Serum-Free Medium Evokes Cyclooxygenase-2 Pathway in A549 Cells

WeiGang Duan*[,1], WenHui Chen[1], Yu Yun[2], DeQuan Liu[2], Cheng Luo[3] and ZhiQiang Shen[2]

[1]*Key Lab. of Molecular Biology for Sinomedicine, Yunnan University of Traditional Chinese Medicine, 1076, Yuhua Road, University City of Chenggong, Kunming650500, P.R. China;* [2]*Yunnan Pharmacological Laboratories of Natural Products, the third affiliated hospital, Kunming Medical College, 191, Western People's Road, Kunming 650031, P.R. China;* [3]*Institute of Molecular & Cell Biology, University of Tartu, Riia St. 23, Tartu 51010, Estonia, EU*

Abstract: *Purpose:* The main aim of the present study was to find out if serum-free treatment was able to up-regulate cyclooxygenase-2 (COX-2) pathway in A549 cells which were treated with serum-free medium for different hours.

Methods: Prostaglandin E_2 (PGE_2, a prostaglandin mainly from COX-2 pathway) in the supernatant synthesized by A549 cells, were determined with PGE_2 enzyme immunoassay kits. The protein expression of COX-1, 2 and PGE synthase (PGES) were detected by Western Blotting.

Results: The yield of PGE_2 increased when A549 cells were treated with serum-free medium for 12 h, and decreased thereafter. The results of western blotting showed that changes in PGE2 were related to the variation of the expression of COX-2 and PGES.

Conclusion: All the results suggested that the COX-2 pathway in A549 cells was up-regulated by serum-free treatment, the mechanism of which was associated with the increase of the expression of COX-2 and PGES.

Keywords: Prostaglandin E_2, prostaglandin E synthase, Cyclooxygenase-2, up-regulation, serum-free medium.

INTRODUCTION

Cyclooxygenase-2 (COX-2), an inducible type of COX, is one of the main enzymes associated with arachidonic acid (AA) metabolism; which is related to inflammation, angiogenesis and carcinogenesis [1,2]. The main product of COX-2 pathway is PGE_2: COX-2 transforms arachidonic acid (AA) to PGH_2, then, PGE synthase (PGES) transforms it to PGE_2 [3,4]. The cell line from NSCLC (non-small cell lung cancer), A549 cell is a common tool cell line in studying lung cancer. It was reported that the amount of PGE_2 (mainly from COX-2 pathway) in A549 cells was far more than that of 6-keto-$PGF_{1\alpha}$ (a metabolite from PGI_2, mainly from COX-1 pathway) whether A549 cells were activated by inflammatory stimuli or not [5]. A recent report [5] suggested that the yield of PGE_2 in A549 cells was sensitive to serum-free medium. However, there are few further understandings of the new effect. The main aim of the present study was to reveal that serum-free medium was an effective factor to up-regulate COX-2 pathway in A549 cells.

MATERIALS AND METHODS

Materials

The cell line A549 cell from American Type Culture Collection (ATCC) was used and the identity of cells was checked by their typical morphology. AA, PGE_2 enzyme immunoassay (EIA) kits, 6-keto-$PGF_{1\alpha}$ EIA assay kits, and rabbit anti- cytosolic PGES (human) were purchased from Cayman Chemical Company, Ann Arbor, USA. Rabbit anti-COX-1 (human) antibody, rabbit anti- COX-2 (human) antibody, and goat anti-rabbit antibody linked with horse radish peroxidase were produced by Boster Biological Engineering Co., Ltd., Wuhan, China. Tecan Reader was manufactured by Tecan Group Ltd., Männedorf, Switzerland. Ham's F12K culture media were produced by Hyclone Company of Thermo Fisher Scientific Inc., Logan, USA. Fetal bovine serum was manufactured by Hangzhou Sijiqing Bio-material Co., Ltd., Hangzhou, China. Chemi-luminescence kit (Pierce) was produced by Thermo Fisher Scientific Inc., Rockford, USA. Semi-Dry Electrophoretic Transfer Cell, Gel Scanner and its system of "Quantity One" were manufactured by Bio-Rad Laboratories, Inc., Farmin-gton, USA. Other reagents used were of analytic purity made in China.

Cell Culture

A549 cells were incubated in F12K medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate and 10% fetal bovine serum (standard medium), at 37°C, in an atmosphere of 95% air and 5% CO_2 [6]. When cells were about to capture 80% area of the flask, they were digested and seeded to other flasks or to 96- well plates with different treatments.

PGE_2 Assay and 6-keto-$PGF_{1\alpha}$ Assay

The A549 cells seeded in 96- well plates (5×10^4/mL) were cultured in standard medium for 12 h. After serum-starved for 0, 6, 12, or 24 h, A549 cells were exposed to AA of 10 μM for 30 min. The supernatant was collected as

*Address correspondence to this author at the Key Lab. of Molecular Biology for Sinomedicine, Yunnan University of Traditional Chinese Medicine, 1076, Yuhua Road, University City of Chenggong, Kunming650500, P.R. China; Tel: +86-159-25146965;
Fax: +86-871-5918014; E-mail: deardwg@126.com

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sample. PGE_2 was determined with PGE_2 EIA kits. The product of 6-keto-$PGF_{1\alpha}$ was determined with 6-keto-$PGF_{1\alpha}$ EIA kits. The cells in every well was lyzed with DMSO and the protein content was determined with Bradford protein assay [7]. The final concentration of PGE_2 or 6-keto-$PGF_{1\alpha}$ was normalized to the protein content of the same well.

Western Blotting

A549 cells seeded in flasks (1×10^4/ml) were cultured with standard medium at first. When they were about to occupy 50% area of the flasks, the medium was renewed with serum-free medium and cultured for 0, 6, 12, or 24 h. The area captured by A549 cells was less than 80% of the flask before cells lysing. The cells were lyzed on ice in a lyse buffer containing 20 mM TrisHCl (pH7.5), 0.1% Triton X-100, 1 mM EDTA, 0.2 mM dithiothreitol, and 2 mM NaF. The lysate was spun ($6{,}000 \times g$) at 4°C for 5 min and the supernatant was collected as protein sample. The protein in the supernatant was determined with Bradford protein assay [7], and the supernatant was diluted with lyse buffer to a solution containing 2.00 mg/ml protein.

Different samples of 20.0 μg protein were applied for SDS-PAGE (sodium dodecylsulphate - polyacrylamide gel electrophoresis). The protein in SDS-PAGE was transferred to a nitrocellulose (NC) filter by an electrical current of 10 V for 60 min in a Semi-DryElectrophoretic Transfer Cell. The filter was blocked with 3% BSA, stained with primary antibodies and secondary antibodies, and developed with a chemiluminescence kit. The results of western blotting were scanned by the software of "Quantity One" and the relative density was calculated by using Formula 1.

$$Relative\ Density = \frac{Density\ of\ Band\ of\ interest}{Density\ of\ Actin\ Band\ of\ the\ Same\ Sample}$$

(Formula 1)

Statistical Analyses

Values are expressed as mean ± S.D. One-way analysis of variance (ANOVA) was performed, and the least significant difference (LSD) method of ***post hoc*** test was performed to compare the means with the control group. Statistically significant differences were accepted at $P < 0.05$ [6].

RESULTS

PGE_2 and 6-keto-$PGF_{1\alpha}$ from A549 Cells with Serum-Free Treatment

In order to observe whether serum-free treatment was able to evoke COX-2 pathway, the amount of PGE_2 was detected at several intervals over 24 h (Fig. **1**). From 0 to 12 h, the yield of PGE_2 increased (Fig. **1**). However, it then decreased if A549 cells were treated with serum-free medium for 24 h. The yield of 6-keto-$PGF_{1\alpha}$ remained at a relative stable level of lower concentration (Fig. **1**).

COX-2 and PGES Expression Evoked by Serum-Free Medium

In order to find the direct cause(s) of the COX-2 pathway activation, the expression of both COX-2 and PGES in A549 cells treated with serum-free medium was detected. The results of western blotting showed that, from 0 to 12 h, the expression of COX-2 and PGES increased (Fig. **2A**). However, the expression of COX-2 and PGES then decreased if A549 cells were treated with serum-free medium longer (between 12 -24 h). Unlike COX-2, COX-1 expression almost kept at a stable state over 24 h. The relative density analysis showed in Fig. (**2B**).

DISCUSSION

There are at least three types of COX discovered, COX-1, COX-2, and COX-3. Therefore, there are at least three COX pathways associated with different physiological functions or pathological functions. Among them, COX-2 was proved to be induced by various stimuli, such as lipopolysaccharide (LPS) [8], phorbol 12-myristate 13-acetate (PMA) [9], and cytokines [10]. PGE_2 is thought to be one of the key molecules involved in many pathological processes including tumor metastasis, immunosuppression,

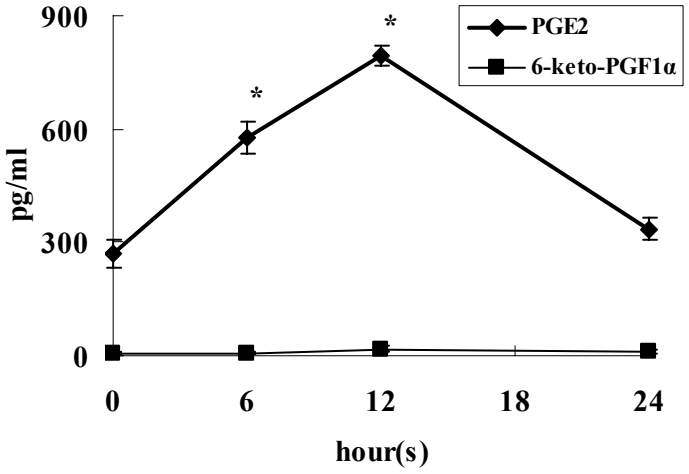

Fig. (1). PGE_2 and 6-keto-$PGF_{1\alpha}$ from A549 cells treated with serum-free medium (Mean ± S.D., $n = 3$).

From 0 to 12 h, the yield of PGE_2 in A549 cells increased then decreased if A549 cells treated longer, but 6-keto-$PGF_{1\alpha}$ remained at a lower stable level. *: $P < 0.05$ *versus* 0 h.

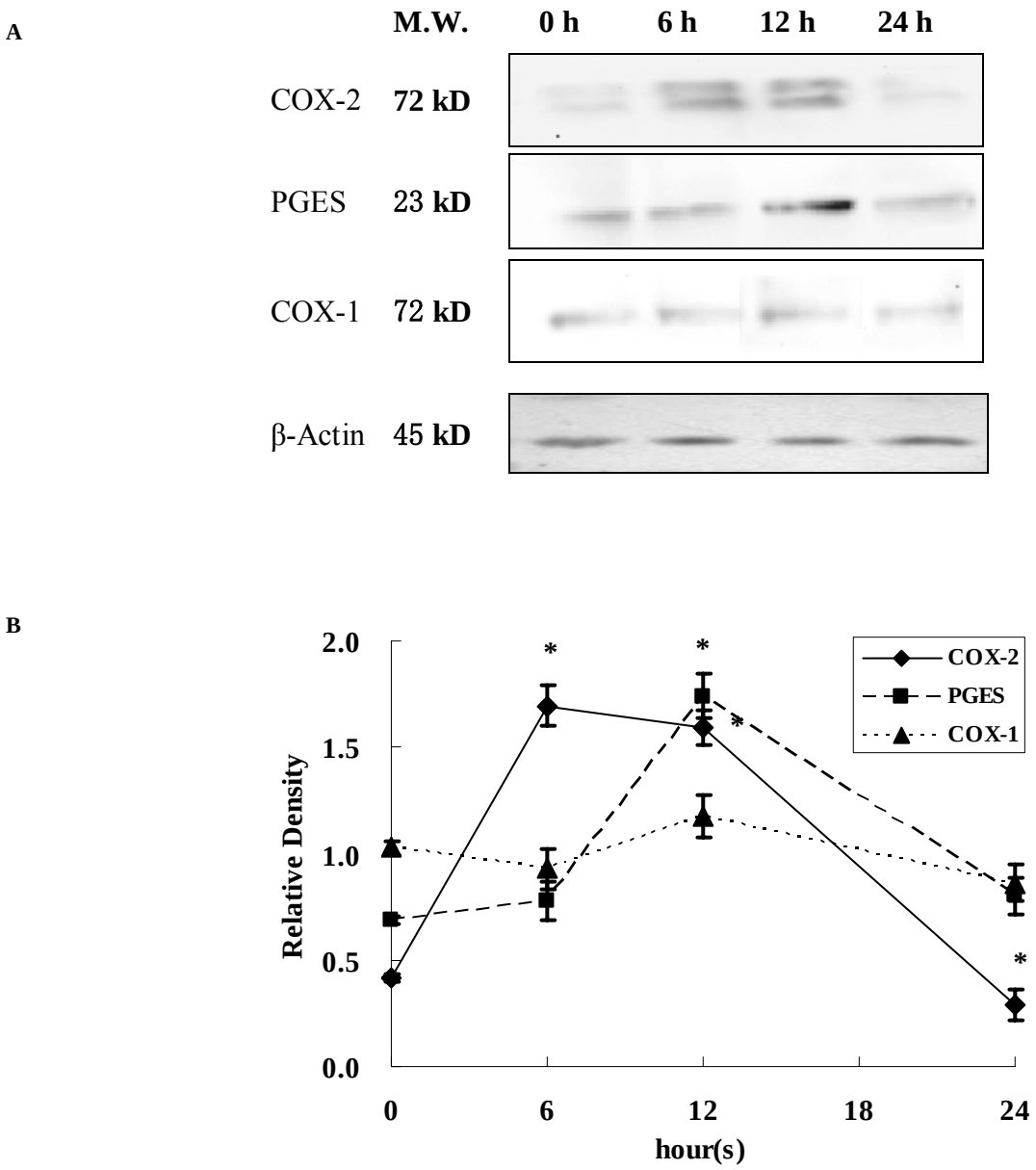

Fig. (2). The expression of COX-2, PGES, and COX-1 in A549 cells treated with serum-free medium.

A549 cells were treated with serum-free medium for 0, 6, 12, or 24 h. The results of western blotting (A) showed that the expression of COX-2 and PGES was up-regulated from 0 to 12 h and down-regulated thereafter; while the expression of COX-1 kept at a relatively stable level all the time. "B" was the result from "A" expressed in relative density (Mean ± S.D. $n = 3$, * $P < 0.05$ versus 0 h).

even hyperplasia [1-4,11]. Recently, Yao and his colleagues [5] established a screening method in A549 cells for non-steroidal anti-inflammatory drugs (NSAIDs). Their results suggested that COX-2 pathway of A549 cells was inducible by serum-free treatment, but detailed information was unknown. In Yao's study [5], the effect of COX-2 pathway up-regulation caused by serum-free treatment for 12 h was even greater than that by LPS of 10 μg/ml.

The results of the present study showed that serum-free culture medium is able to induce the COX-2 pathway. The COX-2 pathway is the main COX pathway in A549 cells, and is believed to be highly associated with tumorigenesis of NSCLC [10,11]. Apart from LPS, PMA, and some cytokines, the present study first discovered that serum-free treatment was also a powerful stimulus to increase PGE_2 in A549 cells; the direct causes of which were serum-free treatment up-regulating the expression of COX-2 and PGES. Since the immediate product of the three types of COX catalyzed from AA was PGH_2, the up-regulation of PGES expression is important in ensuring PGE_2 increase. PGES is an enzyme at the downstream of COX-2 pathway, and the expression of PGES often couples with that of COX-2 [12,13]. Based on the results in the present study, it could be deduced that, the up-regulation of COX-2 expression occurs first, and the up-regulation of PGES expression follows. The results indicted that there is a short lag (about 6 h) from

COX-2 expression up-regulation to PGES expression up-regulation. However, how PGES coupled with COX-2 is not thoroughly clear so far. However, if A549 exposed to serum-free medium too long (24 h), the up-regulation of COX-2 pathway was inhibited; for A549 can be starved to death for too long time in serum-free medium.

Taken together, the present study discovered that COX-2 pathway in A549 cells was able to up-regulated when treated with serum-free medium for 12 h, and the direct mechanism was associated with the activation of the expression of COX-2 and PGES. This finding is useful for COX-2 inhibition model and offers new understanding of lung cancer.

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