both essential for activity of Hh signaling [5,7]. Moreover, metabolites of cholesterol and cholesterol biosynthetic pathway intermediates including oxysterols have been shown to play an intracellular role in Shh signal transduction, inhibition of their biosynthesis at different steps by pharmacological inhibitors suppresses Shh signaling, and leads to defective responses to Shh, both in vitro and in vivo [8–11]. Finally, defect in either 17β-hydroxysteroid dehydrogenase 7 (17β-HSD7) or 3β-HSD, the enzymes of the cholesterol biosynthetic pathway, leads to severe developmental abnormalities in several tissues, resulting from an abnormal sterol profile and deficient responses to Hh signaling [12,13]. Thus, cholesterol

Abbreviations: ACTH, Adrenocorticotropic hormone; caSmo, Constitutively active form of Smo; CK1, Casein kinase 1; DHEA, Dehydroepiandrosterone; E2, Estradiol; GH, Growth hormone; GSK3β, Glycogen synthase kinase-3β; Hh, Hedgehog; Kif7, Kinesin family member 7; PKA, Protein kinase A; P4, Progesterone; P450scc, P450 cholesterol side chain cleavage enzyme; P5, Pregnenolone; Smo, Smoothened; STS, Steroid sulfatase; SuFu, Suppressor of fused; 3β-HSD1, 3β-hydroxysteroid dehydrogenase type 1; 17β-HSD, 17β-hydroxysteroid dehydrogenase.

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http://dx.doi.org/10.1016/j.cellsig.2015.01.004
0898-6568/

modification of Hh ligands is fundamental for Hh activity, and cholesterol biosynthesis is also required for intracellular Hh signal transduction. To date, there is no evidence that Hh signaling regulates the cholesterol metabolism.

Human placenta is a crucial site for the conversion of cholesterol into steroids, and biosynthesis of placental steroids is essential for pregnancy maintenance and embryo development. Among the cholesterol-derived steroids, progesterone (P4) and estradiol (E2) are the most important hormones that are fundamentally involved in the regulation of the menstrual cycle and in the establishment and maintenance of pregnancy [14]. Placental trophoblasts convert the cholesterol to pregnenolone (P5), the first step in the synthesis of all steroids, and then to P4 by P450 cholesterol side chain cleavage enzyme (P450scc) and 3β-HSD1, respectively [15,16]. On the other hand, in fetal adrenal, maternal P5 and P4 are converted into C19 androgens (dehydroepiandrosterone, DHEA) which are further utilized by placental trophoblasts to synthesize the E2 through sequential reactions catalyzed either by 3β-HSD1 and aromatase via the intermediate A-dione, or by aromatase and 17β-HSD1 via C18 estrone [15,17].

In the present study, we have demonstrated that Hh signaling stimulates cholesterol conversion to P4 and E2 by inducing the expression of key steroidogenic enzymes. We further identify the genes encoding these enzymes as direct transcriptional targets of either Gli2 or Gli3. This work establishes Hh signaling as an essential mechanism in conversion of cholesterol into its steroid metabolites.

2. Materials and methods

2.1. Cell culture

All the cell lines used in the present study were obtained from ATCC (Manassas, VA). JEG-3 and BeWo choriocarcinoma cells were cultured as described previously [15], 293 EcR Shh cells (Shh-expressing cells) and control HEK 293 cells were used for the production of biologically active murine Shh conditional medium (SM) and control medium (CM), respectively, in the presence of ecdysone [18]. Human placentas were obtained from uncomplicated normal term (38–40 W) pregnancy after elective cesarean section without labor, following a protocol approved by the Ethics Committee of School of Medicine of Zhejiang University. The primary cytotrophoblasts were purified by using a 5–65% Percoll (Sigma) gradient at step increments of 5%, and cultured to allow syncytialization in vitro as described previously [19].

2.2. Oligonucleotides, plasmids, viruses, and infections

The primers for regular RT-PCR and quantitative RT-PCR were listed in the Supplement Data (Supplementary Table 1 and 2). Retroviruses expressing Smo, hGli2, hGli3 and their constitutively active forms including ca-Smo, ΔN-Gli2 and ΔN-Gli3 were all as previously described [20,21]. Lentiviruses expressing Gli1-, Gli2- and Gli3-shRNA were generated by co-transfecting the 293FT packaging cells with lentiviral shRNA expression vector,Pll3.7, inserted with the hairpin shRNA templates of complementary oligonucleotides (Supplementary Table 4) at the sites of *XbaI* and *NotI*. Either the retrovirus-containing or the lentiviruses-containing supernatants with the titers greater than 1×10^6 cfu/ml was used for infection of JEG-3 cells in the presence of 8 μg/ml polybrene (Sigma) as described previously [16]. The promoter regions of P450scc (nt −2497/+141), 3β-HSD1 (nt −2416/+207), and aromatase (nt −2487/+7), and their deletion mutants potentially interacting with Glis were amplified by PCR (primers in the Supplementary Table 3) in the presence of genomic DNA from JEG-3 cells, and the PCR products were subcloned into pGL3-Basic vector (Promega) to generate the luciferase reporter constructs as described previously [16]. The mutations at potential binding sites of Glis were introduced by site-directed mutagenesis. All of these constructs were verified by DNA a sequencer.

2.3. Antibodies, proteins, and chemicals

Human Gli1 (ab92611), Gli2 (ab26056), Gli3 (ab69838), and Smo (ab72130) antibodies were from Abcam (Cambridge, UK), human Ptc1 (06–1102) and Shh (06–1106) were from Millipore (Billerica, MA), and antibodies for human aromatase (sc-30086), P450scc (sc-292456), 3β-HSD1 (sc-30820), and β-actin (sc-69879) were from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant active human ΔN-Shh (1845-SH) was from R&D Systems, and cyclopamine was obtained from Sigma (St. Louis, MO).

2.4. RT-PCR, quantitative RT-PCR, and chip-PCR

RT-PCR was performed to detect the expression of main components of Hh signaling pathway including Dhh, Ihh, Shh, Ptc1, Smo, Gli1, Gli2, and Gli3, and quantitative RT-PCR was performed to measure the expression of steroidogenic enzymes including P450scc, 3β-HSD1, aromatase, 17β-HSD1, 17 β-HSD2, 17β-HSD5, and hSTS as described previously [15,16]. Chromatin immunoprecipitation (Chip) was conducted by using a commercial kit (Millipore, Billerica, MA), and a method modified from the manufacturer's protocol as described previously [19]. Briefly, the shearing of chromatin DNA was performed by sonication to produce an approximate 500 bp of input DNA, and was subjected to immunoprecipitation with either Gli antibodies or control IgG. After the immunoprecipitates were incubated with protein A agarose/salmon sperm DNA, the antibody-protein-DNA-agarose complex was washed and harvested for subsequent reverse cross-linking. The sheared DNA fragments from reverse cross-linking was extracted with a DNA extraction kit for further PCR amplification by using the primers listed in the Supplemental Data (Supplementary Table 5).

2.5. Transient transfections and duel-luciferase assays

Transient transfections in JEG-3 cells were performed by using Lipofectamine 2000 reagent (Invitrogen) as per the manufacturer's instructions. In each well of 24-well plates, the cells were transfected with 2 μl Lipofectamine 2000 reagent, 2 μg luciferase reporter constructs, and 0.02 μg Renilla luciferase constructs (Promega) for 8 hrs in the absence of serum, then, the cells were cultured in the media containing either CM or SM for 48 hrs. In some experiments, the cells were infected with the viruses for 24 hrs, and then transfected with the luciferase reporters for 8 hrs, and finally cultured in the media containing either CM or SM for 48 hrs. After transfection and treatment, the cellular lysates were prepared in reporter lysis buffer (Promega), and the supernatants were used for dual-luciferase assay according to the manufacturer's instructions (Promega). The firefly luciferase activities were normalized to Renilla luciferase levels.

2.6. Western blots and EIA

Western blots were performed using standard protocols, and the intensity of protein bands ($n = 3$) and statistical analysis was undertaken by NIH ImageJ. EIA assays for P4 and E2 were performed in serum-free culture supernatants harvested from JEG-3 cells and cytotrophobalsts, which were incubated with the substrates of steroidogenic enzymes (25-hydroxycholesterol or testosterone) for 4 hrs, as described previously [15,22].

2.7. Ovariectomy, JEG-3 choriocarcinoma xenografts, and treatments

Female Balb/c-nu/nu mice (6-week-old) were ovariectomized (OVX) and rested for a week, and tumor-bearing mice model was established by subcutaneous inoculation with xenografts of human choriocarcinoma JEG-3 cells (5×10^6 cells/site) into both the left and right armpit. After the tumors grew to a size of $\approx 250\ mm^3$ (6 ~ 7 d),