Expression of hydrogen sulfide, hydrogen-sulfide synthase and cyclooxygenase-2, and their mechanisms of action in amniotic tissues

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Abstract

Purpose: To investigate the expressions of cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and cyclooxygenase-2 (COX-2) and their relationships with premature delivery in amniotic tissues.

Methods: Parturients were divided into three groups: 40 preterm-labor parturients (PTL group), 28 term-labor parturients (TL group), and 28 term non-labor parturients (TNL group). Changes in expressions of CBS, CSE and COX-2 were determined by Western blot (WB) in amniotic tissues of parturients in the three groups. The expression of COX-2 was determined after the amniotic tissues of parturients in the TNL group were cultured in vitro and processed by exogenous hydrogen-sulfide (H2S).

Results: The expression level of COX-2 was significantly lower in the TNL group, when compared with the PTL and TL groups (p < 0.05). The expression of CBS was increased in the order PTL > TL > TNL, with TNL group having the highest level, and there were significant differences in CBS expressions among the three groups (p < 0.05).

Conclusion: These results suggest that when the expressions of CBS and CSE are down-regulated, the decreased H2S synthesis promotes the overexpression of COX-2 by NF-κB signaling pathway, causing increased prostaglandin synthesis which results in premature delivery.

Keywords: Cystathionine β-synthase, Cystathionine γ-lyase, Cyclooxygenase-2; Amniotic tissues; Preterm labor

INTRODUCTION

Preterm labor (PL) is the most common intrapartum complication, and also the first cause of neonatal illness and death [1]. In the world, the incidence of PL are 5 to 18 %, while in China, PL accounts for 7.1 % [2], about two third of which are spontaneous [3]. The precise mechanism involved in the initiation of PL is not totally clear. Hydrogen sulfide (H2S) is a gas with the scent of rotten eggs, and for long, has been regarded as a toxic waste which causes environmental and...
occupational hazards. However, recent studies suggest that endogenous H₂S plays an important role in human pregnancy and delivery. In mammals, endogenous H₂S is produced by the precursor L - cysteine (L-cys) via two pyridoxal 5'-phosphate (PLP) - dependent enzymes: cystathionine β - synthase (CBS) and cystathionine γ-lyase (CSE) [4].

Prostaglandin (PG) plays an important role in the initiation of human labor. It can induce contraction of uterine muscle and premature rupture of membranes, promote cervical ripening, and participate in the last process of initiation of labor. Arachidonic acid (AA) generates prostaglandin under the catalysis of prostaglandin G / H synthase - 2 (PGHS - 2), i.e.cyclooxygenase - 2 (COX - 2) [5]. Studies have found that H₂S reduces production of PG by inhibiting expression of COX - 2 in microglia [6,7].

In this study, the expressions of CBS, CSE and COX - 2 proteins were determined in the amniotic tissues of parturients who had PL, full-term normal cesarean section delivery or full-term normal vaginal delivery. In addition, the regulation of H₂S and its possible mechanism in expression of COX - 2 (i.e. PGHS - 2) were investigated in order to provide new train of thought and therapeutic target for the prevention and cure of PL.

**EXPERIMENTAL**

**Parturients**

In this study, 96 parturients who delivered at the First Maternity and Infant Hospital between September 1, 2014 and June 30, 2016 were recruited as subjects. They included 40 parturients with preterm vaginal delivery (spontaneous preterm - labor group, i.e., PTL group); 28 with full-term normal vaginal delivery (term - labor group, i.e., TL group), and 28 with full- term normal cesarean section delivery (term non - labor group, i.e., TNL group). Parturients with cardiovascular diseases and obesity prior to pregnancy or diabetes and hypertension in pregnancy, and those with complications of pregnancy such as intra-uterine growth retardation were excluded. In the PTL group, the average age was 27.31 ± 6.42 years, with a range of 22 - 34 years, and average gestational period was 34.58 ± 2.17 weeks, with a range of 30 - 36 weeks. In the TL group, the average age was 27.60 ± 6.28 years with a range of 21 - 34, and average gestational period was 40.37 ± 2.35 weeks, with a range of 38 - 42 weeks. In the TNL group, the average age was 27.54 ± 6.15 years with a range of 22 - 36, and average gestational period was 40.36 ± 2.42 weeks, with a range of 37 - 41 weeks. The study was approved by the Independent Ethics Committee of the Shanghai First Maternity and Infant Hospital affiliated to Tongji University (approval no. SH20140109), and followed the guidelines of Helsinki Declaration [8].

**Main reagents and instruments**

The reagents and equipment used in this study and their sources were: Western Blot electrophoretic apparatus (Bio-Rad Laboratories Pty Ltd); Western Blot electrophoresis and transmembrane system (Bio-Rad Laboratories Pty Ltd); UV Gel imaging system (Shanghai Furi Company); pre-stained protein molecular weight markers (Thermo Fisher Scientific); antibodies (Cell Signaling Technology); NaHS (Sigma-Aldrich); protease inhibitors (Sangon Biotech (Shanghai) Co., Ltd).

**Collection and treatment of specimen**

Postoperative amniotic tissues were excised and immediately placed in precooling physiological saline at 4.0 °C. After rinsing 3 times, the tissues were directly and quickly frozen in liquid nitrogen in cryogenic vials, and then transferred and stored at - 80 °C. The tissues were taken out, blotted with absorbent paper, and put into special 2 mL EP tubes. Phosphatase inhibitors and RIPA lysate solution were added to the tubes, and then steel balls were put inside the tubes. The tissues were ground for one minute in a tissue grinder, and cooled in an ice bath for 30 min prior to centrifugation at 13, 000 rpm for 30 min at 4 °C. The supernatant was transferred to a new 1.5 mL EP tube. The amount and composition of proteins in the supernatant protein were quantitatively analyzed using BCA protein quantitative kit.

**Western blot assay**

Electrophoresis was performed under constant voltage of 80 V and electrical current of 300 mA. The electric voltage was modulated to 120 V after the samples entered the separation gel, and electrophoresis was continued until the separation gel was free of bromophenol blue. Then the membrane was transferred in ice bath. After membrane was transferred for two hours under constant current of 300 mA and electric voltage of 100 V, the proteins in gel were transferred to cellulose acetate membranes. Subsequently, the prepared cellulose acetate membranes were taken out and rinsed thrice with 1xTBST solution (10 min each). Then the
membranes were blotted with 5% skimmed milk and rinsed thrice with 1xTBST solution. Antibody for COX-2 / CBS / CSE / P65 antibody (first antibody) was applied at a dilution of 1:500 and the cellulose acetate membranes were incubated at 4 °C overnight. Next, the membranes were taken out again, shaken at room temperature slowly for 15 min, and rinsed thrice with 1xTBST solution. Subsequently, the second antibody which was diluted with 1:1000 with 5% skimmed milk was added and the cellulose acetate membranes were incubated at room temperature for 2 h. Finally, the cellulose acetate membranes were exposed and developed with ECL Western blotting system, scanned and photographed using Bio-Rad chemiluminescence imaging instrument. The protein expressions were analyzed by gel analysis system. The control used to equalize protein in loading sample was GAPDH.

**Evaluation of COX-2 activity**

Amniotic tissues from term non-labor parturients were collected for primary culture in vitro. The tissues were processed using H₂S at different gradient concentrations, and changes in expression of COX - 2 were determined. Extrinsic H₂S was provided by NaHS solution. The amniotic tissues were cultured in vitro for 24 h and processed at concentrations of 1 × 10⁻⁵ M, 2 × 10⁻⁵ M, 4 × 10⁻⁵ M and 8 × 10⁻⁵ M. After 24 h, they were collected for the determination of COX - 2 expression, while the supernatant of culture medium was also collected for the indirect assay of COX - 2 activity with Western blotting method.

**Statistical analysis**

Statistical analysis was performed using the statistical software, SPSS 19.0. Data are expressed as mean ± standard deviation (mean ± SD), and were analyzed by Student t-test for group comparison, analysis of variance was used to analyze the multiple groups comparison. Linear regression analysis was used for the comparison of correlation between two variables. Statistical significance was assumed at p < 0.05.

**RESULTS**

**Expressions of COX - 2, CBS and CSE in amniotic tissues**

The expression level of COX - 2 was lowest in the TNL group, which was significantly different from those in the PTL and TL groups (p < 0.05). There was no significant difference in COX - 2 expression between the PTL and TL groups (p > 0.05). The expression of CBS was increased in the order: PTL > TL > TNL. There were significant differences in CBS expressions amongst the three groups (p < 0.05). The expression level of CSE was the lowest in the PTL group, and was significantly different from those in the TL and TNL groups (p < 0.05). There was no significant difference in CSE expression between the TL and TNL groups (p > 0.05). These results are shown in Figure 1.
Correlation among the expressions of COX-2, CBS and CSE in amniotic tissues

From linear regression model analysis, the expression level of CBS showed negative correlation with that of COX-2 (r = 0.288). However, there was a weak correlation between the expression levels of CSE and COX-2 (r = 9.9×10^{-4}, Figure 2).

Expression and phosphorylation of P65 protein in NF-kB signaling pathway

Among the three groups, there were significant differences in expression of phosphorylated P65 (p < 0.05), but there were no significant differences in expression of un-phosphorylated P65 (p > 0.05). The expressions of phosphorylated P65 in the PTL and TL groups were significantly higher than that in the TNL group, and in the PTL group, phosphorylated P65 expression was significantly higher than that in the TL group (p < 0.05). These results are shown in Figure 3.

Regulatory role of exogenous H_{2}S in expression and activity of COX-2 in human amniotic tissues

With increases in concentration of exogenous...
H\textsubscript{2}S, the expression of COX-2 decreased continuously in human amniotic tissues (Figure 4).

**DISCUSSION**

Hydrogen sulfide (H\textsubscript{2}S) is a colorless gas with the scent of rotten egg and the third signaling gas molecule found after nitric oxide (NO) and carbon monoxide (CO), which can regulate various physiological functions. L-cysteine is the substrate for the synthesis of H\textsubscript{2}S, and the enzymes involved in its synthesis are CSE, CBS and mercaptopyruvate sulfurtransferase (MPST or 3-MST). Amongst these enzymes, CSE and CBS are found in various tissues and systems including uterine smooth muscle [9,10]. In this study, the expressions of CSE and CBS were also detected in amniotic tissues. Comparisons between the PTL, TL and TNL groups showed that the expressions of CSE and CBS in the PTL and TL groups were significantly decreased when compared with corresponding expressions in the TNL group; expressions of CSE and CBS were lowest in the PTL group, while expressions of CSE and CBS in the TL group were significantly lower than those in the TNL group. These results indicate that the expressions of CSE and CBS were significantly down-regulated in the PTL and TL groups, which resulted in the decrease in H\textsubscript{2}S synthesis. In contrast, higher levels of H\textsubscript{2}S delayed the occurrence of delivery and maintained the pregnancy state by inhibiting the contraction of uterine smooth muscle in the TNL group [11].

Regardless of whether parturients underwent premature labor or TL, the processes of initiation of delivery were the same, which included regular uterine contraction, dilatation of cervix and rupture of membranes, all of which involve the important role of PG [12,13]. The main function of PG is to stimulate smooth muscle cells of uterine wall, causing contraction of muscles which then induce the initiation of labor. In human intra-uterine tissues, PG is synthesized mainly in fetal membranes, including amniotic membrane and chorion. The enzyme COX - 2 is an important rate-limiting protein in the synthesis of intra-corporeal PG [14]. It catalyzes the conversion of arachidonic acid to PG. In pregnancy, the functions of fetal membranes are to express COX - 2 lowly, reduce the synthesis of PG, maintain low levels of PG, reduce the transfer of PG into uterine smooth muscle and maintain the pregnancy state [15]. However, as pregnancy progresses, the expression of COX-2 is increased gradually in the third trimester, resulting in increased PG synthesis in fetal membranes which then stimulate the contraction of uterine muscles and induce the initiation of labor [16,17]. In the present study, the
expression of COX - 2 was determined in amniotic tissues in the PTL, TL and TNL groups. The results showed that the expression of COX - 2 was the lowest in the TNL group and higher in the PTL and TL groups, which indicated that the high level of COX - 2 could induce the initiation of labor.

Subsequent correlation analyses between the expression of COX - 2 and the expressions of CSE and CBS showed a good linear negative correlation between the expressions of CBS and COX - 2, and a weak correlation between the expressions of CSE and COX - 2, which indicates that a regulatory role may exist between the expression of COX - 2 and the expressions of CSE and CBS.

However, it was not clear whether H$_2$S can inhibit the expression of COX - 2. In this study, the amniotic tissues from term non-labor parturients were collected for primary culture in vitro. The tissues were processed with H$_2$S at different gradient concentrations, and the changes in expression of COX - 2 were assayed. From the results, with increase in NaHS concentration, the expression of COX - 2 was gradually decreased and showed a dose-dependent effect, indicating that H$_2$S could regulate the synthesis and secretion of PG by regulating the expression of COX - 2.

Hydrogen sulfide (H$_2$S) plays its physiological function by activating multiple signaling pathways such as MAPK and NF - kB signaling pathways [18,19]. P65 is an important subunit of nuclear factor kappa B (NF - kB) family. NF - kB is an important intra-nuclear transcriptional factor (TF), which can be activated by lipopolysaccharide (LPS) and multiple inflammatory factors (e.g. IL - 1 and TNF - α) and transposed into the nucleus to activate transcription of target genes. A large number of studies have shown that NF- kB is linked to the synthesis of PG in intra-uterine tissues [20].

There are many binding sites of transcriptional factors in the promoter region of COX - 2, of which two NF - kB binding sites are associated with PG synthesis in intrauterine tissues [21]. In immortalized human amnion epithelial (WISH) cells, IL - 1 beta promotes the synthesis of PGs by activating NF- kB to up-regulate the expression of COX - 2 [22]. In the present study, the expression and phosphorylation of P65 protein was assayed in NF - kB signaling pathway in the PTL, TL and TNL groups, and the effects of the related genes on premature labor were analyzed. The results showed significant differences in expression of phosphorylated P65 but no significant differences were seen in expressions of un-phosphorylated P65 among the three groups; expressions of phosphorylated P65 in the PTL and TL groups were significantly higher than those in the TNL group, while the expression in the PTL group was highest and significantly different from that in the TL group. These results indicate that the phosphorylation of P65 was related to the initiation of labor.

CONCLUSION

The expression levels of CBS, CSE and COX - 2 are closely related to the initiation of premature labor. The down-regulation of expressions of CBS and CSE causes a decrease in H$_2$S synthesis and reduction of inhibitory effect of H$_2$S on COX - 2 in human amniotic tissues. The up-regulation of expression of COX - 2 results in increased PG synthesis and activation of PG in placenta and fetal membranes, thereby inducing premature delivery. The findings of this study may lay the foundation for developing new methods of inhibiting the initiation of PL, and provide a new basis for the effective treatment of premature delivery. However, because of the complexity of mechanisms involved in the initiation of premature labor, the specific molecular mechanisms underlying the effects of H$_2$S, CBS, CSE and COX - 2 on the initiation of preterm delivery in amniotic membranes need to be further investigated.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors, all authors read and approved the manuscript for publication. Xin Ni conceived and designed the study, Dan Liu, Tao Duan, Xing - ji You and Lu Gao collected and analyzed the data, Ming Liu wrote the manuscript. Ming Liu and Dan Liu contributed equally to the work.

REFERENCES


