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Full Length Research Paper

The effect of extracted bacterial LPS from *Salmonella enteritidis* on COX-2 in hepg2 cell line in induction and inhibition conditions

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Hepatocellular carcinoma is the fifth most common neoplasm and the most important cause of death in patients with liver cirrhosis in the world. lipopolysaccharide (LPS) stimulates the hepatocyte cells and increases the production of inducible cytokine with the high production of nitric oxide and ROS. Chronic inflammation caused by lipopolysaccharide directly activates immune system and indirectly, through COX-2 interferes in the formation of malignant disease. In this study, the rate change of COX-2 was searched by adding LPS and also the effect of inhibition of this inflammatory enzyme by Celecoxib and combined effect of this in HepG2 cancer cells was assessed. *Salmonella enteritidis* lipopolysaccharide is extracted by methanol-chloroform method and the Sodium dodesyl sulfate poly acryl-amide gel electrophoresis (SDS-PAGE) electrophoresis bands were stained by silver nitrate. Four treatment groups of HepG2 cells were stimulated with 100 ng/ml LPS, 500 µM Celecoxib as inhibitor and were incubated for 12 and 24 h. Variables including both increase and decrease inflammatory factor, COX-2, was assayed. The obtained results showed that initial activity of COX-2 in not stimulated in HepG2 cell, 1.957 ng/ml after stimulation with LPS for 12 and 24 h (1.383 and 0.618 ng/ml) decreased and the effect of this inhibitor was also studied. The data showed that the reduction of COX-2 in HepG2 cells was correlated with the cell density and duration of incubation with LPS. Inhibition of enzyme associated with inflammation, with inhibitor substance; celecoxib, observed as well as LPS. We can potentially design drugs to treat a variety of diseases and cancers.

Key words: Cyclooxygenase-2, HepG2 Cell, *Salmonella enteritidis*, lipopolysaccharide, Sodium dodesyl sulfate poly acryl-amide gel electrophoresis (SDS-PAGE).

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm, the major cause of death in patients with liver cirrhosis and the third most common cause of cancer-related death in the world. Ninety percent of HCC is developed in the context of chronic liver disease and mainly in patients with cirrhosis. The hepatitis virus is the commonest cause of HCC in the world and every year kills more than 1.25 million people and 500000 new causes are recognized in one year (Sripathi et al., 2006). Human liver hepatocellular carcinoma cell lines (HepG2) are immortalized, repeatedly proliferable, culturable and available. So, they can be easily saved, proliferated,

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cultured and tested and are good targets for gene therapy in hemophilic patients. They are used in cellular and genetic tests in hepatic diseases, HIV, liver and breast cancer and many genetic diseases such as zeloger syndrome and metabolic studies (Xiao et al., 2008; Tan et al., 2009). One of the stimulating factors of growth and proliferation and causing malign diseases in cell is lipopolysaccharide (LPS) which is glycolipid part of outer membrane of negative gram bacteria which produces cytokine and vasodilator such as nitric oxide (Melanie et al., 2005). The produced nitric oxide by iNOS increases gene expression of cyclooxygenase 2, prostaglandin E2 and cell proliferation through activating p38 route and mitogen-activated protein kinases and JNK1/2 (Kant et al., 2006). Chronic inflammation by lipopolysaccharide interferes in the appearance of malign diseases directly by activating immune system and indirectly by the help of Cyclooxygenase-2 (COX-2) (Sharma et al., 2003). Cyclooxygenase (COX) is an enzyme which is responsible for the formation of important biological mediators called prostanoids, including prostaglandins, prostacyclin and thromboxane. The cyclooxygenase enzyme also is known as the prostaglandin H-synthase, catalyses the rate-limiting step in prostanoid biosynthesis (Queiroga et al., 2007). Cyclooxygenase 2 enzyme is an inducible enzyme and in normal conditions, it is expressed in low level in frequent tissues, while under the effect of inducible factors like growth factors, cytokines and tumor inducing is expressed in high level (Mehmet et al., 2008) and Cox-2 is usually absent in normal cells but it can be induced by the growth factors, inflammatory reactions, tumour promoters and oncogenes (Queiroga et al., 2007). Exposed to lipopolysaccharide in a chronic way, it causes a frequent cycle in which pre-inflammated cytokine and Th1 cytokine such as IL-2, IFN-γ, TNF-α are induced and resulted in the increase of expression of cyclooxygenase 2 and also the chronic production of Th2 cytokines like IL-4, IL-6, IL-10 and at last causes the decrease in the function of CMI (Sharma et al., 2003). Unlike the increase of 2 inflammatory enzymes of COX-2 and iNOS in cells, the inhibitor drugs are used that in this study celecoxib and L-NAME were used. Celecoxib is a non-steroidal anti-inflammatory drug which selectively inhibits COX-2 enzyme, causing the inhibition of synthesis of prostaglandin E2 in an inflammatory area and can stop the cell cycle in G1/G0 level and avoid the movement of cell to S-level (Taketo, 1998; Hu et al., 2003; Praveen et al., 2003; Fiorucci et al., 2001; Grosch et al., 2001). Two inflammatory enzymes of COX-2 and iNOS are inducible and have the synergy effect on each other and the increase in one result in the increase in the other and vice-versa. However, the effect of lipopolysacharide upon increase and decrease of activity of inflammatory factor of COX-2 and also the effect of specific inhibitor of COX-2 in HepG2 cells was not studied completely.

In this study, we tried to stimulate this inflammatory factor by using of extracted lipopolysaccharide out of *Salmonella enteritidis* and by using COX-2 inhibitor inspecting the results in HepG2 cancer cells in inducing condition with lipopolysaccharide in these cells.

MATERIALS AND METHODS

Materials

In this study the following materials were used:

Medium culture DMEM+L-glutamine (Gibco, code 12800-116), COX-2 EIA kit (assay designs and Stressgen company, Michigan, America), QC-Celecoxib substance (Arastoo chemical drug industrial company, RD/0480508), *S. enteritidis* bacteria of mutant rough pour H.g.m (Iran Pastour institute), and HepG2 liver cells (Iran Pastour institute).

METHODS

Lipopolysaccharide was extracted by the methanol-chloroform method which is a simple, cheap and safe method. Then Sodium dodesyl sulfate poly acryl-amide gel electrophoresis (SDS-PAGE) and silver staining, was used for purity and accuracy checking of the extract.

Cell culture and laboratory experiments

For the growth of HepG2 cell line:

Cells were collected by centrifugation, resuspended in Dulbecco's modified Eagle's medium containing amino acid mixtures including 4 mmol L-glutamine and 10% fetal bovine serum, 1000 unit/ml penicillin and 100 µg/ml streptomycin with humid atmosphere 95, 5% CO_2 in 37°C (Das et al., 2008) and then the cells were incubated.

After culture in T75 model flask and approaching to the density of 80%, they are cultured in the plate and 3 h after the stimulation of cells with lipopolysaccharide, Celecoxib was added. Then one time for 12 h and the other time for 24 h, the treated cell groups were incubated. From each group, the amount of 10^6 cells was placed in glass container and they were lysated and its activity of COX-2 was quickly measured by the intended microplate kits and the results were read by the ELISA reader.

Four treatment groups were categorized as follows

1) HepG2 liver cells
2) LPS + HepG2 liver cells
3) Celecoxib + HepG2 liver cells
4) Celecoxib+ LPS + HepG2 liver cells

Group 1: In this phase, we perused activity of COX-2 in HepG2 cells without inhibitor or LPS.
Group 2: In this phase, we perused activity of COX-2 in HepG2 cells, 3 h after the stimulation of cells with *S. enteritidis* lipopolysaccharide.
Group 3: In this phase, we perused activity of COX-2 in HepG2 cells, 500 µM Celecoxib as inhibitor were added and incubated for 12 and 24 h.
Group 4: In this phase, we perused activity of COX-2 in HepG2

Table 1. The results of 4 treatment groups of cancerous cells of HepG2 with lipopolysacharide and inhibitors during 12 and 24 h.

24 h	12 h	Treatment groups
COX-2 (ng/ml)	COX-2 (ng/ml)	
1.977	1.957	1- HepG2 liver cells
0.618	1.383	2- LPS + HepG2 liver cells
0.836	1.87	3- Celecoxib + HepG2 liver cells
0.727	1.823	4- Celecoxib+ LPS + HepG2 liver cells

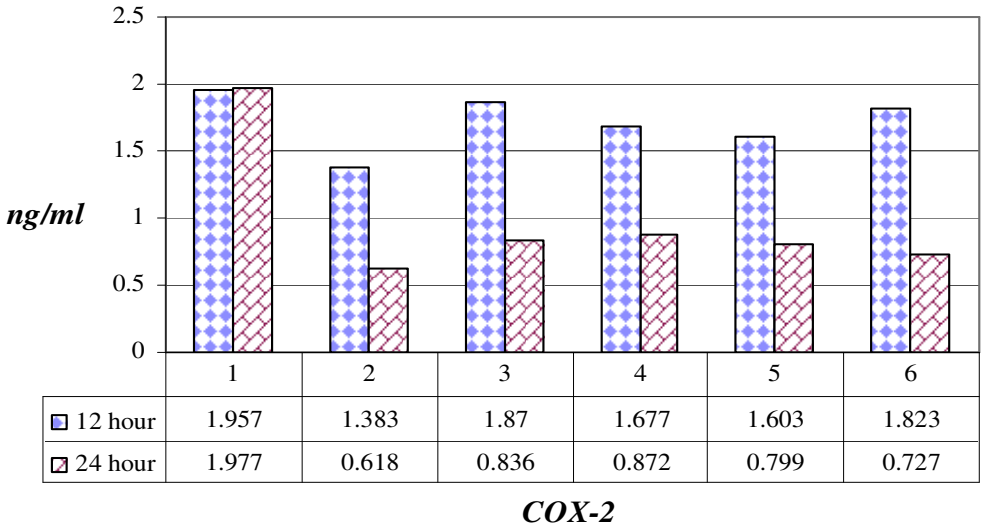

Figure 1. The activity of COX-2 in cancerous cells of HepG2 after 12 and 24 h of incubation after and before stimulation with lipopolysacharide.

cells, 3 h after the stimulation of cells with *S. enteritidis* lipopolysaccharide; 500 µM Celecoxib was added and incubated for 12 and 24 h.

Statistical analysis

The results of this study were analyzed by using two-way ANOVA for comparing the means. The differences between the means were determined with $P<0.05$ level in different experimentations. Data are expressed as means ± standard errors (SEM) and all of the computations were assessed by the software SPSS.

RESULTS

Findings of the study showed that activity of COX-2 in the culture media, COX-2 in unstimulated HepG2 cells was 1.957 ng/ml. This amount after the stimulation of the HepG2 cells with lipopolysaccharide for 12 and 24 h decreases as 1.383 ng/ml. Therefore, in the other experiments, lipopolysaccharide was used as an inhibitor of COX-2 producing. The inhibitor effect of Celecoxib was investigated and the results are shown (Table 1). Treatment with Celecoxib as specific inhibitors of COX-2 enzyme significantly decreases the activity of COX-2 in stimulated HepG2 cells with LPS, while the same substance does not change the activity of produced COX-2 in unstimulated cells (Figure 1).

DISCUSSION

In the present study, activity of COX-2 in the culture media COX-2 in unstimulated HepG2 cells was 1.957 ng/ml. This amount after the stimulation of the HepG2 cells with lipopolysaccharide for 12 and 24 h decreases as 1.383 ng/ml. Treatment of HepG2 cells with Celecoxib as specific inhibitors of COX-2 enzyme significantly decreases the activity of COX-2 in stimulated HepG2 cells with LPS from *S. enteritidis*.

Of course, Fukata and his colleges in 2006, Housset and his colleges in 2009 and also Schlachetzki and his colleges in 2010 found out that LPS did not induce COX-2 up-regulation significantly until 4 h after stimulation (Fukata et al., 2006; Housset et al., 2009; Johannes et

al., 2010). In 2006, Inada et al. found out In osteoblasts collected from C3H/HeJ mice, however, LPS did not induce the expression of COX-2 or mPGES-1, and PGE 2 synthesis was not elevated by LPS at all (Masaki et al., 2006). In 2009, Liu and et al showed that LPS or IFN alone did not induce COX-2 (Tongzheng et al., 2009) and these are in agreement with the results of this study. The stimulation of the intended cells and inducing of inflammative enzyme was done in this study with 100 ng/ml of the extracted LPS out of salmonella bacterium which this amount was suggested in a standard way by Nemeth and his colleges in 2003 (Nemeth et al., 2003). In 2008, Ziwen Liu and et al in their experiments investigated that lipopolysaccharide in RAW264.7 cells, stimulates the gene expression of COX-2 AND iNOS with releasing MAPK and NF-κB, and some of inhibitors which stop the signal route of MAPK and the activity of NF-κB, it also inhibits the gene expression of COX-2 and iNOS (Ziwen et al., 2008). Nuria and et al in 2009 found out that in the primary cultures of germinal hepatocytes, COX-2 is expressed as soon as it is stimulated with lipopolysaccharide. The amount of COX-2 protein can be increased for five times after activation with lipopolysaccharide (Nuria et al., 2009) and it is opposite of result of this study. In 2005, Melanie J Scott and et al found out that lipopolysaccharide is the starter of an intra cell signaling cascade (Melanie et al., 2005).

In 2000, Di Popolo and et al explained that in culture environment of cancer cells of caco-2, the level of mRNA, COX-2, PGG2, is much more in the proliferating and undistinguished cells compared with the distinguished cells (Di Popolo et al., 2000). Also in 2003, Ai-wen Wu and et al found out that COX-2 has an important role in carcinogenesis and development of many kinds of human cancers such as Colorectal cancer, Gastric cancer, Lung cancer and Esophageal cancer and some of the factors and anti-inflammative drugs which can decrease COX-2 expression, level of enzyme and its activity (Ai-Wen et al., 2003; Park et al., 2006).

Regarding the measured activity of inflammatory enzyme of COX-2 in cancerous cells of HepG2 in 12 and 24 h, we found out that the activity of this enzyme in cancerous cells is very high and it is in line with the results of studies done by Dipopolo and Aiwen in 2000 and 2003 (Di Popolo et al., 2000; Ai-Wen et al., 2003). In 1999, Nanbo and et al found out that Lipopolysaccharide (LPS)-binding protein (LBP) is synthesized in hepatocytes and is known to be an acute phase protein. Cytokine-induced production of LBP was reported to increase 10-fold in hepatocytes isolated from LPS-treated rats, compared with those from normal rats. HepG2 cells were shown to express CD14. Pre-stimulation of HepG2 cells with LPS/LBP were augmented cytokine-induced production and gene expression of LBP and CD14 (Nanbo et al., 1999; Ziwen et al., 2008).

In 2006, Park and his colleges studied the effect of Celecoxibe and NS398 which are two inhibitors of COX-2 upon cancerous cells of HuH7 and HCC and they found out that celecoxibe reduces the production of prostaglandin which is related to the amount of COX-2. Also, celecoxibe noticeably reduces the amount of phosphorylation (protein available in HCC cells) and increases the amount of apoptosis and activity of caspase 3 and 9 (Sripathi et al., 2006; Melanie et al., 2005). The activity of COX-2 in HepG2 cancerous cells during 12 h of incubation with lipopolysacharide decreased from 1.957 to 1.383 ng/ml regarding this fact that celecoxibe is inhibitor and reducing factors of inflammative enzyme of COX-2 but lipopolysacharide of *S. enteritidis* highly reduces the activity of inflammative enzymes of COX-2. HepG2 cell groups which receive celecoxibe for inhibition of COX-2; it is observed that by adding lipopolysacharide to COX-2 and incubation, not only lipopolysacharide does not induce COX-2 but of course helps the inhibitor and reduce COX-2 in higher activity. Celecoxibe properly decreased the activity of COX-2 after 12 and 24 h before and after the stimulation of HepG2 cancerous cells with LPS. In groups which received LPS, the decrease activity was more noticeable. Celecoxib can properly be used in the treatment of many diseases and cancers because of their positive effects on both inflamative enzymes. Also lipopolysacharide is a decreasing factor of this enzyme. Regarding this fact, group 4, lipopolysacharide helps more to inhibitors in the stimulation of enzyme, LPS of *S. enteritidis* that has a special sugar linkage in its Antigen O, can be used in reducing the activity of inflamative enzyme of COX-2 as the helper of inhibitors.

Conclusion

This study confirmed that lipopolysacharide of *S. enteritidis* has decreasing effects on COX-2, and it can be used in reducing the activity of inflammative enzyme of COX-2 as the helper of inhibitors such as celecoxib and NS-398.

Therefore, the suggested mechanism of this decrease is that a lipid of lipopolysaccharide consists of abnormal structure including unique fat acids which are accompanied with hydroxyl groups and they can cause the inhibition of COX-2 with competition by arashidonate. We can potentially design drugs to treat a variety disease and cancer to use.

Also studies on the expression of Cox-2 in tumors of companion human and its inhibition with LPS from *S. enteritidis* should authorize further investigation in order to gain insights into the mechanisms involved at a gene level and to have better therapeutic protocols for cancerous patients.

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REFERENCES

Ai-Wen Wu, Jin Gu, Jia-Fu Ji, Zhen-Fu Li, Guang-Wei Xu (2003). Role of COX-2 in carcinogenesis of colorectal cancer and its relationship with tumor biological characteristics and patients' prognosis. World J. Gastroenterol., 9(9): 1990-1994.

Das S, Hashimoto T, Kanazawa K (2008). Growth inhibition of human hepatic carcinoma HepG2 cells by fucoxanthin is associated with down-regulation of cyclin D. Biochemical et Biophysica Acta (BBA), 1780(4): 743-749

Di Popolo A, Annamaria Memoli, Apicella A, Tuccillo C, Palma1 AD, Ricchi P, Acquaviva AM, Zarrilli R (2000). IGFII/IGF-I receptor pathway up-regulates COX-2mRNA expression and PGE2 synthesis in Caco-2 human colon carcinoma cells. Oncogene, 19: 5517-5524.

Fiorucci S, Meli R, Bucci M, Cirino G (2001). Dual inhibitors of cyclooxygenase and 5-lipoxygenase. A new avenue in anti-inflammatory therapy? Biochem Pharmacol., 62: 1433-1438.

Fukata M, Chen A, Klepper S, Krishnareddy AS, Vamadevan LS, Thomas R, Inoue H, Arditi M, Dannenberg AJ and Abreu MT (2006). COX-2is regulated by Toll-like receptor-4 (TLR4) signaling: role in proliferation and apoptosis in the intestine. Gastroenterol., 131: 862-877.

Grosch S, Tegeder I, Niederberger E, Brautigam L, Geisslinger G (2001). COX-2independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2inhibitor celecoxib. FASEB, 15: 2742-44.

Housset A, Nadel Laetitia F, Matt XG, Shao F, Chantal P (2009). Carcinoma Cells Prostaglandin E 2 in Human Biliary Factor Receptor Signaling by Feedback of Epidermal Growth Lipopolysaccharide Initiates a Positive. J. Immunol., 2269-2276.

Hu W, Guo Z, Yi X, Guo C, Chu F, Cheng G (2003). Discovery of 2-phenyl-3-sulfonylphenyl-indole derivatives as a new class of selective COX-2inhibitors.Bioorg. Med. Chem., 11: 5539-5544.

Kant S, Schumacher S, Singh MK, Kispert A, Kotlyarov A, Gaestel M (2006). "Characterization of the atypical MAPK ERK4 and its activation of the MAPK-activated protein kinase MK5". J Biol Chem. 281(46): 35511-35519.

Masaki I, Chiho M, Satoshi U, Shizuo A, Chisato M (2006). Membrane-Bound Prostaglandin E Synthase-1-Mediated Prostaglandin E2 Production by Osteoblast Plays a Critical Role in Lipopolysaccharide-Induced Bone Loss Associated with Inflammation. J. Immunol., pp. 1879-1885.

Mehmet S¸ Emel S¸ Saadet G (2008). Cyclooxygenase-2 in Cancer and Angiogenesis. Angiology Online First: 18505747 Cit, 5

Melanie S, Shubing L, Grace S, Yoram V, Timothy B (2005) .Hepatocytes Enhance Effects of Lipopolysaccharide on Liver Nonparenchymal Cells Through Close Cell Interactions. Shock, 23(5): 453-458.

Nanbo A, Nishimura H, Muta T, Nagasawa S (1999). Lipopolysaccharide stimulates HepG2 human hepatoma cells in the presence of lipopolysaccharide binding protein via CD14. Eur. J. Biochem., 260(1): 183 -91.

Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A,Ganz T, Hepcidin A (2003). Putative mediator of anemia of inflammation is a type II acute-phase protein. Blood, 101: 2461-2463.

Nuria C, Antonio C, Lisardo B, Paloma MS (2009). Inhibition of Prostaglandin Synthesis Up-Regulates Cyclooxygenase-2 Induced by Lipopolysaccharide and Peroxisomal Proliferators. J. Pharmacol. Exp. Therapeutics., 288(3).

Park SW, Sung MW, Heo DS, Inoue H, Shim SH, Kim KH (2006). Nitric oxide up regulates the cyclooxygenase-2 expression through the cAMP-response element in its promoter in several cancer cell lines. Oncogene, 24: 6689-6698.

Park JW, Park J, lee J (2006). Cyclooxylgenase (COX-2) Is Directly Involved but not Decisive in Proliferation of HCC Cells. J. Cancer Res. Clin. Oncol., 132(3):184-92.

Praveen PN, Amini M, Li H, Habeeb AG, Knaus EE (2003). 6-Alkyl, alkoxy, or alkylthio- substituted 3- (4-methanesulfonylphenyl) - 4-phenylpyran-2-ones: a novel class of diarylheterocyclicselective cyclooxygenase-2 inhibitors.Bioorg Med. Chem. Lett., 13: 2205-2209.

Queiroga FL, Alves A, Pires I, Lopes C (2007). Expression of Cox-1 and Cox-2 in Canine Mammary Tumours. J. Comp. Path., 136: 177-185.

Schlachetzki J, Bernd F, Elisabeth H, Antonio O, Eduardo CJ, Michael H, Michael H (2010). Norepinephrine enhances the LPS-induced expression of COX-2and secretion of PGE2 in primary rat microglia. J. Neuroinflammation., 7: 2.

Sharma RA, Dalgleish AG, Steward WP, O'byrne KJ (2003). Angiogenesis and the immune response as targets for the prevention and treatment of colorectal cancer. Oncol. Rep., 10: 1625-31.

Sripathi MS, Dharmalingam S, Paramasivam R, Rama PR, Brian KD, Courtney WH, Shrikant A (2006). Therapeutic Effects of Phyllanthus Species: Induction of TNF-α-mediated Apoptosis in HepG2 Hepatocellular Carcinoma Cells. Am. J. Pharmacol. Toxicol., 1(4): 65-71.

Tan XW, Xia H, Xu JH, Cao JG (2009). Induction of apoptosis in human liver carcinoma HepG2 cell line by 5-allyl-7-gendifluoromethylenechrysin. World J. Gastroenterol., 15(18): 2234-2239.

Taketo MM (1998). Cyclooxygenase-2 Inhibitors in Tumorigenesis (Part I). J. Nat. Cancer Inst., 90: 1529-36.

Tongzheng L, Schneider RA, Vaibhav S, Yongcheng H, Rostislav IL, Lakhu K, Dale GH (2009). Protein Never in Mitosis Gene A Interacting-1 regulates calpain activity and the degradation of cyclooxygenase-2 in endothelial cells. J. Inflamm. (Lond), 6(1): 20.

Xiao Y, Yang FQ, Li SP, Hu G, Lee SM, Wang YT (2008). Essential oil of Curcuma wenyujin inhibits growth and induces apoptosis in human HepG2 hepatoma cells through mitochondria-caspase pathway. World J. Gastroenterol., 14(27): 4309-4318.

Ziwen L, Yumei F, Yu W, Cui H, Yu P, Huang H, Ying Y, Lan L, Zhimin Y (2008). Dipyrithione inhibits lipopolysaccharide-induced iNOS and COX-2up-regulation in macrophages and protects against endotoxic shock in mice. FEBS Letters, 582: 1643-1650.