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• *BRIEF REPORTS* •

# Effect of peroxisome proliferator-activated receptor-gamma ligand on inflammation of human gallbladder epithelial cells

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## Abstract

**AIM:** To investigate the effect of peroxisome proliferator-activated receptor gamma (PPAR-γ) and its ligand, ciglitazone, on inflammatory regulation of human gallbladder epithelial cells (HGBECs) and to assess the effect of human epithelial growth factor (hEGF) on growth of HGBECs.

**METHODS:** HGBECs were cultured in media containing hEGF or in hEGF-free media. HGBECs were divided into normal control group, inflammatory control group and ciglitazone group (test group). Inflammatory control group and ciglitazone group were treated with 5 μg/L of human interleukin-1β (hIL-1β) to make inflammatory model of HGBECs. The ciglitazone group was treated with various concentrations of ciglitazone, a potent ligand of PPAR-γ. Subsequently, interleukin-8 (IL-8), IL-6, and tumor necrosis factor-α (TNF-α) concentrations in all groups were measured. The data were analyzed statistically.

**RESULTS:** HGBECs were cultured in medium successfully. The longevity of HGBECs in groups containing hEGF was longer than that in hEGF-free groups. So was the number of HGBECs. The longest survival time of HGBEC was 25 d. The inflammatory model of HGBECs was obtained by treating with hIL-1β. The concentrations of IL-6 and IL-8 in ciglitazone group were lower than those in inflammatory control group ($P<0.05$). The secretion of IL-6 in inflammatory control group was higher (350.31±37.05 μg/L) than that in normal control group (50.0±0.00 μg/L, $P<0.001$). Compared to normal control group, IL-8 concentration in inflammatory control was higher ($P<0.05$).

**CONCLUSION:** hEGF improves the growth of HGBECs *in vitro*. Ciglitazone inhibits the inflammation of HGBECs *in vitro* and has potential therapeutic effect on cholecystitis *in vivo*.



**Key words:** PPAR-γ; Human gallbladder epithelial cells; Inflammation; Effect

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## INTRODUCTION

The peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors[1]. With ligand binding, PPAR-γ forms a heterodimer with the retinoid X receptor-α (RXR-α) and joins to the PPAR response element in promoters of target genes, thus directly regulating their expressions[2]. PPAR-γ plays an important role in cell growth, differentiation, inflammation, and apoptosis. Binding to its ligand, PPAR-γ is associated with the regulation of cardiovascular disease, diabetes, and carcinogenesis[3]. Expression in many kinds of cells such as colon epithelial cells, monocytes, hepatic stellate cells, aortic smooth-muscle cells, thyrocytes, and airway epithelial cells, PPAR-γ gene can regulate their inflammation[4-8].

The commonly used method to elucidate the physiological role of PPAR-γ is to investigate the effects of its well-characterized ligands. The most specific PPAR-γ agonists are thiazolidinediones (TZDs)-insulin-sensitizing drugs including pioglitazone, rosiglitazone, troglitazone, and ciglitazone[9]. Previously, cyclopentenone prostanoids were thought to be the most potent one among the naturally occurring PPAR-γ activators[10]. However, 15d-PGJ2 is proved to be a potent inducer of interleukin-6 (IL-6) and IL-8 production and can be a mediator of inflammatory response recently, but this effect is independent of PPAR-γ activation[11].

In activated macrophages and vascular smooth muscle cells, the agonists of PPAR-γ inhibit the expression of many proinflammatory genes, like inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), IL-6 or metalloproteinase-9. Endothelium lines, the inner surface of blood vessels, and is a primary target for inflammatory agents. Exposure of endothelial cells to cytokines or bacterial lipopolysaccharide (LPS) induces the secretion of proinflammatory mediators, among them both IL-6 and IL-8 are crucial to acute inflammation[12,13]. IL-6 and IL-8 are produced either by endothelial cells directly or by endothelial cells activated by human interleukin-1β (hIL-1β) and TNF-α[14].

The present study was to investigate whether and how the PPAR-γ agonist, ciglitazone, one of the TZDs, affected the inflammatory regulation of human gallbladder epithelial cells (HGBECs). The effect was assessed by changes of IL-6, IL-8, and TNF-α in media after being treated with ciglitazone and IL-1β in order to determine the potential therapeutic function of PPAR-γ agonists for cholecystitis.

## MATERIALS AND METHODS

### Materials

Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic solution, trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco BRL Life Technologies. Epidermal growth factor (EGF), type IV collagenase were purchased from Sigma Chemical Co. hIL-1β was purchased from Roche Biology Co. Ciglitazone was purchased from Cayman Chemical Co. IL-8, IL-6 RIA kit and TNF-α RIA kit were purchased from East Asia Radio-immunology Institution, Beijing, China. The lower detection limit of the assay was 0.3 μg/L for TNF-α, 50 μg/L for IL-6, and 0.2 μg/L for IL-8.

### Cell isolation and primary culture

The isolation of HGBECs was processed immediately after its surgical removal. Bile was aspirated through a cannula placed in the cystic duct and the mucosal cavity was washed repeatedly with cold PBS containing $10^5$ U/L penicillin and 100 mg/L streptomycin. Subsequently, 15 mL of 2.5 g/L trypsin-EDTA was introduced into the gallbladder and then placed in a sterile glass container immersed in a water bath at 37 ¡æ for 20 min. Trypsin treatment led to a clear separation of the lining columnar epithelial cells from the underlying fibrous connective tissue. The cells were further dissociated by agitating in a turbulent stream of culture medium containing the pepsin for 5-10 min. The cell suspension was then centrifuged at 4 000 r/min for 5 min and the pellet was washed twice with DMEM containing 100 mL/L FCS. Subsequently, the cells were counted and tested for viability using concentrated trypan blue solution. In a typical study, >95% of cells would exclude trypan blue. Cells were placed on the Vitrogen-100 coated 24-well culture plate with DMEM containing 100 mL/L FCS, 10 μg/L human epithelial growth factor (hEGF) as test groups. Another 24-well cells were cultured in media without hEGF as control groups. Cell concentration was $(5\text{-}10)\times10^5$/mL. After being incubated for 2 h, media were changed to exclude non-epithelial cells. Media were changed after 48 h and subsequently, the cells were fed every 3 d.

Cells in three wells were digested by 2.5 g/L trypsin/ 0.1 g/L EDTA and counted every 24 h for 3 d to protract cell growth curve. Cells were studied serially by inverted phase microscopy and immunohistochemical reaction with epithelial keratins.

### Cell group and treatment

HGBECs obtained through the above procedure were incubated in DMEM supplemented with 100 mL/L FCS, 10 μg/L hEGF, $10^5$ U/L of penicillin, 10 mg/L of streptomycin at 37 ¡æ in 50 mL/L $CO_2$. They were placed into 48-well culture plates in $5\times10^5$/mL of cell density. Then, they were randomly divided into normal control group, inflammatory control group, test group 1, test group 2, test group 3, and test group 4, among which each group had 8 wells containing 2 mL of media in a well. On the 5th d of culture, media were changed to DMEM without hEGF. Various final concentrations including 10, 20, 30, 50 mmol/L of ciglitazone were added into test groups 1-4, respectively. Cells were incubated in DMEM at 37 ¡æ in 50 mL/L $CO_2$. The cells were treated with ciglitazone and incubated for 24 h, then inflammatory control group and all test groups were treated with the final concentration 5 μg/L of hIL-1β. Media were collected after 2-h incubation for measuring concentration of IL-6, TNF-α, and IL-8 by radioimmunoassay. Cell morphology was studied serially by inverted phase contrast microscopy.

### Statistical analysis

The data were analyzed by Student's *t*-test for paired sample, by ANOVA for multiple comparison between groups. The statistical significance of the difference between mean values was determined by the *P* value less than 0.05.

## RESULTS

### Growth and conformation of cultured cells

HGBECs were cultured successfully in DMEM containing hEGF or without hEGF. In groups containing hEGF, the number of HGBECs reached the peak on the 5th d of culture and maintained for 10-12 d. After 20 d, apoptosis of HGBECs was noted. The longest longevity of HGBECs was 25 d. Compared to hEGF-free group, the growth and number of HGBECs were increased by hEGF (Figure 1).

After 6 h of culture, HGBECs attached to the monolayer were flat and multiangular in morphology. Some of the HGBECs were columnar with vigorous growth (Figure 2A). After 20 d of culture, vacuoles and drawbenches were found in cytoplast.

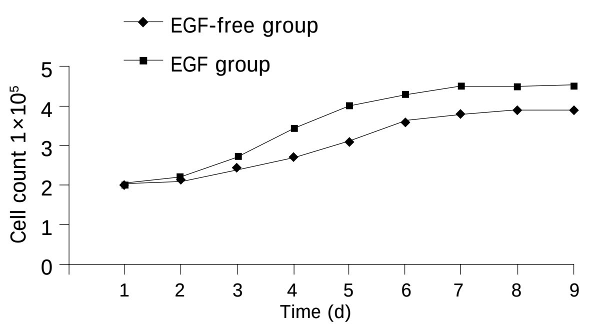


Figure 1 Growth curve of HGBECs.

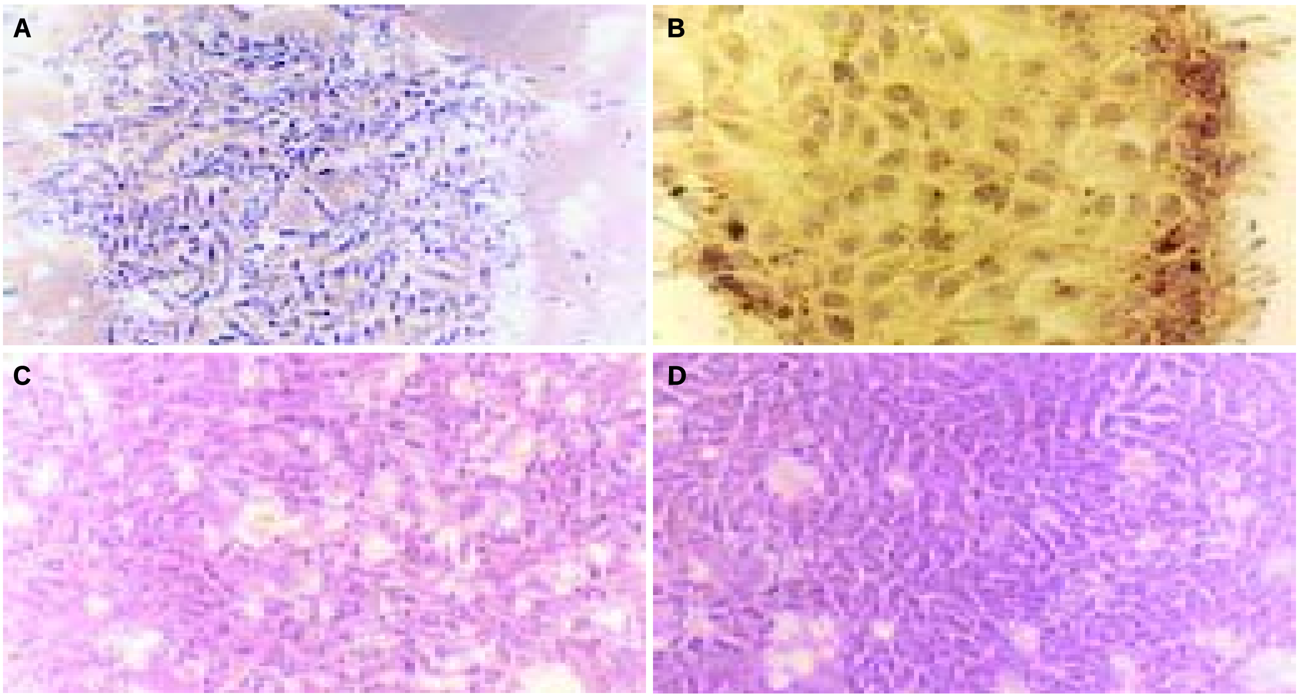


**Figure 2** Attachment to monolayer (A), positive response (B), normal shape and structure (C) and edema (D) of HGBEC.

### *Identification of HGBECs*

The cultured cells were identified by immunohistochemistry as keratin CK19. Specific positive reaction that could identify HGBECs was found in the cultured cells (Figure 2B).

### *Morphology of inflammatory HGBECs and inflammatory model of HGBECs*

After HGBECs were treated with hIL-1β for 2 h, inflammatory changes of HGBECs such as swelling, unclear edge and irregular shape were found in test groups and inflammatory control group compared to normal control groups (Figures 2C and D).

### *Concentration of IL-6, IL-8 and TNF-α*

Concentration of IL-6, IL-8 and TNF-α was measured by radioimmunoassay (Table 1). The data of TNF-α concentration were not shown because of its error and dispersion.

**Table 1** IL-6 and IL-8 concentration in all groups (mean±SE)

| Group | IL-6 (μg/L) | IL-8 (μg/L) |
|---|---|---|
| Normal control | 50.0±0.00 | 0.20±0.00 |
| Inflammatory control | 350.31±37.05[b] | 13.97±0.63[b] |
| Ciglitazone 10 mmol/L | 231.46±39.41[a] | 10.94±1.59[a] |
| 20 mmol/L | 207.22±26.72[a] | 11.74±2.01[a] |
| 30 mmol/L | 188.89±29.33[a,d] | 9.62±1.71[a] |
| 50 mmol/L | 170.46±20.24[a,e] | 9.34±2.91[a,e] |

[a]*P*<0.05 *vs* inflammatory control group; [b]*P*<0.001 *vs* normal control group; [d]*P*<0.001, [e]*P*<0.025 *vs* 10 mmol/L group.

## DISCUSSION

The isolation and culture of HGBECs play a crucial role in studying biliary tract and liver disease. HGBECs have been cultured successfully since 1993[15], but its short duration limits the related research about the pathophysiology of biliary tract and liver. hEGF is a potent proliferation-activated factor of epithelial cells[16]. The first part of this study was performed to determine the hypothesis that hEGF could improve the growth of HGBECs. The proliferative activity of HGBECs promoted by hEGF was assessed by calculating the number and the life span of HGBECs, and compared to those in hEGF-free group. Results are in agreement with the hypothesis because hEGF increased the number of HGBECs and prolonged the longevity of HGBECs in which the longest was 25 d (8.2 d in EGF-free group). Also, the effect of hEGF on the growth of HGBECs is in agreement with the light microscopic findings. Thus, HGBECs cultured in medium containing hEGF are beneficial to the biological study of HGBECs.

The discovery that the insulin-sensitizing TZDs-specific PPAR-γ agonists have antiproliferative, anti-inflammatory and immunomodulatory effects has led to the evaluation of their potential use in the treatment of diabetic complications and inflammatory, proliferative diseases in non-insulin-resistant, euglycemic individuals. Apart from improving insulin resistance, plasma lipids and systemic inflammatory markers, ameliorating atherosclerosis and preventing coronary artery restenosis in diabetic subjects, currently approved TZDs that have been shown to improve psoriasis and ulcerative colitis in euglycemic human subjects[17]. In endothelial cells, troglitazone reduces expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, which are adhesion molecules that facilitate monocyte attachment and migration[18].

In addition to their impact on TNF-α and IL-6, IL -8, PPAR-γ agonists have also been reported by Pasceri *et al*.[18], to inhibit other macrophage proinflammatory mediators, including iNOS, gelatinase B, and the macrophage scavenger receptor-A. PPAR-γ activation suppresses gastric mucosal inflammatory responses to *Helicobacter pylori* (*H pylori*) LPSs, suggesting that pharmacological manipulation of PPAR-γ activation may provide therapeutic benefits in the resolution of inflammation associated with *H pylori* infection[19]. In airway, PPAR-α and -γ (co) agonists might be of therapeutic interest for the regulation of allergic or inflammatory reactions by targeting both regulatory and effector cells involved in the immune response[20,21]. An important step in the monthly turnover of the endometrial lining during the menstrual cycle is the cyclical recruitment and activation of inflammatory cells. The use of PPAR-γ ligands to reduce chemokine production and inflammation may be a productive strategy for future therapy of endometrial disorders, such as endometriosis[22,23].

The mechanism of PPAR-γ and its ligand to regulate cellular inflammation may involve multiple pathways in different kinds of cells and the state of differentiation/activation of the same source of cells. In macrophages and epithelial cells, the effect of 15 d-PGJ2 is targeted to the NF-κB/I-κB pathway and to the mitogen-activated protein kinase ERK1/2. The role of PPAR-γ activation in tissue factor inhibition by 15 d-PGJ2 is excluded[24]. 15 d-PGJ2 and rosiglitazone rapidly induce the transcription of suppressor of cytokine signalings 1 and 3, which in turn inhibit Janus kinase (JAK) activity in activated glial cells. In addition, Src homology 2 domain-containing protein phosphatase 2, another negative regulator of JAK activity, is also involved in their anti-inflammatory action[25]. Although it is not a direct causal effect, the insufficient PPAR-γ activity contributes to ongoing dysregulated inflammation in pulmonary sarcoidosis by failing to suppress NF-κB[26]. Dendritic cells (DCs), the most potent antigen-presenting cells, involve the anti-inflammatory activation of PPAR-γ. Recent reports showed that activation of PPAR-γ alters the maturation process of DCs, prevents induction of Th2-dependent eosinophilic airway inflammation and contributes to immune homeostasis in the lung[27]. However, conflicting findings on the consequences of PPAR-γ activation on inflammatory responses have led to a confusion regarding the role of the transcription factor in inflammation. Cyclopentenone prostaglandins, synthetic ligand of PPAR-γ, induce apoptosis of human DCs in a PPAR-γ-independent manner. Since these compounds are released during an inflammatory event and show anti-inflammatory properties, they may contribute to the downregulation of DC function through apoptotic cell death[28]. In a murine model of asthma, IL-8 release and activation of NF-κB-responsive reporter gene are inhibited only at micromolar concentrations, suggesting that these effects are not mediated by PPAR[29].

Based on the hypothesis that PPAR-γ expresses in HGBECs and inhibits inflammation of HGBECs, the following part of this study was designed to investigate whether the activation of PPAR-γ inhibited inflammation of HGBECs. IL-1β is a potent proinflammation factor that can induce multiple inflammatory mediators. After being treated with IL-1β for 2 h, HGBECs secreted a higher concentration of IL-6 and IL-8 in media ($P<0.001$). An inflammatory model of HGBECs was achieved successfully and showed a higher concentration of IL-6 and IL-8 in media, and inflammatory morphological changes such as edema and unclear edge in cellular formation. TNF-α, a potent inflammatory mediator, is often produced by white blood cells and smooth muscle cells in the early stage of inflammation. TNF-α could not be detected in the present study because HGBECs did not secrete it.

*In vivo*, IL-1β, TNF-α, and LPS initiate the secretion of cytokines including IL-6, IL-8, and IL-2. IL-1 can induce inflammation of gallbladder epithelial cells and biliary epithelial cells. Gallbladder inflammation is an early feature of gallstone formation[30]. These findings have been proved by molecular biology at mRNA level[31,32]. Also, these cytokines interact to form a network, which is named as cytokine storm[33]. The storm activates inflammatory cells and mediates inflammatory cell chemotaxis, then causes systemic inflammation. This process is defined as systemic inflammation response syndrome by the Association of American Physician and Critical Care in 1992[34].

PPAR-γ is a nuclear hormone receptor, with a well-established role in adipogenesis and glucose metabolism. Over the past 3 years several laboratories have reported that this protein can influence macrophage responses to a variety of inflammatory stimuli[32]. Immunolocalization of PPAR-γ primarily to colonocytes, especially in the presence of inflammation, strongly suggests that these epithelial cells are the target of PPAR-γ ligands[35-37]. In order to verify that HGBECs could express PPAR-γ and that PPAR-γ could affect the inflammation of HGBECs after binding to the ligand, we investigated the characteristics of PPAR-γ receptor using traditional endocrinological technique to study unknown receptor by binding to known ligand. We noted that ciglitazone with a final concentration of 10-50 mmol/L could suppress the IL-6 and IL-8 gene expression induced by IL-1β in a dose-dependent manner. This is consistent with our experimental hypothesis. Our results suggest that activation of PPAR-γ downregulates inflammation of HGBECs *in vitro*.

PPARs are nuclear receptor isoforms with key roles in the regulation of lipid and glucose metabolism. Synthetic ligands for PPAR-γ promote insulin sensitization in the context of obesity. In this study, ciglitazone showed potential therapeutic effects on inflammation of HGBECs *in vivo*.

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